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Glucocorticoid steroids such as prednisone are prescribed for chronic muscle conditions such as Duchenne muscular dystrophy, where their use is associated with prolonged ambulation. The positive effects of chronic steroid treatment in muscular dystrophy are paradoxical because these steroids are also known to trigger muscle atrophy. Chronic steroid use usually involves once-daily dosing, although weekly dosing in children has been suggested for its reduced side effects on behavior. In this work, we tested steroid dosing in mice and found that a single pulse of glucocorticoid steroids improved sarcolemmal repair through increased expression of annexins A1 and A6, which mediate myofiber repair. This increased expression was dependent on glucocorticoid response elements upstream of annexins and was reinforced by the expression of forkhead box O1 (FOXO1). We compared weekly versus daily steroid treatment in mouse models of acute muscle injury and in muscular dystrophy and determined that both regimens provided comparable benefits in terms of annexin gene expression and muscle repair. However, daily dosing activated atrophic pathways, including F-box protein 32 (*Fbxo32*), which encodes atrogin-1. Conversely, weekly steroid treatment in *mdx* mice improved muscle function and histopathology and concomitantly induced the ergogenic transcription factor Krüppel-like factor 15 (*Klf15*) while decreasing *Fbxo32*. These findings suggest that intermittent, rather than daily, glucocorticoid steroid regimen promotes sarcolemmal repair and muscle recovery from injury while limiting […]

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Glucocorticoid steroids such as prednisone are prescribed for chronic muscle conditions such as Duchenne muscular dystrophy, where their use is associated with prolonged ambulation. The positive effects of chronic steroid treatment in muscular dystrophy are paradoxical because these steroids are also known to trigger muscle atrophy. Chronic steroid use usually involves once-daily dosing, although weekly dosing in children has been suggested for its reduced side effects on behavior. In this work, we tested steroid dosing in mice and found that a single pulse of glucocorticoid steroids improved sarcolemmal repair through increased expression of annexins A1 and A6, which mediate myofiber repair. This increased expression was dependent on glucocorticoid response elements upstream of annexins and was reinforced by the expression of forkhead box O1 (FOXO1). We compared weekly versus daily steroid treatment in mouse models of acute muscle injury and in muscular dystrophy and determined that both regimens provided comparable benefits in terms of annexin gene expression and muscle repair. However, daily dosing activated atrophic pathways, including F-box protein 32 (Fbxo32), which encodes atrogin-1. Conversely, weekly steroid treatment in mdx mice improved muscle function and histopathology and concomitantly induced the ergogenic transcription factor Krüppel-like factor 15 (Klf15) while decreasing Fbxo32. These findings suggest that intermittent, rather than daily, glucocorticoid steroid regimen promotes sarcolemmal repair and muscle recovery from injury while limiting atrophic remodeling.

Introduction
Glucocorticoid (GC) steroids are used to treat many chronic conditions ranging from lung disease to autoimmune disorders and muscle disease. It is estimated that more than 1% of the US population is treated chronically with GC steroids. Although the side effect profile from GC steroids is extensive, muscle weakness and myopathy can occur in response to chronic GC steroid use and can be extremely debilitating (1). Curiously, chronic GC steroids are used to treat Duchenne muscular dystrophy (DMD), a primary muscle disease, and the use of steroids is associated with prolonged ambulation (2, 3).

The mechanisms underlying the beneficial effects of steroid use in muscular dystrophy are still largely unknown. In the US, the most commonly used agent is prednisone, while deflazacort is used in Europe and Asia. In muscular dystrophy, steroids are thought in part to act through immune system modulation, as a direct reduction in immune cell infiltration into muscle has been observed (4). However, GC steroids also directly affect muscle itself by shifting metabolism and gene expression (5). Chronic GC steroid use has been seen to reduce fibrosis in human DMD muscle (6). In mdx mice, a genetic model of DMD, deflazacort was observed to improve muscle regeneration and growth after injury (7, 8). An 8-week study of prednisolone in mdx mice improved specific force and decreased the number of centrally nucleated myofibers (9). However, 3 months of daily prednisone in mdx mice resulted in weight loss, reduced strength, and increased fibrosis in the heart, suggesting that longer, chronic administration may be problematic (10). Recently, it has been shown that GC steroids rely on Krüppel-like factor 15 (Klf15), a Krüppel like transcription factor, to mediate ergogenic muscle performance effects (11). Deletion of Klf15 in the mouse exacerbated the mdx muscle phenotype, and muscle-specific overexpression of Klf15 improved muscle performance in the mdx mouse. In addition, it is still unclear whether prednisone and deflazacort vary in their effects in DMD. Deflazacort is a synthetic oxazine derivative of prednisolone. A recent comparative study in boys with DMD reported that deflazacort associated with higher efficacy, measured as later age at loss of ambulation, but also may have more side effects, although differences in dosing could account for these findings (2). A second recent study showed comparable benefit between deflazacort and prednisone, but greater weight gain with prednisone (12). The benefit of GC steroids on DMD muscle likely arises from multiple mechanisms and a balance between beneficial and detrimental effects.

One hallmark of muscle injury is disruption of the muscle plasma membrane. In DMD, the sarcolemma is weakened and more readily disrupted, thus representing a model of chronic muscle injury (13). Anxa6, which encodes annexin A6, was identified as a genetic modifier of muscular dystrophy in mice (14), and annexins A1 and A6 are directly implicated in sarcolemmal repair and resealing after muscle membrane injury (15, 16). Furthermore, annexins are known regulators of the immune system where they are thought to improve muscle regeneration and growth after injury (17, 18). Moreover, recent work has shown that annexins can directly mediate sarcolemmal repair through increased expression of annexins A1 and A6, which mediate myofiber repair. This increased expression was dependent on glucocorticoid response elements upstream of annexins and was reinforced by the expression of forkhead box O1 (FOXO1). We compared weekly versus daily steroid treatment in mouse models of acute muscle injury and in muscular dystrophy and determined that both regimens provided comparable benefits in terms of annexin gene expression and muscle repair. However, daily dosing activated atrophic pathways, including F-box protein 32 (Fbxo32), which encodes atrogin-1. Conversely, weekly steroid treatment in mdx mice improved muscle function and histopathology and concomitantly induced the ergogenic transcription factor Krüppel-like factor 15 (Klf15) while decreasing Fbxo32. These findings suggest that intermittent, rather than daily, glucocorticoid steroid regimen promotes sarcolemmal repair and muscle recovery from injury while limiting atrophic remodeling.
sequences of muscle atrophy. Repair and muscle recovery while avoiding the deleterious consequences of muscle atrophy is one mechanism by which GC steroids exert their effect (18). Here, we compared the effect of prednisone and deflazacort on sarcolemmal repair in both normal and dystrophic myofibers. We found that a single pulse dose of either GC steroid enhanced sarcolemmal repair after laser injury. This effect was mediated by a direct upregulation of Anxa1 and Anxa6 through both direct transcriptional regulation and microRNA-regulated mRNA stability. We compared weekly versus daily GC steroid administration in murine models of acute muscle injury and chronic muscular dystrophy. We compared weekly versus daily GC steroid administration in murine models of acute muscle injury and chronic muscular dystrophy. We compared weekly versus daily GC steroid administration in murine models of acute muscle injury and chronic muscular dystrophy. We compared weekly versus daily GC steroid administration in murine models of acute muscle injury and chronic muscular dystrophy.

Results
A single pulse of GC steroids improves sarcolemmal repair after injury in normal muscle. We visualized muscle membrane repair in real time using high-resolution microscopy (16, 19). In this assay, a laser was used to disrupt the myofiber sarcolemma. Using this microwounding assay, it was previously shown that annexins assemble into a repair cap forming within seconds specifically at the site of laser ablation. FM4-64, a phosphatidylserine-binding dye, is used to mark the region of muscle injury. The flexor digitorum brevis (FDB) muscle of 8-week-old 129T2/SvEmsJ WT mice was electroporated with a plasmid to express GFP-tagged annexin A6 (ANXA6). Electroporation was conducted 7 days prior to injury protocol in order to allow time for plasmid expression. Mice were treated with a single dose of steroids, either prednisone or deflazacort (1 μg/g body weight; ref. 20), one day prior to harvesting myofibers for laser injury and live-cell imaging. Control mice were treated with vehicle or eplerenone (100 μg/g body weight), an antimineralocorticoid steroid, as an additional pharmacological control.

FM4-64 dye accumulation, which measures the extent of membrane injury and resealing, was significantly lower in both prednisone- and deflazacort-treated myofibers compared with either eplerenone-treated or vehicle-treated myofibers (Figure 1A). The area of FM4-64 fluorescence was significantly reduced in both prednisone- and deflazacort-treated mice, as compared with vehicle- or eplerenone-treated myofibers (Figure 1A). This smaller injury area correlated with more efficient annexin repair cap formation. The annexin A6 cap appeared sooner and was smaller in steroid-treated myofibers compared with vehicle- or eplerenone-treated myofibers (Figure 1A). Together, these findings demonstrate that a single pulse of GC steroids prior to injury accelerates and improves injury repair.

To verify the effectiveness of in vivo steroid dosing, splenocytes from treated animals were isolated and analyzed for markers of immune cell activation. In prednisone- and deflazacort-treated mice, Gzmb (encoding granzyme B) and Ifng (encoding IFN-γ) expression levels were downregulated compared with those of control animals, consistent with the anticipated effect of steroids to promote resolution of the immune response (17). In leukocytes, Anxa1 is induced by GC steroids, suggesting that its upregulation is one mechanism by which GC steroids exert their effect (18).
GCs act through GC receptor (GR, also known as NR3C1). We hypothesized that activated GR would drive Anxa1 and Anxa6 expression in muscle (Figure 2A). We identified GR-responsive elements (GRE; GACAnnTGnTnG; ref. 23) upstream of the Anxa1 transcriptional start site at –7610 bp and upstream of Anxa1 elements (GRE; GnACAnnnTGTnC; ref. 23) upstream of the expression in muscle (Figure 2A). We hypothesized that activated GR would drive GR-responsive GRE sites by GR was significantly increased after a single dose of prednisone or deflazacort, as compared with vehicle (Figure 2C).

We generated both normal reporter constructs and those in which the GRE sequences were ablated (ΔGRE). Plasmids were then electroporated into muscles of normal mice. One week following electroporation, animals were treated with a single dose of prednisone and muscles were collected for luciferase assays. Data were normalized to luciferase activity measured from muscles of vehicle-injected animals. The GRE upstream of Klf15 was used as a positive control of GC responsiveness in muscle (11). A prednisone pulse induced increased luciferase activity from WT constructs as well as the Klf15 construct (Figure 2D). Conversely, the prednisone pulse failed to elicit a luminescence signal when the GRE sequences had been ablated (Figure 2D). These data are highly consistent with the studies conducted in C2C12 cells and document that GCs promote the transcription of Anxa1 and Anxa6 in muscle.

In addition to direct transcriptional activation, GR-dependent transactivation of Anxa1 and Anxa6 was further reinforced through indirect activation of forkhead box O1 (Foxo1), which encodes a transcriptional activator that regulates muscle growth and atrophy (Supplemental Figure 1B) (24). The single pulse of GC steroids, but not of eplerenone, increased Foxo1 expression in muscle and primary myoblasts as compared with vehicle-treated muscles (Supplemental Figure 1C). We found the same results when primary myoblasts were exposed to GC steroids in culture. We isolated primary myoblasts from WT mice that were age- and background-matched with mice used in previous analyses and cultured them for 24 hours (passage 0) in the presence of either vehicle, prednisone, or deflazacort (25 μg/ml). Chromatin immunoprecipitation (ChIP) using antibodies to GR and quantitative PCR (qPCR) was performed on the predicted GRE sites within Anxa1 and Anxa6 genomic loci. Occupancy of GR and quantitative PCR (qPCR) was performed on the predicted GRE sites by GR was significantly increased after a single dose of prednisone or deflazacort, as compared with vehicle (Figure 2C).

To confirm the role of GCs directly inducing annexin expression in skeletal muscle, we ligated promoter regions of Anxa1 and Anxa6 containing GREs upstream of a luciferase reporter plasmid. We generated both normal reporter constructs and those in which the GRE sequences were ablated (ΔGRE). Plasmids were then electroporated into muscles of normal mice. One week following electroporation, animals were treated with a single dose of prednisone and muscles were collected for luciferase assays. Data were normalized to luciferase activity measured from muscles of vehicle-injected animals. The GRE upstream of Klf15 was used as a positive control of GC responsiveness in muscle (11). A prednisone pulse induced increased luciferase activity from WT constructs as well as the Klf15 construct (Figure 2D). Conversely, the prednisone pulse failed to elicit a luminescence signal when the GRE sequences had been ablated (Figure 2D). These data are highly consistent with the studies conducted in C2C12 cells and document that GCs promote the transcription of Anxa1 and Anxa6 in muscle.

In addition to direct transcriptional activation, GR-dependent transactivation of Anxa1 and Anxa6 was further reinforced through indirect activation of forkhead box O1 (Foxo1), which encodes a transcriptional activator that regulates muscle growth and atrophy (Supplemental Figure 1B) (24). The single pulse of GC steroids, but not of eplerenone, increased Foxo1 expression in muscle and primary myoblasts as compared with vehicle-treated muscles (Supplemental Figure 1C). We found the same results when primary myoblasts were exposed to GC steroids in culture. We isolated primary myoblasts from WT mice that were age- and background-matched with mice used in previous analyses and cultured them for 24 hours (passage 0) in the presence of either vehicle, prednisone, or deflazacort (25 μg/ml). Expression of Anxa1, Anxa6, and Foxo1 was significantly upregulated in cells exposed to GCs as compared with those treated with vehicle (Supplemental Figure 1D).

Furthermore, a GRE site was identified within the first Foxo1 intron (+55284), and FOXO1-binding sites (GTAAACA; ref. 25) were
found upstream of Anxa6 (−1872 bp), Foxo1 itself (−266 bp), and in the 5′ UTR of Anxa1 (+118 bp). In GC-treated C2C12 myoblasts, GR binding to Foxo1 GRE appeared increased as compared with that in vehicle-treated cells (Supplemental Figure 1E). In C2C12 cells, GC steroids induced occupancy of FOXO sites by FOXO1 at the Anxa1, Anxa6, and Foxo1 loci (Supplemental Figure 1E). GC steroid activation of the Foxo1 GRE was also confirmed in intact muscle using luciferase assays on electroporated muscles and was dependent on the GRE as ablating the GRE sequence reduced reporter activity (Supplemental Figure 1F). GC steroids also induced activity of candidate FOXO sites in Anxa1, Anxa6, and Foxo1, and this also was dependent on the presence of FOXO sites (Supplemental Figure 1G). Thus, a single steroid pulse leads to the upregulation of Anxa1 and Anxa6 in muscle through direct GR-driven transactivation and this response is reinforced through upregulation of Foxo1, which in turn further drives annexin gene expression.

**GC steroids sustain Anxa6 upregulation via miR-383 modulation.** In silico prediction showed a putative binding site for the microRNA miR-383 in Anxa6 3′ UTR (+228–247 bp from 3′ UTR start). This binding site was hypothesized as an additional route by which GC steroids may contribute to Anxa6 upregulation. In this alternative pathway, nuclear receptor subfamily 0, group B, member 1 (Nr0b1), a reported target of the GR (NR3C1) in the adrenal cortex (26), would decrease miR-383 levels, thereby reducing miR-383–driven targeting of Anxa6 mRNA (Figure 3A).

Consistent with this pathway, Nr0b1 was significantly upregulated and miR-383 significantly downregulated in muscle and in primary myoblasts after prednisone or deflazacort pulse, but not control treatments (Figure 3B).

To further validate miR-383 interaction with Anxa6′s 3′ UTR in muscle, we electroporated WT muscles with synthetic miR-383–mimic and anti–miR-383 and interrogated the muscles for gene expression and sarcolemmal repair. Experiments were controlled with scramble miR-mimic and anti-miR. Expression levels of miR-383 were significantly higher after miR-mimic and lower after anti-miR electroporation (Figure 3C).

Anxa6 mRNA was reduced with miR-mimic and increased after anti-miR (Figure 3C). Sarcolemmal injury, area of injury, and rate of FM4-64 accumulation were significantly greater after miR-mimic and significantly smaller after anti-miR electroporation (Figure 3D).

**Figure 3. miR-383 contributes to GC-associated Anxa6 regulation.** (A) Model of pathway linking GC uptake in muscle to regulation of miR-383 and Anxa6 expression. Solid lines, reported interactions; dashed lines, interrogated interactions. (B) Nr0b1 and miR-383 were upregulated and downregulated, respectively, in GC-pulse dosed skeletal muscle (quadriceps) and primary myoblasts. (C) Electroporation directly into muscle with targeting oligonucleotides altered miR-383 and Anxa6 levels. (D) miR-383–mimic correlated with worse sarcolemmal injury, whereas anti–miR-383 correlated with smaller injury. (Left) Z-stack rendering of laser-injured sarcolemmal sites at 300 seconds after injury; (center) quantitation of FM4-64 dye accumulation at sarcolemmal site after laser injury; (right) end-point area of injury. (E) Luciferase assay to validate GC-dependent regulation of Anxa6′s 3′ UTR targeting by miR-383 in skeletal muscle. Diagram depicting experimental design after introducing 3′ UTR reporter constructs into muscle followed by *ex vivo* Fluc measurement. miR-383 reduced luminescence, while 3′ UTR without the miR-383–binding site (∆miR-383) increased luminescence. *n* = 5 mice/group (B); *n* = 3 mice (30 myofibers)/group (C–E). \*P < 0.05 vs. vehicle, 1-way ANOVA test with Bonferroni’s multiple comparison; \#P < 0.05 vs. vehicle, 2-way ANOVA test with Bonferroni’s multiple comparison.
To confirm responsiveness of Anxa6 3′ UTR to miR-383 and GCs, we then tested whether mutation of the miR-383 site in Anxa6 3′ UTR would remove steroid-dependent modulation. The Anxa6 3′ UTR site with the intact or ablated miR-383 site (Δ miR-383) was placed downstream of a luciferase (Fluc) reporter gene. We then electroporated the constructs into muscles and evaluated the luminescence levels 24 hours after prednisone pulse. In the presence of the miR-383 site, the prednisone pulse was associated with increased luminescence (Figure 3E). Conversely, ablation of the binding site (Δ miR-383 constructs) induced significantly higher luminescence levels, resulting in no difference between vehicle and prednisone treatments (Figure 3E). Thus, in addition to direct effects of steroids on Anxa6 gene expression, the GC steroid pulse reinforces Anxa6 upregulation by reducing miR-383–dependent targeting of Anxa6 3′ UTR.

**GC steroids improve recovery from muscle injury.** The studies described above demonstrate that GC steroids enhance recovery from laser-induced disruption of the sarcolemma by upregulating annexins. We next assessed whether GC steroids improved recovery from acute focal in vivo injury by injuring muscles using an intramuscular injection of cardiotoxin (27). WT mice were given prednisone or deflazacort i.p. at 1 μg/g body weight 1 day before injury and then given daily or weekly steroids for 14 days (Figure 4A). Injury area and macrophage infiltration were comparably reduced with all steroid regimens (Figure 4, B and C, and Supplemental Figure 2A). Serum creatine kinase (CK) was elevated at 24 hours after injury and was significantly reduced by all steroid treatments (Figure 4D). By 7 days after injury, serum CK was no longer elevated. Injury-associated fibrosis, measured by hydroxyproline content and Masson’s trichrome staining, was variably reduced by all steroid regimens at both 7 and 14 days after injury (Figure 4E). All steroid treatments, including the single predose, reduced inflammation and fibrosis after acute injury. These data indicate that a pulse of GC steroids improves recovery from muscle injury in vivo.

**Daily GC steroid dosing drives atrophy.** Chronic steroid treatment has been associated with muscle atrophy (28). To understand how differential GC dosing altered muscle repair, we compared weekly steroid administration versus daily administration in WT mice after acute injury. We assessed how steroid dosing affected muscle performance and molecular profile. A single weekly steroid dose increased running distance to exhaustion, while daily steroid dosing reduced exercise capacity compared with that of vehicle controls (Figure 5A). Ex vivo muscle performance was monitored in the tibialis anterior muscles. Consistent with in vivo performance, tetanic force was increased in weekly
dosed and decreased in daily dosed mice (Figure 5B). Contraction
time to maximum force was reduced after weekly dosing
and, correspondingly, delayed in muscles from mice that received
daily GC administration (Figure 5B). These outcomes were depen-
dent on repetitive dosing of GCs, since the predose group did not
significantly differ from vehicle controls (Figure 5, A and B). We
evaluated the myofiber cross-sectional area (CSA) in injured mus-
cles. We separately analyzed the CSA of myofibers within the inju-
ry area, as marked by myofibers with centrally placed nuclei, and
those fibers remote from injury area (Supplemental Figure 2C).
While the CSA of centronucleated myofibers did not significantly
differ among groups, the CSA of remote myofibers was increased
after weekly dosing and decreased after daily steroid administra-
tion (Figure 5C). To evaluate systemic effects on glucose metabo-
lism, serum glucose from peripheral blood was measured and did
not change among groups at 14 days after injury (Supplemental
Figure 3A). These data demonstrate that weekly steroid dosing
enhanced muscle recovery from injury. In contrast, daily steroid
dosing impaired muscle performance, indicating that enhanced
sarcolemmal repair was insufficient to overcome atrophy driven
by daily GC steroid dosing.

To better understand the differential effects of steroid-dos-
ing regimens, we examined gene expression and phosphorylated
AKT levels in injured muscles 14 days after cardiotoxin injection.
Both weekly and daily steroid treatments were associated with
increased levels of Anxa1 and Anxa6 expression, accounting for
the reduced injury response (Supplemental Figure 3B). Phosphor-
ylated AKT (Ser473; p-AKT) is a marker of hypertrophic muscle
remodeling and is reduced when muscle mass is lost (29). p-AKT
levels were significantly increased after weekly regimens and
significantly reduced after daily GC dosing (Figure 5D). We ana-
yzed Fbxo32, which encodes atrogin-1 (also known as MAFbx),
an important mediator of muscle atrophy (30, 31). We also moni-
tored Klf15, a transcription factor that interacts with GR pathways
and mediates steroid-dependent ergogenic effects in skeletal
muscle (11, 32). Fbxo32 was upregulated in muscle of daily dosed
mice, paralleling atrophic remodeling, and suppressed by weekly
dosing. Klf15 was suppressed by daily dosing and upregulated in
weekly dosed mice (Figure 5E). Consistent with the expression
pattern of Klf15, genes within its downstream metabolic pathway
were upregulated by weekly administration and downregulated by
daily dosing 14 days after injury (Supplemental Figure 3C).

To confirm that GC-associated molecular changes derived
from myofibers, we isolated the myofiber-enriched fraction from
GC- and vehicle-treated injured muscles 14 days after injury (Sup-
plemental Figure 3D). qPCR of the myofiber fraction recapitulated
the findings observed in whole muscle. Namely, these data showed
that both daily and weekly steroid dosing was sufficient to upreg-
ulate repair pathways, but that daily dosing induced Fbxo32 and
repressed Klf15 (Supplemental Figure 3E). Therefore, although
daily GC steroid dosing improved repair after acute injury, this
beneficial effect was negated by profound atrophic remodeling.
Weekly and daily steroid treatments promote sarcolemmal repair in mdx dystrophic mice. Chronic daily steroid administration is associated with adverse effects including obesity and, in children, growth suppression (33). Studies of prednisone administration in children with DMD have suggested that weekend dosing has comparable beneficial effects compared with daily dosing (34). In the mdx model, weekly dosing was associated with a sustained long-term benefit compared with that in control-treated mice (35). However, there has been limited widespread uptake of weekly steroid regimens, so we investigated the effects of weekly and daily steroid dosing in an animal model of muscular dystrophy. These studies were conducted in 6-month-old mdx male mice on a DBA/2J background, since these mice bear a closer resemblance to human DMD pathology and specifically the age at which steroid dosing is often initiated (36). Mice were given prednisone and deflazacort i.p. at 1 μg/g body weight either weekly or daily, and results were analyzed after 4 weeks of treatment. Sarcolemmal injury and repair of isolated dystrophic myofibers were improved comparably by both steroid-dosing treatments, with both groups showing reduced FM4-64 dye accumulation and area of injury when compared with vehicle (Figure 6A). There were no differences between prednisone and deflazacort, indicating that the types of GC steroid and dosing regimen were comparable in their effect on response to laser-induced sarcolemmal injury.

We correlated steroid-induced repair enhancement with serum CK levels, fibrosis, and immune infiltration within dystrophic skeletal muscle. Serum CK was comparably reduced by all steroid regimens (Supplemental Figure 4A). Fibrotic infiltrates were comparably reduced in gastrocnemius muscles among steroid groups, as compared with vehicle controls (Figure 6B). Quantitation of hydroxyproline levels in quadriceps muscles revealed a significant reduction of hydroxyproline content in all steroid-treated mice compared with vehicle-treated controls (Figure 6C). Inflammation is a prominent feature in many forms of muscular dystrophy, and we found that macrophage infiltration was reduced after all GC regimens (Figure 6D). Diaphragm muscles followed trends similar to those of hind limb muscles, as fibrosis (blue scars; arrows) appeared comparably reduced after all GC regimens (10 replicates). n = 5 mice (50 myofibers)/group. *P < 0.05 vs. vehicle, 1-way ANOVA test with Bonferroni’s multiple comparison; #P < 0.05 vs. vehicle, 2-way ANOVA test with Bonferroni’s multiple comparison.
Daily steroid dosing triggers atrophy, while weekly dosing ameliorates muscular dystrophy features in mdx mice. To monitor for muscle atrophy, body weight, muscle performance, and histopathology were assessed in steroid-treated mdx mice. Body weight significantly declined during daily steroid treatment, while mdx mice receiving weekly steroids did not lose body mass compared with vehicle controls (Figure 7A). Grip strength significantly increased after weekly steroid treatments compared with vehicle controls, while daily dosing resulted in decreased grip strength compared with controls (Figure 7B), although, when results were normalized to body weight, no differences were found among groups (Supplemental Table 1). To further examine muscle performance, mice were subjected to incline treadmill run test until exhaustion. Weekly steroid-injected mdx mice ran further than controls, while daily dosed mice ran less than controls, indicating that weekly and daily treatments had opposing effects on muscle performance (Figure 7B).

These functional changes were reflected in muscle size and fiber type. Myofiber CSA analysis of the gastrocnemius muscle revealed a significant increase in myofiber size after weekly steroid treatments compared with vehicle-treated control. There was a corresponding decrease in myofiber CSA after daily steroid dosing compared with vehicle-treated muscles (Figure 7C). Myofiber typing of quadriceps muscle revealed that weekly steroid treatment was associated with an expansion of fast glycolytic type 2B fibers and a decrease in the number of fast oxidative type 2A, while daily steroid treatments correlated with the opposite trend compared with vehicle (Supplemental Figure 4, B and C). Moreover, daily steroids induced a shift of type 2A myofiber CSA toward lower values compared with weekly dosed and vehicle treated. Conversely, weekly dosing shifted toward larger type 2B myofibers. However, these trends were not significant when fiber type CSA was compared across treatments for both variables (Supplemental Figure 4D).

Force, fatigue, and contraction/relaxation time analyses were analyzed in tibialis anterior muscles in live, anesthetized mice. Tetanic force increased after weekly steroid treatment compared with vehicle controls (Figure 6D). Fibrosis was reduced by all GC regimens in the diaphragm muscle (Figure 6E), which was analogous to what occurred in hind limb muscles. Thus, both weekly and daily steroid treatments enhanced sarcolemmal repair and reduced fibrosis and immune cell infiltration to a comparable extent in dystrophic muscles.

Daily steroid dosing elicits atrophic mdx skeletal muscles, while weekly GC dosing does not. (A) Daily, but not weekly, GC steroid dosing caused loss of body mass. (B) Weekly GC steroid dosing enhanced grip strength and run-to-exhaustion performance, while daily GC steroid-dosing treatments correlated with reduced grip strength and run performance. (C) Myofiber CSA was increased after weekly, but decreased after daily GC administration in gastrocnemius muscle sections. (D) Max tetanic force of tibialis anterior muscles was increased after weekly treatments and decreased after daily GC administration. (E) Fatigue analysis showed that tetanic force was increased over consecutive contraction bouts after weekly dosing, while daily dosing induced opposite trends. (F) Weekly GC regimen correlated with improved respiratory function, as assessed by WBP. Minute volume was increased and inspiration time was decreased in weekly GC dosing. Daily dosing reversed these beneficial trends. (G) Weekly GC treatments promoted increased CSA and diaphragm thickness, while daily GC dosing reduced CSA and diaphragm thickness. n = 5 mice/group. *P < 0.05 vs. mdx vehicle, 1-way ANOVA test with Bonferroni’s multiple comparison; #P < 0.05 vs. mdx vehicle, 2-way ANOVA test with Bonferroni’s multiple comparison.
Resting was measured utilizing whole-body plethysmography (WBP) on mice by daily steroid dosing. This treatment triggered atrophic remodeling. These data indicate that dysregulation of dystrophic muscle, like normal muscle, benefits from weekly dosing, with lower relative resistance to fatigue. Conversely, daily steroid treatment reduced tetanic force compared with vehicle control. Time of inspiration was decreased in weekly GC-treated mice and increased in daily GC-treated mice (Figure 7F). Myofiber CSA and transverse diaphragm muscle thickness were increased after weekly GC injection and decreased after daily GC administration (Figure 7G). Thus, similarly to what occurred in hind limb muscles, weekly GC treatment resulted in improved respiratory function and diaphragm pathology, while daily GC treatment correlated with atrophy, irrespective of reduced fibrosis.

We monitored heart fibrosis and blood pressure in mdx mice to assess possible effects of GC steroid treatments. Histologic assessment of left and right ventricle walls showed that all GC treatments correlated with decreased fibrosis in both ventricle walls, paralleling the effects observed in muscle (Supplemental Figure 5A). Myocardial fibrosis associates with increased ventricle strain (38). Left ventricle strain was assessed by echocardiography. Consistent with the reduction in fibrosis, longitudinal strain and circumferential strain were reduced in all steroid-treated mice compared with vehicle-treated mice, with no significant changes recorded between weekly and daily regimens (Supplemental Figure 5B). Systolic and diastolic blood pressure, although variable within each cohort, did not significantly change with treatment (Supplemental Figure 5C). Thus, fibrosis and strain were comparably reduced in dystrophic ventricles after 4 weeks of either weekly or daily GC treatments, with no significant effects on blood pressure.

Gene regulation is associated with regimen-specific remodeling of dystrophic muscle. We next interrogated dystrophic muscles of treated and control mice for expression levels of candidate genes involved in sarcolemmal repair and atrophy. We assessed the expression of specific genes from whole muscle tissue as well as the isolated myofiber fractions from vehicle and weekly and daily GC steroid regimens. As in WT muscle, Anxa1 and Anxa6 were upregulated by all GC regimens (Supplemental Figure 6A). Fbxo32 expression levels were significantly higher after daily steroid treatments and lower after weekly steroid treatments, while Klf15 levels were increased by weekly steroids and suppressed by daily steroid treatment (Supplemental Figure 6A).

To gain further insight into differential regulation of these genes, we examined chromatin marks and GR occupancy on the GREs of Anxa1, Anxa6, and Klf15. Studies were conducted at treatment end point using the myofiber fraction of vehicle and treated mdx muscles. We monitored genomic elements for enrichment in the repressive histone mark histone-3 tri-methyl-lysine-9.
a GC dose–dependent increase in NR3C1 occupancy, which was significantly higher after daily dosing than weekly dosing (Figure 8B). Taken together, the expression and chromatin data demonstrate that GC administration, irrespective of dosing, correlated with positive transcriptional activation of Anxa1 and Anxa6. Klf15 chromatin marks followed regimen-specific patterns consistent with overall gene expression changes.

**Transcriptomic profile of prednisone-treated mdx muscles.** We conducted broad transcriptional profiling of quadriceps muscles from the mdx mice cohorts treated with daily or weekly prednisone using RNA sequencing (RNA-seq) (n = 5 mice/group). Unbiased hierarchical clustering based on differentially expressed genes also separated samples in regimen-specific groups. Analysis of differentially expressed genes in paired comparisons for enriched GO terms. Highlighted are GO term groups of interest. Both daily and weekly GC steroids enriched for GO terms of membrane repair, growth regulation, immune response, and steroid metabolism. Weekly versus daily GC steroids only enriched for steroid metabolism. (D–F) Heat maps of annotated gene lists. Green denotes higher values, while red indicates lower values of gene mRNA quantitation, as compared with row mean value. n = 5 mice/group. (B–F) Adjusted P value < 0.05 for all genes and GO terms was reported using edgeR differential expression (treated vs. vehicle paired comparisons) and Panther GO enrichment tests (details in Supplemental Methods).
ased clustering of samples using principal component analysis segregated transcriptional profiles according to dosing regimen (Figure 9A and Supplemental Data Set 1). Unsupervised hierarchical clustering confirmed clustering based on dosing regimen (Figure 9B). The differential gene subsets from each binary comparison (vehicle versus weekly, vehicle versus daily, weekly versus daily) were analyzed for enrichment of gene ontology (GO) terms of interest. Among all enriched GO terms, GO terms related to membrane repair, tissue growth, immune response, and steroid metabolism were significantly enriched when comparing prednisone-treated muscles with vehicle-treated muscles (Figure 9C; Supplemental Data Set 2). When comparing weekly versus daily treated muscle to vehicle-treated muscle for the GO terms listed above, only the GO terms related to steroid metabolism were significantly enriched (Figure 9C; Supplemental Data Set 2). Thus, both weekly and daily prednisone regimens induced gene expression changes in membrane repair and tissue growth pathways in muscle. However, the metabolic response to steroids differed between weekly and daily GC administration.

Heat maps of gene-relative increases in sequencing reads (reads per kilobase of transcript per million reads [RPKM]) showed that sarcolemmal repair genes, including Anxa1 and Anxa6, were upregulated in both GC-treated muscles, as compared with vehicle-treated muscles (Figure 9D; Supplemental Data Set 3). Genes associated with muscle atrophy were generally downregulated in weekly treated, but upregulated in daily treated muscles, including Fbxo32 (Figure 9E; Supplemental Data Set 3) (40). Conversely, genes associated with the KLF15 pathway were upregulated after weekly administration, but downregulated after daily dosing, as compared with vehicle-treated muscles (Figure 9F; Supplemental Data Set 3). Thus, the regimen-associated trends in sarcolemmal repair and muscle remodeling were recapitulated in the whole-muscle transcriptome analysis.

We compared profiles from weekly versus daily treated mdx muscle. No significant changes in Anxa1 and Anxa6 transcript abundance were seen between weekly and daily dosing. Compared with weekly dosing, daily prednisone administration was associated with upregulation of Fbxo32 and Mstn, encoding the negative regulator of muscle mass myostatin (Supplemental Figure 7; Supplemental Data Set 4) (41). Conversely, weekly GC dosing was associated with upregulation of Klf15 and, to a lesser extent, Ifgl. Thus, weekly dosing activates key aspects of the beneficial effects of steroids in muscle and recovery from injury without triggering atrophy.

Discussion

**GC steroids improve muscle repair after injury.** Skeletal muscle myofibers are susceptible to plasma membrane disruption, likely due to elongated shape and contraction-induced stress (42). Here, we report direct effects of GC steroids on sarcolemmal repair. We evaluated both prednisone and deflazacort and found that both stimulated annexin gene expression equally. Although it has been suggested that the properties of deflazacort may be associated with fewer metabolic adverse effects (43), we found little difference between prednisone and deflazacort in their ability to improve recovery from injury. A single pulse of GC steroids correlated with upregulation of annexin genes in muscle, faster formation of the annexin repair cap, and faster recovery of annexins to the injury site. GC treatment, whether daily or weekly, reduced the extent of injury in both WT and dystrophic muscles. GC steroids improved sarcolemmal repair not only in muscular dystrophy, but also after acute muscle injury in normal muscle.

Notably, GC steroid treatment in dystrophic muscle upregulated many genes encoding proteins implicated in muscle repair (16); thus, the effect of GC steroids on recovery from injury is not just limited to annexin gene expression. Transcriptome analysis showed that genes including cavelolin 3 (Cav3), myoferlin (Mfyf), dysferlin (Dysf), and Trim72 (encoding MG53) were all increased (19, 44, 45). More refined studies are needed to shed light on whether transcriptional regulation of those genes is directly stimulated by GCs or is an indirect effect of enhanced muscle repair. It is also important to consider that other molecular pathways likely contribute to improving repair. For example, GC steroids are known to alter membrane lipid composition, and this altered composition may facilitate repair after injury (46).

**Daily steroid dosing elicits atrophy, while weekly dosing does not.** Weekly dosing of GC steroids improved muscle recovery from injury in both the acute setting of normal muscle and the chronic setting of muscular dystrophy. We did not observe significant differences in the type of CG steroid in that both prednisone and deflazacort yielded similar results in repair assays. A recently published phase III clinical trial found comparable effects on the muscle strength of boys with DMD treated with prednisone and deflazacort over 12 weeks, using dose ranges that were comparable to those used in our study, although prednisone was associated with more pronounced weight gain than deflazacort (12). Both weekly and daily steroid regimens induced a comparable reduction in fibrosis and ventricle strain in the heart of treated mice. Additional studies are needed, as GC steroid effects in DMD cardiomyopathy are debated, with contrasting reports of beneficial effects in DMD patients and detrimental effects in DMD models (47, 48). In skeletal muscle, molecular pathways of atrophy were triggered by daily dosing and this was accompanied by reduced performance of voluntary hind limb and respiratory skeletal muscles. In contrast, weekly dosing did not trigger atrophy and instead was associated with improved muscle performance.

Weekly GC treatment increased Klf15, while daily dosing suppressed Klf15. KLF15 is a known target of GR (32), and loss of Klf15 is known to exacerbate the skeletal muscle phenotype in mdx mice (11). Muscle-specific upregulation of KLF15 was shown to improve muscle performance in mdx mice, and it is known that KLF15 regulates not only glucose and amino acid use, but also lipid flux in muscle (11, 49). The striking divergent Klf15 transcriptional pattern elicited from weekly versus daily GC steroid dosing was reflected in a similar pattern of KLF15-regulated genes, seen in both candidate gene expression and in broad transcriptional profiling. KLF15 has also been implicated in diurnal regulation of gene expression in the heart (50), and so it is notable that all injections in this study were performed at 7 am, when mice have naturally low levels of cortisol (51). This pattern is the opposite of that in humans, where the highest peak of cortisol is seen in the early morning (52), and so in translating this finding to humans, time-of-day dosing may be relevant.

Ergogenic factors such as KLF15 improve muscle performance through a number of mechanisms, including substrate use (49).
In this work, we extend this concept to gene-encoding proteins essential for repair. Thus, an important aspect of muscle performance includes the ability of muscle to recover from injury, including exercise-induced injury. In addition to weekly GC dosing increasing Klf15, upregulation of Igf1 and Mef2a was also seen. Thus, efficient ergogenesis likely relies on fuel substrates, growth, and sarcolemmal repair processes.

Regimen-associated divergence in elicited muscle response. Klf15 is a direct target of the GR (52, 53–55). We found divergent epigenetic chromatin marks on the Klf15 GRE that paralleled the change in gene expression elicited by differential GC steroid dosing. Interestingly, GR (NR3C1) occupancy demonstrated a dose-dependent difference in occupancy of the Klf15 promoter, suggesting that the level of GR occupancy elicits differential effects. Muscle-specific deletion of the GR results in larger muscle mass and a reduction in adipose tissue, demonstrating crosstalk between these tissues (56). Furthermore, mTOR can counterbalance GR effects in muscle and thus may also represent a means of subverting aspects of the atrophy pathway triggered by daily steroid dosing (55).

In summary, our data indicate that intermittent weekly dosing of GC steroids better harnesses the dual beneficial effects on skeletal muscle, i.e., (a) improved sarcolemmal repair and (b) ergogenic remodeling. When chronic treatment is required, weekly dosing was associated with benefit without driving atrophic pathways. Furthermore, single pulse dosing of GC steroids in normal muscle improved repair by increasing repair pathways in muscle, indicating that repair pathways are relevant for the ergogenic process of both normal and diseased muscle.

Methods

Animals. The WT mice on a 129T2/SvEmsJ background were used for studies involving acute injury protocols. The mdx mice on a DBA/2J background were obtained from The Jackson Laboratory (stock 013141) and interbred. Male mice were used for reported experiments. Mice were housed in a specific pathogen–free facility.

Plasmids. GFP-tagged annexins A1 and A6 were described previously (14, 16). The 3′ UTR of Anxa6 was PCR cloned from cDNA from 129T2/SvEmsJ skeletal muscle and ligated into the pmirGlo plasmid (catalog 1330, Promega) using the ScaI-Xhol sites; the mR-383–binding site (5′-CUGCAUUCACUCUGAUC-3′) was ablated by means of a Phusion Site-Directed Mutagenesis Kit (catalog F541, Thermo Scientific). Luciferase plasmids containing promoter fragments were obtained by cloning genomic sequences into pGL4.23 (E8411, Promega) using the KpmI-Xhol sites. Cloned genomic sequences from 129T2/SvEmsJ mice were used for GRE/FOXO site analysis (Supplemental Table 2).

Electroporation, myofiber isolation, and laser injury. FDB fibers were transfected by in vivo electroporation. Methods were as described previously (57) with modifications also described (58). For quantitative analysis of FM dye, fluorescence was measured at the site of injury in individual fibers using ImageJ (NIH) and adjusted to baseline fluorescence at time 0 calculated at the membrane prior to damage (F/F0). This method allows comparisons of all strains and reduces variability introduced by differences in dye uptake or binding at time 0. Analysis was conducted with the investigator blinded to treatment group.

Drug treatments. Prednisone, deflazacort, and eplerenone (catalog P6254, SML0123, E6657, Sigma-Aldrich) were resuspended in DMSO (catalog D2650, Sigma-Aldrich) at 5 mg/ml, 5 mg/ml, and 100 mg/ml, respectively. Dosing was based on pretreatment weights (1 mg/kg body weight; ref. 20) in 30 μl total PBS volume for daily treatments and in 210 μl total saline volume for weekly treatments. Mice were injected daily via i.p. injection at 7 am. On injection days, stock solutions stored at -20°C were diluted into sterile Eppendorf tubes containing sterile PBS (catalog 14190, Life Technologies). Sterile BD Micro-Fine IV Insulin Syringes (catalog 14-829-1A, Fisher Scientific) were used to inject the i.p. cavity of nonsestated animals. Mice were weighed 3 times per week over the course of 4 weeks at 7 am. Body mass was expressed as fold change from day 0. Cardiotoxin injury was performed injecting 20 μl of a 10 μM cardiotoxin (discontinued, Sigma-Aldrich) solution in PBS in target muscles in sedated animals (3% isoflurane, 0.8 l/min O2). Cardiotoxin was released in the center of the muscle through the whole major axis in order to have a homogenous area of injury at the center of the muscle.

Grip strength, treadmill, muscle mechanics, blood drawing, and plethysmography. Forelimb grip strength was monitored using a meter (catalog 1027SM, Columbus Instruments) with the investigator blinded to treatment group. Animals performed 10 pulls with 5 seconds rest on a flat surface between pulls. Mice were run on a treadmill (Exer3/6 without electrical stimulation grills, Columbus Instruments) with a 15° incline with a start speed of 1 m/min with 1 m/min2 acceleration. The assay was interrupted if the animal stopped for longer than 15 seconds on the rest pad. Immediately before sacrifice, in situ tetanic force from tibialis anterior muscle was measured using a Whole Mouse Test System (catalog 1300A, Aurora Scientific) with a 1 N dual-action lever arm force transducer (300C-LR, Aurora Scientific) in anesthetized animals (3% isoflurane in 100% O2). Tetanic isometric contraction was induced with the following specifications: initial delay, 0.1 seconds; frequency, 200 Hz; pulse width, 0.5 msec; duration, 0.5 seconds; using 100 mA stimulation (59). Length was adjusted to a fixed baseline of 30 mN resting tension for all muscles/conditions. Fatigue analysis was conducted by repeating tetanic contractions every 10 seconds until complete exhaustion of the muscle (50 cycles). Time of contraction was assessed as time to maximum tetanic value within the 0.0–0.5 second range of each tetanic contraction, while time of relaxation was assessed as time to 90% minimum tetanic value within the 0.5–0.8 second range of every tetanus.

Unanesthetized WBP was used to measure respiratory function using a Buxco FinePointe 4-site apparatus (Data Sciences International). Individual mice were placed in a calibrated cylindrical chamber at room temperature. Each mouse was allowed to acclimate to the plethysmography chamber for 120 minutes before recording was initiated. Data were recorded for a total of 15 minutes broken into 3 consecutive 5-minute periods. All physiological studies were conducted by investigators blinded to treatment group.

Histology, immunofluorescence microscopy, and antibodies. Microscope detection used anti-F4/80 conjugated to Alexa Fluor 488 (catalog ab6640, Abcam) at a dilution of 1:100 overnight at 4°C; nuclei were counterstained with 0.5 μg/ml Hoechst PBS (45 minutes, room temperature). For fiber typing, sections were incubated with primary antibodies BA-F8 (1:10), SC-71 (1:30), and BF-F3 (1:10, all by Developmental Studies Hybridoma Bank) overnight at 4°C. Then sections were incubated with secondary antibodies Alexa Fluor 350 anti-IgG2b, Alexa Fluor 488 anti-IgG1, and Alexa Fluor 594 anti-IgM (catalog A21140, A21121, 1010111, Life Technologies). Type 1 fibers were stained blue, type 2A stained green, and type 2B stained red (60).
Imaging was performed with a Zeiss Axio Observer A1 microscope, using 10× and 20× objectives. Brightfield pictures were acquired via Gryphax software (version 1.0.6.598, Jenoptik), while immunofluorescence pictures were acquired via ZEN 2 software (version 2011, Zeiss). Area quantitation, length measurement, and cell counting were performed by means of ImageJ (NIH).

Hydroxyproline quantification. Hydroxyproline content was measured on 100 mg portions of frozen quadriceps muscles as previously described (61). Results were reported as nM hydroxyproline/mg (tissue).

Serum collection and CK analysis. After completion of the physiological analyses, serum was acquired and processed as previously described (19). Serum CK was analyzed in triplicate for each mouse using the EnzyChrom Creatine Kinase Assay (catalog ECPK-100, BioAssay Systems) following the manufacturer’s instructions. Results were acquired with the Synergy HTX Multi-Mode Plate Reader (BioTek). Results are expressed as U/ml.

Transcription factor-binding site prediction. Prediction of transcription factor-binding sites was conducted by interrogating genomic sequences of query genes (UCSC, mm 10 assembly, including 10 kb upstream of TSS) for predicted binding sequences as reported by the JASPAR database and described previously (62). Alignments were conducted in both strand directions with ApE software (v2.0.47, M.W. Davis, University of Utah, Salt Lake City, Utah, USA). One mismatch in noncritical positions, i.e., less than 95% conserved, was allowed. Prediction of miRNA-binding sites was conducted using the miR-16

Quantitative RT-PCR. Gene expression analysis was conducted on total RNA extracted with Trizol (catalog 15596018, Life Technologies) from 30 mg tissue per the manufacturer’s instructions. RNA from the myofiber fraction was obtained after separating muscle tissue in myofiber- and mononuclear-enriched fractions as follows. Whole muscle (tibialis anterior) was cut in approximately 2-mm–thick myofiber bundles, following the natural myofiber orientation. Bundles were then incubated in collagenase II and gently mechanically dissociated, following the conditions used for myofiber isolation from FDB muscles (as detailed above). The mononuclear-enriched fraction was filtered out through a 40-μm cell strainer (catalog 22363547, Fisher Scientific), while the myofiber-enriched fraction was recovered as the unfiltered fraction and further processed with Trizol. Efficient separation of the 2 fractions was confirmed via microscopy and qPCR of dedicated distinctive markers, and 2 μg of RNA was reverse transcribed by means of the qScript cDNA Kit (catalog 95048, Quanta Biosciences) following the manufacturer’s instructions. cDNA was diluted 1:7, and 2 μl was used per 10 μl qPCR reaction. Each qPCR reaction contained 100 nM primers and 5 μl iTaq SYBR Green Mix (catalog 1725124, Bio-Rad). A detailed list of primers and sequences is provided in Supplemental Table 3. Fluorescence was quantitated using the CFX96 Real-Time System (Bio-Rad). miR-16 was used as internal normalizer.

miRNA expression studies. miRNA expression analyses were performed from total RNA from tissue. miRNAs were reverse transcribed by means of the TaqMan MicroRNA Reverse Transcription Kit (catalog 4366596, Life Technologies) following the manufacturer’s protocol and using 4 μg of total tissue RNA. cDNA was diluted 1:15, and 2 μl was used per 10 μl qPCR reaction. Each qPCR reaction contained 100 nM primers and 5 μl of TaqMan Master Mix (catalog 4427788, Life Technologies). Both reverse transcription and qPCR reactions were conducted using miRNA-specific TaqMan Assays (catalog 4427975, Life Technologies). Fluorescence was quantitated using the CFX96 Real-Time System (Bio-Rad). miR-16 was used as internal control.

ChIP-qPCR and luciferase assays. ChIP-qPCR was performed according to a previously reported protocol (64) and adjusted conditions (59). Results were expressed as percentages of raw expression of the respective input. ChIP-qPCR assays on isolated myofibers were performed as above, with the following steps before chromatin sonication. Freshly isolated whole gastrocnemius muscles were finely minced and digested in 5 ml/muscle of PBS supplemented with 1 mM CaCl2 and 100 U/ml collagenase II (catalog 17101, Life Technologies) at 37°C for 1 hour with shaking. The suspension was then filtered through a 40-μm strainer (catalog 22363547, Fisher Scientific) and the unfiltered fraction (enriched in myofibers) was kept for further steps. Separation of mononuclear fraction in the filtered fraction was confirmed at the microscope. Myofibers were lysed in lysis buffer, using 700 μl per muscle, with approximately 250 μl 2.3-mm zirconia/silica beads (catalog 11079125z, BioSpec). Lysis buffer consisted of 10 mM HEPES (pH 7.3, catalog H3375), 10 mM KCl (catalog P9541), 5 mM MgCl2 (catalog M8266), 0.5 mM DTT (catalog 646563), and 3 μg/ml cytochalasin B (C6762, all reagents from Sigma-Aldrich) and protease inhibitor cocktail (catalog 11852700, Roche). Myofibers were them homogenized by means of Mini-BeadBeater-16 (catalog 607, Biospec) for 30 seconds, then by rotating at 4°C for 30 minutes. Samples were centrifuged at 3,000 g for 5 minutes at 4°C; supernatant was removed. The pellet was resuspended in lysis buffer supplemented with 3 μg/ml cytochalasin B, as per reported conditions (64), and incubated on ice for 10 minutes. Nuclei were pelleted at 300 g for 10 minutes at 4°C and resuspended in 1 ml 1% PFA for 5 minutes at room temperature. Fixation was quenched with 100 μl of 1.375 M glycine (catalog BP381-5, Fisher Scientific). Nuclei were repelleted as before and subsequently processed following reported protocol (64) and the procedures mentioned for myoblasts, with the adjustment of adding 3 μg/ml cytochalasin B into all solutions for chromatin preparation and sonication, antibody incubation, and wash steps.

Ex vivo luciferase assay was performed on whole, electroporated FDB muscles. Muscles were minced and homogenized in the lysate buffer, and experiments were performed according to the Dual Luciferase Assay Kit (catalog 1910, Promega) instructions. Luminescence was recorded using the Synergy HTX Multi-Mode 96-Well Plate Reader (BioTek). Raw values were normalized to Renilla luciferase, then to protein content (MyHC), and finally either to empty pGL4.23 vector (unregulated negative control) and to vehicle-treated muscles with the same plasmids (Figure 3) or to pMiR empty vector-transfected muscles (unregulated, positive control) and to vehicle-treated muscles with the same plasmids (Figure 4) or to pMiR empty vector-transfected muscles (unregulated, positive control) and to vehicle-treated muscles with the same plasmids (Figure 4) or to pMiR empty vector-transfected muscles (unregulated, positive control). For promoter site analysis, raw values were normalized to Renilla luciferase, protein content (MyHC), and empty pGL4.23-transfected muscles. Results are expressed as fold change to average vehicle.

RNA-seq analysis. A detailed explanation of RNA-seq protocols and analyses can be found in Supplemental Methods. All original microarray data were deposited in the NCBI’s Gene Expression Omnibus (GSE95682).

Primary myoblast isolation. Primary myoblasts were sorted as CD56+ cells from the mononuclear preparation obtained from tibialis anterior muscles, adapting previously reported conditions (22).

Statistics. Statistical analyses were performed with Prism (GraphPad). Most comparisons relied on ANOVA with Bonferroni’s multi-comparison appropriate for the variables tested (1-way ANOVA for 1


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