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Authors

Morrison-Nozik, Alexander
Anand, Priti
Zhu, Han
et al.

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Glucocorticoids enhance muscle endurance and ameliorate Duchenne muscular dystrophy through a defined metabolic program

Alexander Morrison-Nozik[a], Priti Anand[a,b], Han Zhu[a], Qiming Duan[a,b], Mohamad Sabeh[a,c], Domenick A. Prosdocimo[a], Madeleine E. Lemieux[d], Nikolai Nordsborg[e], Aaron P. Russell[f], Calum A. MacRae[g], Anthony N. Gerber[h], Mukesh K. Jain[a,c], and Saptarsi M. Haldar[a,b,c,i,1]

[a]Case Cardiovascular Research Institute, Department of Medicine, Case Western Reserve University School of Medicine, Cleveland, OH 44106; [b]Gladstone Institutes, San Francisco, CA 94158; [c]Harrington Heart & Vascular Institute, University Hospitals Case Medical Center, Cleveland, OH 44106; [d]Bioinfo, Plantagenet, Ontario, Canada K0B IL0; [e]Department of Nutrition, Exercise and Sports Sciences, University of Copenhagen, DK-200 Copenhagen, Denmark; [f]Centre for Physical Activity and Nutrition Research, School of Exercise and Nutrition Science, Deakin University, Burwood, VIC 3125, Australia; [g]Cardiovascular Division, Brigham & Women's Hospital, Harvard Medical School, Boston, MA 02115; [h]Department of Pulmonary Medicine, National Jewish Health and University of Colorado Denver School of Medicine, Denver, CO 80206; and [i]Department of Medicine and Cardiovascular Research Institute, University of California, San Francisco, CA 94158

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Classic physiology studies dating to the 1930s demonstrate that moderate or transient glucocorticoid (GC) exposure improves muscle performance. The ergogenic properties of GCs are further evidenced by their surreptitious use as doping agents by endurance athletes and poorly understood efficacy in Duchenne muscular dystrophy (DMD), a genetic muscle-wasting disease. A defined molecular basis underlying these performance-enhancing properties of GCs in skeletal muscle remains obscure. Here, we demonstrate that ergogenic effects of GCs are mediated by direct induction of the metabolic transcription factor KLF15, defining a downstream pathway distinct from that resulting in GC-related muscle atrophy. Furthermore, we establish that KLF15 deficiency exacerbates dystrophic severity and muscle GC–KLF15 signaling mediates salutary therapeutic effects in the *mdx* mouse model of DMD. Thus, although glucocorticoid receptor (GR)-mediated transactivation is often associated with muscle atrophy and other adverse effects of pharmacologic GC administration, our data define a distinct GR-induced gene regulatory pathway that contributes to therapeutic effects of GCs in DMD through proergogenic metabolic programming.

skeletal muscle metabolism | glucocorticoid | exercise | Duchenne muscular dystrophy | steroid hormone nuclear receptor

Synthetic derivatives of the glucocorticoid (GC) class of steroid hormones, which are ligands for the nuclear receptor NR3C1 (also known as the glucocorticoid receptor; GR), are widely used as antiinflammatory drugs (1). The vast majority of literature on muscle GR signaling has focused on muscle wasting, a side effect of excessive or sustained GC exposure that is mediated, in part, by direct GR-dependent transactivation of genes that drive myocyte atrophy (e.g., Foxo3a, Gdf8/Myostatin, Fbxo32/Atrogin1; also known as atrogenes) (1, 2). However, literature dating back to the 1930s, including the classic physiological studies from the laboratory of Dwight Ingle (3, 4), have documented that moderate or transient exposure to GCs can enhance muscle performance and produce ergogenic effects in animals and humans (5–13). Consistent with this known physiological role of GCs in anticipatory metabolic adaptation, surreptitious GC ingestion is a well-known doping strategy used by elite endurance athletes, an act that has prompted disqualifications and has led to the universal banning of these drugs by sports regulatory agencies (14). Although mechanisms governing GC-mediated muscle atrophy have been extensively studied (1, 2), the molecular basis for their ergogenic effects remains poorly understood.

In addition to these ergogenic physiological effects, low-dose GC therapy also improves muscle function, quality of life, and survival in patients with Duchenne muscular dystrophy (DMD) (15–17), a progressive muscle-wasting disease caused by X-linked inheritance of nonsense mutations in the gene encoding Dystrophin (18). Although GCs have been widely used in the treatment of DMD for nearly 25 years (15), the mechanism of action underlying their salutary effects in this condition remains obscure. Several studies in patients and animal models have called into question whether the antiinflammatory properties of GCs adequately explain their therapeutic effect in DMD (15, 19). We hypothesized that the ergogenic properties of GCs and aspects of their therapeutic efficacy in DMD might be mediated, in part, by a defined GR-dependent metabolic transcriptional pathway distinct from that resulting in GC-related muscle atrophy. As GCs cause a myriad of systemic side effects that limit their therapeutic index in DMD, elucidation of such a downstream signaling pathway could inform novel steroid-sparing treatment strategies, a major unmet need for patients suffering from this devastating and currently incurable disease (15, 20).

Significance

Classic physiological studies have documented the endurance-promoting effects of glucocorticoid (GC) hormones on skeletal muscle. Pharmacologic GC therapy also improves muscle function in patients with Duchenne muscular dystrophy (DMD), a genetic muscle-wasting disease. Despite these well-established physiological and clinical observations, the molecular basis underlying the beneficial effects of GCs in skeletal muscle remains obscure. This study shows that physiological effects of GCs on muscle endurance and their therapeutic effect in DMD are mediated, in part, via activation of a potent metabolic gene called Kruppel-like factor 15 (KLF15). Importantly, KLF15 does not drive GC-mediated muscle wasting. These data shed light on the poorly understood ergogenic properties of GCs, findings that may inform steroid-sparing therapies for DMD and other muscle diseases.

Author contributions: A.M.-N., P.A., H.Z., C.A.M., A.N.G., and S.M.H. designed research; A.M.-N., P.A., H.Z., Q.D., M.S., and D.A.P. performed research; N.N., A.P.R., C.A.M., M.K.J., and S.M.H. contributed new reagents/analytic tools; A.M.-N., P.A., H.Z., M.E.L., A.N.G., and S.M.H. analyzed data; and A.M.-N., A.N.G., and S.M.H. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE74625).

[1]To whom correspondence should be addressed. Email: saptarsi.haldar@gladstone.ucsf.edu.

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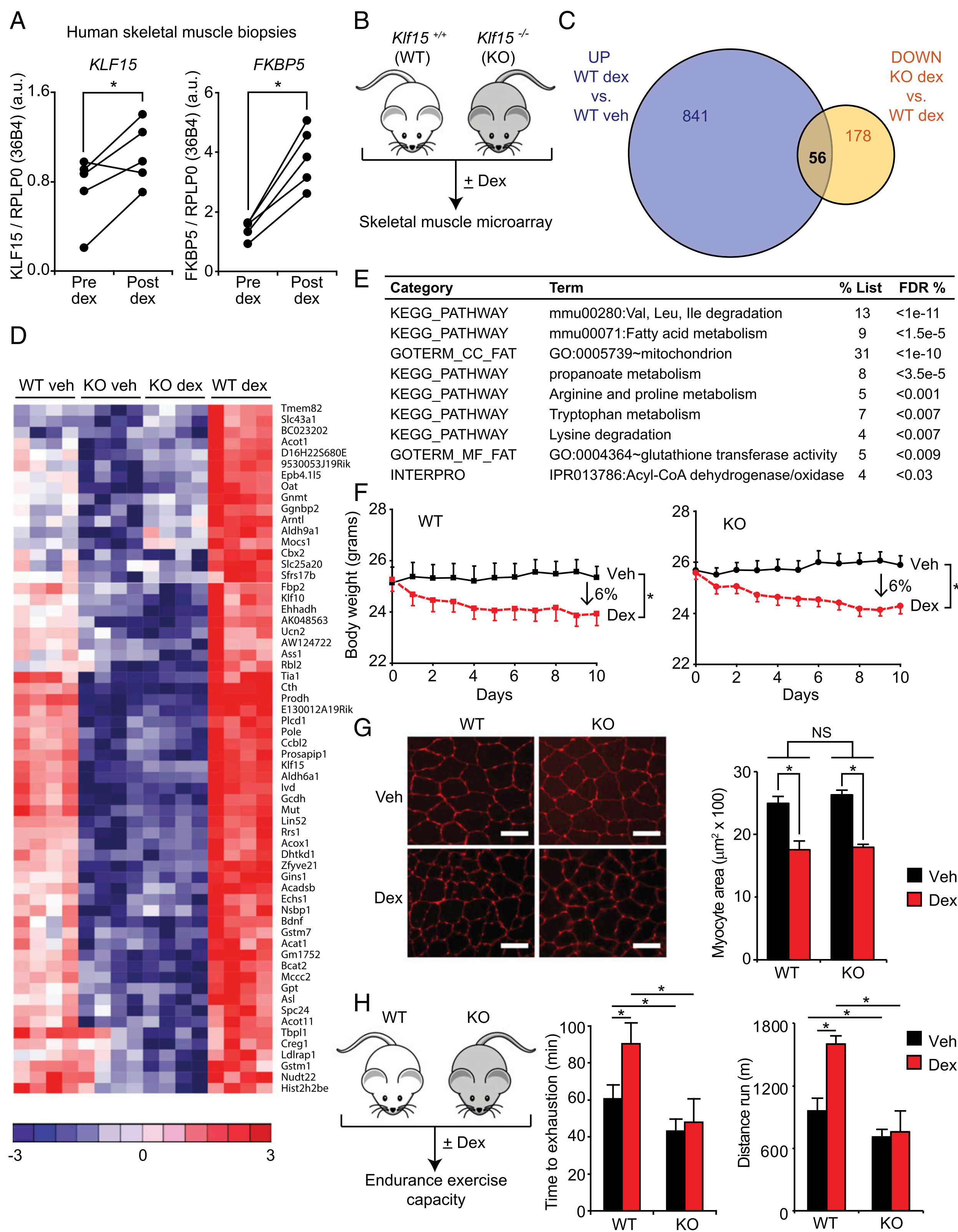


Category	Term	% List	FDR %
KEGG_PATHWAY	mmu00280:Val, Leu, Ile degradation	13	<1e-11
KEGG_PATHWAY	mmu00071:Fatty acid metabolism	9	<1.5e-5
GOTERM_CC_FAT	GO:0005739~mitochondrion	31	<1e-10
KEGG_PATHWAY	propanoate metabolism	8	<3.5e-5
KEGG_PATHWAY	Arginine and proline metabolism	5	<0.001
KEGG_PATHWAY	Tryptophan metabolism	7	<0.007
KEGG_PATHWAY	Lysine degradation	4	<0.007
GOTERM_MF_FAT	GO:0004364~glutathione transferase activity	5	<0.009
INTERPRO	IPR013786:Acyl-CoA dehydrogenase/oxidase	4	<0.03

Fig. 1. The GC–KLF15 axis dissociates ergogenic physiology from muscle atrophy. (*A*) *KLF15* expression in skeletal muscle of healthy human subjects pre- and postdex administration. *FKBP5* is a known direct GR target shown as a positive control (n = 5; *P < 0.05 for indicated comparisons). (*B*) Schematic for skeletal muscle (quadriceps) microarray study (n = 4 per group). (*C*) Venn diagram of dex-inducible and KLF15-regulated genes [2 mg/kg i.p. × 1 dose, 8 h time point; fold change > 1.5 and false discovery rate (FDR) < 0.05]. (*D*) Heat map of genes robustly regulated [family-wise error rate (FWER) < 0.05] by GC–KLF15 axis in mouse skeletal muscle. (*E*) Functional annotation [Database for Annotation, Visualization, and Integrated Discovery (DAVID)] of genes regulated by GC–KLF15 axis. (*F*) Body weight and (*G*) muscle (tibialis anterior) histology [wheat-germ agglutinin (WGA) stain] and cross-sectional area in a mouse model of chronic dex-induced atrophy (1 mg/kg·d s.c. × 10 d; n = 8; *P < 0.05 for indicated comparisons). (Scale bar, 50 μm.) (*H*) Schematic of dex-induced doping experiment (2 mg/kg i.p. × 1 dose) with time to exhaustion and distance run on a motorized treadmill 18 h post-dex administration (n = 7–9; *P < 0.05 for indicated comparisons). Data are shown as mean ± SEM.

The transcription factor KLF15, a direct GR-inducible target in multiple cell types (21, 22), is an attractive candidate for mediating important ergogenic effects of GCs in muscle. Studies from our group using systemic KLF15-deficient mice have demonstrated KLF15 to be an important transcriptional regulator of a gene program governing stress-dependent metabolic adaptation in muscle (23, 24). KLF15-deficient mice have impaired ability to catabolize muscle branched-chain amino acids as fuel for gluconeogenic flux during fasting (25, 26). In addition, we have demonstrated that during sustained aerobic exercise, KLF15-deficient mice fail to augment muscle lipid utilization and consequently have impaired endurance exercise capacity (23). Our detailed characterization of KLF15-deficient mice has revealed that these functional defects in muscle substrate flux occur without abnormalities in muscle development, gross histologic structure, fiber type distribution, mitochondrial number, or muscle mass (23).

Although KLF15 is a direct GR target (21, 22, 27, 28), the function of the GC–KLF15 axis in physiology and disease remains largely unknown. The observation that GCs induce KLF15 in rodent myocytes and that adenoviral KLF15 overexpression increases expression of Atrogin-1 has led to the speculation that KLF15 might promote muscle wasting via activation of an atrophy-promoting transcriptional program (22). However, these studies used supraphysiologic levels of KLF15 overexpression and have thus left unanswered whether the physiologic effects of the GC–KLF15 axis are harmful or beneficial in skeletal muscle. Here, we use unbiased transcriptomic profiling and physiologic analysis of mice harboring KLF15 deficiency and skeletal muscle-specific KLF15 overexpression at physiologic levels to gain a deeper understanding of the GC–KLF15 axis in muscle function. We find that the GC–KLF15 axis does not regulate muscle atrophy in vivo but rather activates a metabolic gene program that mediates ergogenic effects in wild-type (WT) mice and ameliorates dystrophic severity in the *mdx* mouse model of DMD. These studies establish KLF15 as a critical metabolic effector of physiological GC signaling in vivo and suggest that GR-dependent target gene transactivation in skeletal muscle can, in certain contexts, have a therapeutic role.

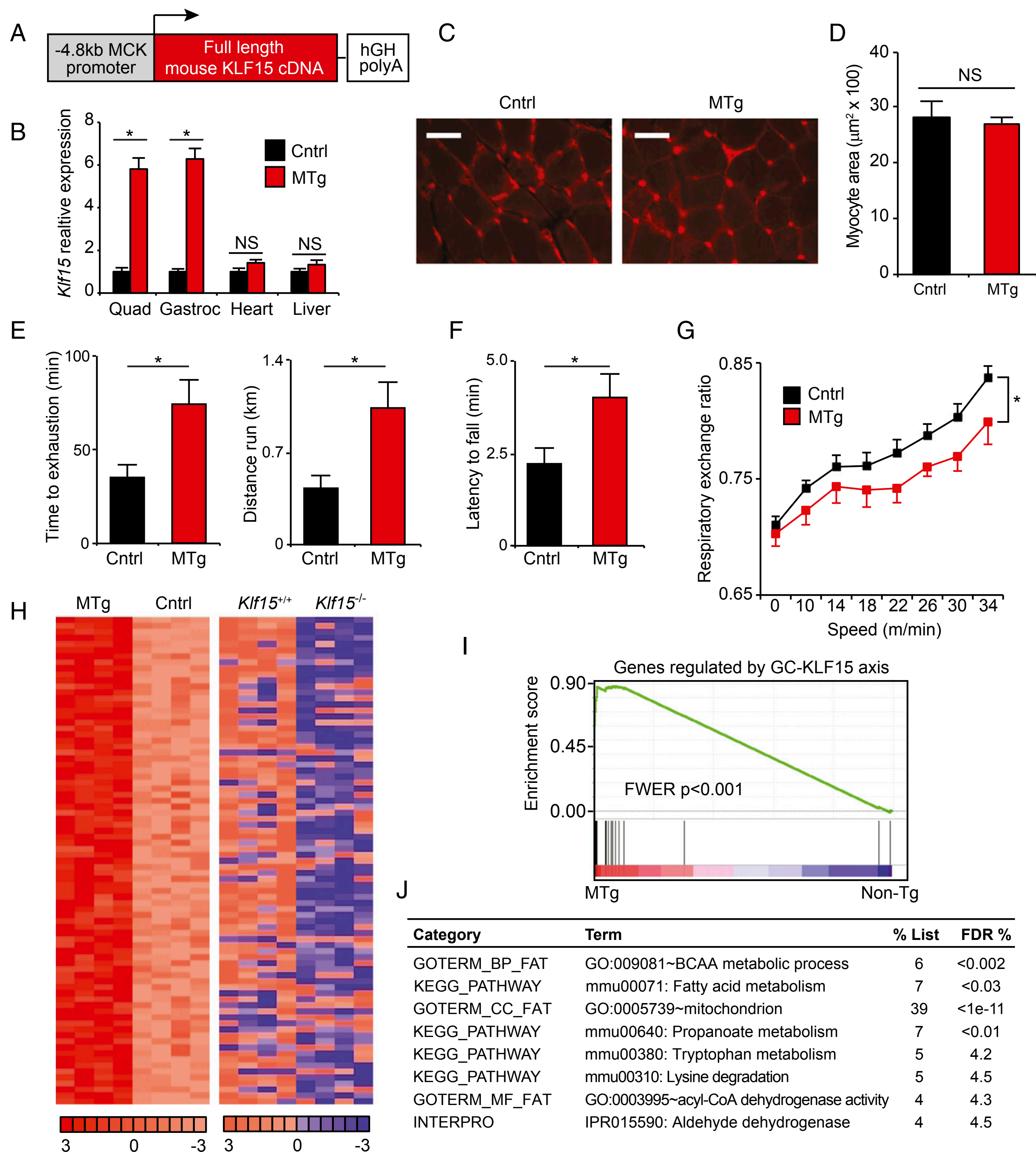
Results

KLF15 Is Induced by GCs in Live Human Subjects. Although GCs can induce KLF15 in rodent muscle tissue (22), it is not known whether GCs regulate KLF15 in human subjects in vivo. Healthy human volunteers underwent skeletal muscle biopsies before and after ingesting the potent GR agonist dexamethasone (dex; 2 mg orally twice daily × 5 d). Quantitative RT-PCR (qRT-PCR) revealed a significant induction of *KLF15* expression in human skeletal muscle tissue in vivo (Fig. 1*A*; *FKBP5* is a well-established GR target that serves as a positive control). Similarly, we found that dex rapidly and robustly increased *KLF15* expression in primary human myotubes and mouse C2C12 myotubes in vitro (Fig. S1 *A* and *B*) and mouse quadriceps in vivo (Fig. S1*C*). The mammalian *KLF15* locus contains two adjacent glucocorticoid response elements (GREs) in the first intron that are highly conserved (schematized in Fig. S1*D*). We performed ChIP-qPCR in quadriceps tissue of adult mice and treated with or without dex and found dynamic enrichment of endogenous GR at these intronic GREs in the *Klf15* locus (Fig. S1*E*). These data confirm prior observations that KLF15 is a direct GR target in muscle and demonstrate that GC-mediated induction of muscle KLF15 occurs in live human subjects.

The GC–KLF15 Axis Regulates an Ergogenic Gene Program That Is Not Involved in Muscle Atrophy. In an effort to definitively resolve the gene program regulated by the GC–KLF15 axis in vivo, we performed unbiased transcriptomic profiling from skeletal muscle tissue of WT and $Klf15^{-/-}$ (KO) mice treated with or without dex. WT and KO mice were given a single dose of dex (2 mg/kg i.p.) versus vehicle, and quadriceps tissue was isolated for transcript expression profiling by cDNA microarrays (Fig. 1*B*). Dex induced a total of 897 genes, of which 7% were highly KLF15-dependent, as shown by Venn diagram (Fig. 1*C*) and heat map of representative genes (Fig. 1*D*; full list of differential gene expression provided in Excel format in Dataset S1). To our surprise, the microarray study revealed that KLF15 was not required for the robust GC-meditated induction of the canonical atrogene program, findings that were confirmed independently by qRT-PCR (Fig. S1*F*). Rather, functional annotation of the GC–KLF15-regulated transcriptome revealed highly significant enrichment for genes critical for metabolism of several amino acids, fatty acids, and propanoate (Fig. 1 *D* and *E*, Fig. S1*G*, and Dataset S1).

Based on these unbiased expression profiles, we hypothesized that KLF15 was not involved in muscle wasting but, in contrast, was required for GC-mediated ergogenic effects in vivo. To test this hypothesis, we first subjected mice to a standard model of chronic dex-mediated muscle atrophy (dex 1 mg/kg given daily s.c. × 10 d) (29, 30). We found that both WT mice and KLF15 KO mice had an identical degree of muscle atrophy, as assessed by changes in body weight, muscle weight, and myofiber cross-sectional area (Fig. 1 *F* and *G* and Fig. S1*H*). Thus, KLF15 is not required for GC-mediated muscle atrophy in vivo. To test the requirement of KLF15 in GC-mediated ergogenesis, WT and KO mice were given a single dose of dex and challenged to an endurance exercise trial 16 h after dex treatment (Fig. 1*H*). Consistent with the gene expression profiling, WT mice treated with dex were able to augment endurance exercise capacity by 50%, whereas KO mice did not demonstrate any significant ergogenic response to dex (Fig. 1*H*). Hence, these data demonstrate that KLF15 is dispensable for GC-mediated muscle atrophy but required for the ergogenic effects of GCs.

Skeletal Muscle-Specific KLF15 Induction Is Sufficient to Drive an Ergogenic Gene Program in Vivo. As the data in Fig. 1 were generated using mice harboring systemic KLF15 deficiency, we next asked whether skeletal muscle-specific augmentation of KLF15 expression was sufficient to activate an ergogenic metabolic program in vivo. We used the –4.8 kb MCK promoter/enhancer to generate transgenic mice expressing a full-length mouse KLF15 cDNA in a skeletal muscle-specific manner (Fig. 2*A*). We studied a transgenic mouse line that expressed KLF15 levels at 5–6 times higher than control exclusively in skeletal muscle with no induction in the heart or other tissues (muscle transgenic, MTg; Fig. 2*B*). This level of transgenic overexpression of KLF15 is in the physiological range (23) and similar to the increase in KLF15 seen with nanomolar concentrations of dex (Fig. S1 *A* and *B*). MTg mice showed no evidence of muscle atrophy as assessed by gravimetry (Fig. S2 *A* and *B*) or histology (Fig. 2 *C* and *D* and Fig. S2*C*). In contrast, MTg mice had significantly increased endurance exercise capacity (Fig. 2 *E* and *F*). Metabolic exercise testing demonstrated that MTg mice had a decreased respiratory exchange ratio (Fig. 2*G*), signifying that muscle-specific KLF15 induction leads to a global shift in substrate preference toward amino acids and lipids. To confirm that these physiological observations were associated with KLF15-dependent transcriptional control in skeletal muscle, we performed genome-wide expression profiling in quadriceps of MTg mice using cDNA microarrays. These analyses revealed that skeletal muscle-specific KLF15 overexpression in the physiological range resulted in a signature that was dominated by gene induction (90 induced genes out of 109 total differentially expressed genes; 83%). A representative heat map of the top 50 genes induced in the MTg skeletal muscle is provided in Fig. S2*D*. As visualized in the global heat maps from MTg mice (Fig. 2*H*), the transcriptomic profile of MTg mice reflected significant reversal of the metabolic gene expression abnormalities observed in KLF15 KO skeletal muscle. This highly significant overlap between genes



Category	Term	% List	FDR %
GOTERM_BP_FAT	GO:009081~BCAA metabolic process	6	<0.002
KEGG_PATHWAY	mmu00071: Fatty acid metabolism	7	<0.03
GOTERM_CC_FAT	GO:0005739~mitochondrion	39	<1e-11
KEGG_PATHWAY	mmu00640: Propanoate metabolism	7	<0.01
KEGG_PATHWAY	mmu00380: Tryptophan metabolism	5	4.2
KEGG_PATHWAY	mmu00310: Lysine degradation	5	4.5
GOTERM_MF_FAT	GO:0003995~acyl-CoA dehydrogenase activity	4	4.3
INTERPRO	IPR015590: Aldehyde dehydrogenase	4	4.5

Fig. 2. Skeletal muscle-specific KLF15 overexpression is sufficient to drive an ergogenic metabolic program in vivo. (*A*) Schematic of transgenic construct. (*B*) qRT-PCR from indicated tissues demonstrating skeletal muscle-specific KLF15 overexpression in MTg mice versus control (littermate non-Tg mice; $n = 5$). (*C*) Representative histology from quadriceps (WGA staining) and (*D*) quantification of myocyte cross-sectional area ($n = 3$ independent mice per group). (Scale bar, 50 μm.) (*E*) Treadmill exercise ($n = 6$), (*F*) wire-hang assay ($n = 7$–10), and (*G*) metabolic exercise test ($n = 7$–10) in MTg and control mice. (*H*) Heat map of all genes induced in MTg versus non-Tg muscle (*Left* panel; $n = 4$). Heat map for the same genes in WT versus KLF15 KO muscle (*Right* panel, WT levels colored as non-Tg in *Left* panel) illustrates the reversal in expression versus control in KLF15 KO relative to MTg. (*I*) GSEA of genes regulated by the GC–KLF15 axis in MTg versus non-Tg muscle. (*J*) Functional annotation (DAVID) of genes regulated by KLF15 overexpression (*$P < 0.05$ for indicated comparisons). Data are shown as mean ± SEM.

induced in the MTg and genes reduced in KLF15 KO muscle was statistically confirmed by gene set enrichment analysis (GSEA; Fig. 2*I*). Functional annotation of differentially expressed genes in MTg mice (Fig. 2*J*) revealed robust enrichment for the same

pathways regulated by the GC–KLF15 axis (Fig. 1*E*), with significant induction of genes critical for amino acid, lipid, and propanoate metabolism (Fig. 2*J* and Fig. S2*E*; full list of differential gene expression provided in Excel format as Dataset S2). Consistent with our published (23) and current observations in KO mice (Fig. 1), the MTg microarrays did not demonstrate significant regulation of a gene program governing fiber type specification, mitochondrial biogenesis, autophagic flux, or angiogenesis. Again, expression profiles and direct qRT-PCR showed no induction of the canonical atrogene program in MTg mice (Fig. S2*E* and Dataset S2). Thus, the data in Figs. 1 and 2 demonstrate that KLF15 is not an atrogene but rather functions as a critical downstream transcriptional effector of muscle GR signaling that regulates amino acid and lipid metabolic programs. Furthermore, these data demonstrate that KLF15 is both necessary and sufficient to mediate ergogenic effects of GCs in vivo.

The GC–KLF15 Transcriptional Axis Modulates Disease Phenotype in the *mdx* Mouse Model of DMD. As the GC–KLF15 axis controls a gene program governing muscle substrate utilization and ergogenesis (Figs. 1 and 2), we asked whether this metabolic signaling pathway might, in part, mediate the salubrious effects of GCs in DMD. Landmark clinical studies have demonstrated that low and intermittent dosing of GCs such as prednisolone can slow disease progression and improve outcomes in DMD patients (15–17). Despite this established efficacy, a defined molecular basis for the salutary effects of GCs in DMD remains poorly understood (15, 20). Although GCs are known to exert antiinflammatory effects via transrepression of NF-κB signaling (1), several clinical and experimental studies cast significant doubt on the contention that GCs ameliorate DMD via a primary antiinflammatory mechanism (15, 19, 31–34). First, non-GC antiinflammatory or immunosuppressant drugs, such as cyclosporine-A or azathioprine, fail to show efficacy in DMD patients (32–34). Second, GC therapy provides benefit in DMD patients even when administered via low or intermittent dosing schemes that do not provide a potent and sustained antiinflammatory effect (15). Third, GC therapy does not dramatically alter the inflammatory cell infiltrate seen in dystrophic muscle (31, 34). Fourth, in the *mdx* mouse model of DMD (35), the therapeutic effects of GCs are fully preserved in a *Rag2*$^{-/-}$ background, in which B and T lymphocytes are absent (19).

In contrast, several studies in DMD patients and animal models have documented abnormalities in muscle substrate and energy metabolism (36–47), prompting the term "metabolic crisis" as a hallmark feature of this disease (41). Given the important role for KLF15 in muscle metabolism, we hypothesized that DMD might be characterized by a state of relative KLF15 deficiency and that GCs may mediate important therapeutic effects in DMD, in part, via induction of KLF15. Skeletal muscle biopsies from DMD patients (Fig. 3*A*) and muscle tissue from male *mdx* mice (Fig. 3*B*) had significantly decreased *KLF15* expression compared with control tissues. We next used GSEA to statistically test whether the metabolic gene program regulated by the GC–KLF15 axis was deficient in dystrophic muscle. These GSEAs confirmed in an unbiased and quantitative manner that the set of genes decreased in *mdx* skeletal muscle tissue (curated from ref. 44) was highly enriched for the set of genes regulated by the GC–KLF15 axis (Fig. 3*C*). These findings were also confirmed by direct qRT-PCR analysis of representative genes (Fig. S3*A*). Hence, DMD is characterized by decreased expression of KLF15 and its downstream metabolic targets.

Prior studies from our group demonstrate that mice with KLF15 deficiency, which have been extensively characterized in a nondystrophic genetic background, are viable into adulthood, are fertile, have normal muscle development and histology, and show no evidence of muscle damage (23). We confirmed this lack of baseline histopathology in an independent cohort of mice and found that male KLF15-deficient mice in a nondystrophic background showed no evidence of elevated plasma creatine phosphokinase (CPK) concentration or histologic muscle damage (Fig. S3 *B* and *C*). Although KLF15 deficiency alone does not cause dystrophic muscle pathology, we hypothesized that KLF15 deficiency might exacerbate dystrophic severity in the *mdx* mouse model of DMD. To test this hypothesis, we bred KLF15-deficient mice into the *mdx* background by intercrossing *Klf15*$^{+/-}$/*Dmd*$^{\mathrm{mdx}}$ *Y* males with *Klf15*$^{+/-}$/*Dmd*$^{\mathrm{mdx}}$ *Dmd*$^{\mathrm{mdx}}$ females. Although pups of all three expected genotypes were born in predicted Mendelian ratios, we observed significant premature death of male *Klf15*$^{-/-}$ *mdx* mice postnatally with only ∼20% surviving to adulthood (Fig. 3*D*). We did not observe any statistically significant loss of *Klf15*$^{-/-}$/*Dmd*$^{\mathrm{mdx}}$ *Dmd*$^{\mathrm{mdx}}$ female mice, a finding that may be related to gender-specific metabolic consequences of KLF15 deficiency or milder aspects of disease severity in *Dmd*$^{\mathrm{mdx}}$ *Dmd*$^{\mathrm{mdx}}$ versus *Dmd*$^{\mathrm{mdx}}$ *Y* mice (48). Analysis of male *Klf15*$^{-/-}$ *mdx* mice before death revealed severe dystrophic pathology in skeletal muscle tissue and massively elevated plasma CPK concentration, an unbiased and quantitative clinical biomarker of global muscle damage in DMD patients (15) (Fig. 3 *E* and *F*). Importantly, single-mutant intercrosses of mice harboring either the null *Klf15* allele or the *mdx* allele alone do not produce such mortality or muscle damage (23, 49). Our observations in these compound mutant strains support the contention that there is a genetic interaction between KLF15 deficiency and the dystrophic pathology of the *mdx* mouse model.

Given the low survival of male *Klf15*$^{-/-}$ *mdx* mice, we were unable to perform longitudinal studies with appropriately matched littermate controls. Therefore, we focused our attention on *Klf15* haploinsufficient mice, which survive into adulthood (Fig. 3*D*) and reflect the partial loss of KLF15 expression seen in muscle tissue from DMD patients and *mdx* mice (Fig. 3 *A* and *B*). We subjected male *Klf15*$^{+/-}$ *mdx* mice and *Klf15*$^{+/+}$ *mdx* littermate controls to a therapeutic trial of prednisolone using a low and intermittent dosing regimen similar to that used clinically in DMD patients and in prior studies of *mdx* mice (Fig. 3*G*) (15, 19, 49). We confirmed that this regimen of chronic prednisolone produced the expected increase in muscle KLF15 and its downstream targets in *Klf15*$^{+/+}$ *mdx* control mice, with blunted expression in *Klf15*$^{+/-}$ *mdx* mice (Fig. 3*H* and Fig. S3*D*). *Klf15*$^{+/-}$ *mdx* mice had minimal baseline perturbations in muscle strength and endurance but had significantly attenuated therapeutic responses to prednisolone therapy, as demonstrated by decreased endurance exercise capacity on treadmill (Fig. 3*I*) and wire-hang tests (Fig. 3*J*) and decreased grip strength (Fig. 3*K*). *Klf15*$^{+/-}$ *mdx* mice had histologic evidence of excessive muscle damage and plasma CPK elevation at baseline with no significant therapeutic response to prednisolone therapy (Fig. 3 *L* and *M*). Utrophin expression, which is known to compensate for mutant Dystrophin in mice (50), was not affected by prednisolone therapy or KLF15 deficiency (Fig. S3*E*). The data in Fig. 3 reveal that KLF15 deficiency, which does not cause significant muscle damage in a nondystrophic background (Fig. S3 *B* and *C*) (23), exacerbates features of dystrophic severity in the *mdx* background. Furthermore, the data in the *Klf15* haploinsufficient mice demonstrate that partial deficiency of this gene attenuates the therapeutic effect of GCs in the *mdx* mouse model.

We next asked whether effects of KLF15 in the *mdx* model were muscle-specific in vivo and whether KLF15 induction was sufficient to exert beneficial effects in the absence of exogenous GCs. To address both of these questions, we crossed KLF15 MTg mice into the *mdx* background to produce male mice of genotype MTg$^{+/-}$ *Dmd*$^{\mathrm{mdx}}$ and non-Tg *Dmd*$^{\mathrm{mdx}}$ control littermates. The transgene increased total KLF15 expression in *mdx* skeletal muscle by fivefold, a level of induction that approximates the effect of GCs (Fig. 4*A*). To test whether the gene expression program induced by the KLF15 transgene was statistically

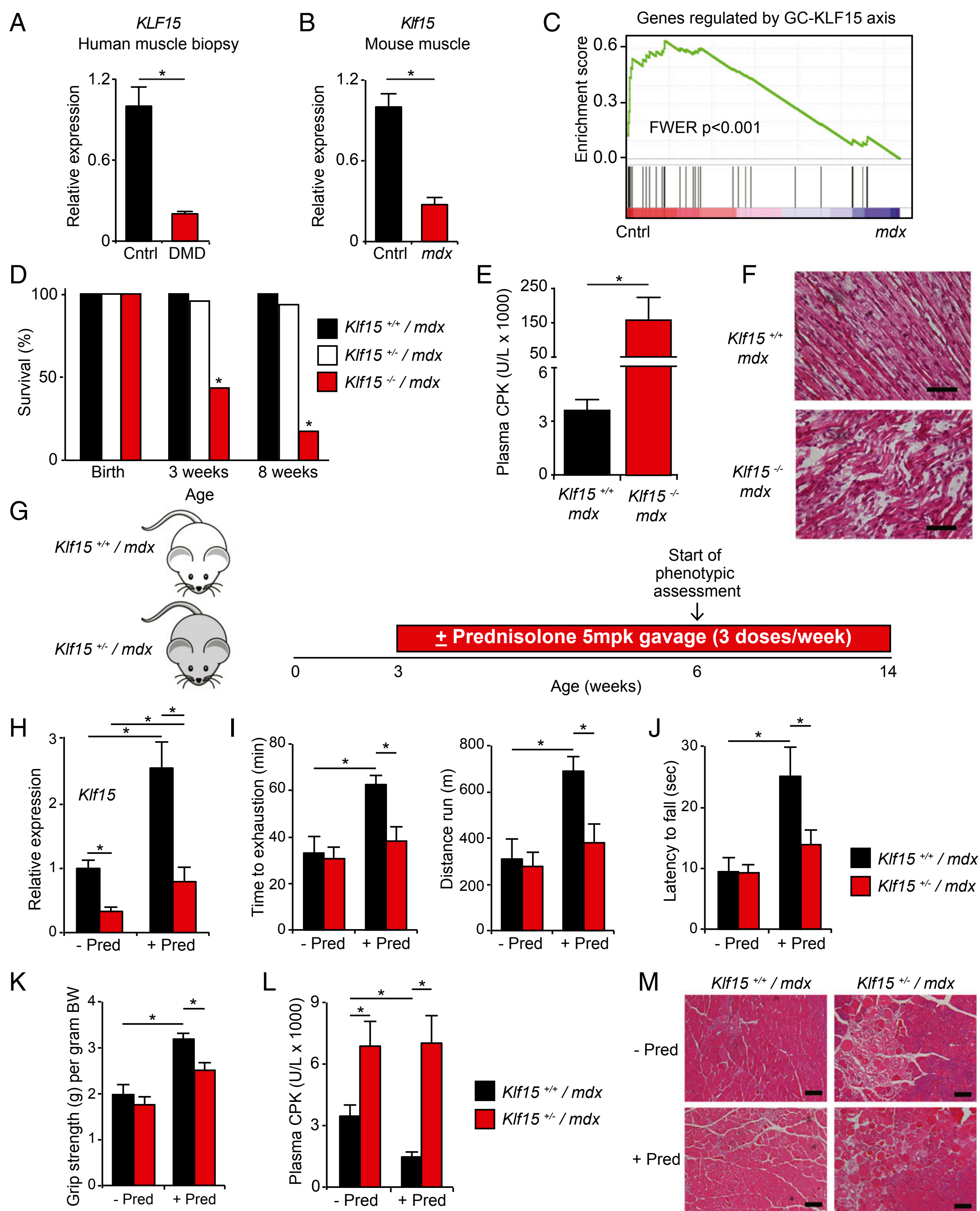


Fig. 3. KLF15 deficiency severely exacerbates DMD, and KLF15 is required for therapeutic efficacy of GCs. qRT-PCR of KLF15 from (*A*) vastus lateralis biopsies of human subjects with DMD versus healthy controls ($n = 10$) and (*B*) quadriceps tissue of *mdx* versus control mice ($n = 5$; $*P < 0.05$ for indicated comparisons). (*C*) GSEA demonstrating that genes reduced in *mdx* skeletal muscle tissue (curated from ref. 44) are highly enriched for those regulated by the GC–KLF15 axis. (*D*) Survival by genotype of male offspring generated from crosses between $Klf15^{+/-}$ *mdx* males and females. There is significant postnatal death of $Klf15^{-/-}$ *mdx* males ($P < 0.05$; $n = 157$ total mice). (*E*) Plasma CPK concentration from mice of indicated genotypes ($n = 6$–7; $*P < 0.05$ for indicated comparison). (*F*) Representative H&E-stained section of hindlimb muscle tissue from mice of indicated genotypes before death (age P4–5; representative of $n = 3$). (Scale bar, 20 μm.) (*G*) Schematic of experimental design for GC therapeutic trial (± prednisolone 5 mg/kg·dose oral gavage given 3 times weekly). (*H*) *Klf15* expression in quadriceps ($n = 4$–7; $*P < 0.05$ for indicated comparisons). (*I*) Treadmill endurance exercise capacity ($n = 6$–15), (*J*) wire-hang endurance assay ($n = 6$–13), and (*K*) grip strength ($n = 6$–11) of indicated genotypes treated ± prednisolone ($*P < 0.05$ for indicated comparisons). (*L*) Plasma CPK concentration ($n = 6$–9; $*P < 0.05$ for indicated comparisons). (*M*) Representative H&E-stained sections of quadriceps from mice of indicated genotypes and treatments (representative of $n = 3$). (Scale bar, 100 μm). Data are shown as mean ± SEM.

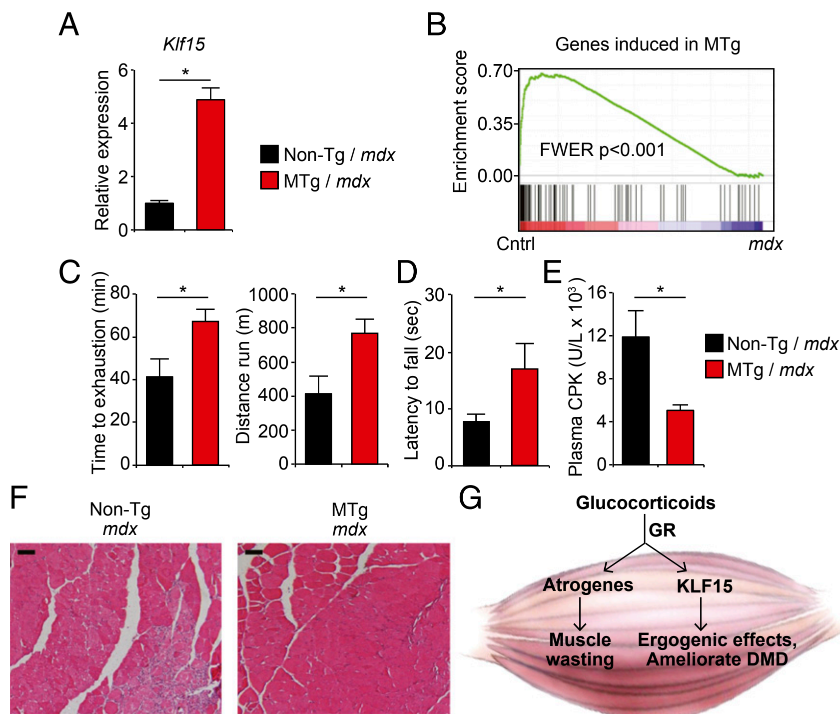


Fig. 4. Muscle-specific KLF15 overexpression ameliorates DMD phenotype in *mdx* mice. (*A*) qRT-PCR of *Klf15* in quadriceps from mice of indicated genotypes ($n = 5$). (*B*) GSEA demonstrating that genes reduced in *mdx* skeletal muscle tissue (curated from ref. 44) are highly enriched for those induced by the muscle-specific KLF15 transgene. (*C*) Treadmill exercise capacity ($n = 9$ MTg *mdx*, $n = 5$ non-Tg *mdx*) and (*D*) wire-hang test ($n = 9$ MTg *mdx*, $n = 15$ non-Tg *mdx*) in mice of indicated genotypes. (*E*) Plasma CPK concentration ($n = 7$ MTg *mdx*, $n = 5$ non-Tg *mdx*). (*F*) Representative H&E-stained quadriceps sections demonstrating histologic improvement of muscle damage in MTg *mdx* mice (representative of $n = 3$ per group; $^*P < 0.05$ for indicated comparisons). (Scale bar, 100 μm.) (*G*) Schematic depicting the role of the GC–KLF15 axis in skeletal muscle. Data are shown as mean ± SEM.

enriched for genes down-regulated in *mdx* mice in an unbiased manner, we performed GSEA between our transgenic microarray data (Fig. 2) and a curated profile of differentially expressed genes in *mdx* mouse skeletal muscle (44). GSEA revealed that the metabolic gene program induced in MTg mice was significantly enriched for genes deficient in *mdx* skeletal muscle (Fig. 4*B*), findings that were confirmed by qRT-PCR of representative genes (Fig. S4*A*). There was no differential expression of Utrophin in MTg *mdx* mice (Fig. S4*B*). Phenotypic assessment revealed that MTg *mdx* mice had significantly increased endurance exercise capacity on a treadmill (Fig. 4*C*) and wire-hang test (Fig. 4*D*). In addition, MTg *mdx* mice had lower plasma CPK concentration (Fig. 4*E*), a reduction in histologic muscle damage (Fig. 4*F*), and decreased Evans blue dye extravasation (Fig. S4*C*) compared with non-Tg *mdx* littermate controls. Taken together, the data in Figs. 3 and 4 demonstrate that DMD tissues have low KLF15 expression and that genetic *Klf15* deletion exacerbates dystrophic pathology and attenuates the therapeutic response to GCs in *mdx* mice. Furthermore, genetic induction of KLF15 in a skeletal muscle-specific and GC-independent manner can ameliorate several pathologic features in *mdx* mice.

Discussion

This study defines a molecular mechanism that mediates ergogenic effects of GCs and contributes to their therapeutic efficacy in DMD (schematized in Fig. 4*G*). The GR directly transactivates KLF15 and regulates a KLF15-dependent metabolic gene program in skeletal muscle that increases endurance exercise capacity in a nondystrophic background and attenuates features of disease severity in the *mdx* mouse model of DMD. Classic physiological studies from Dwight Ingle and colleagues documented that optimal muscle endurance required an intact adrenal cortex (3, 4). In fact, this ergogenic bioactivity formed the basis of a bioassay that directly facilitated the eventual purification, characterization, and synthesis of cortisone from the adrenal cortex by the teams led by Edward Kendall and Tadeus Reichstein (51). When considered alongside these seminal observations, our current data highlight the evolutionarily conserved role of GC signaling in anticipatory metabolic adaptation to stress and establish the GC–KLF15 axis as a critical transcriptional effector of muscle physiology.

Our in vivo gain- and loss-of-function data also support the contention that KLF15 does not directly participate in skeletal muscle atrophy. Although previous studies have demonstrated that adenoviral overexpression of KLF15 can induce Atrogin-1 and affect cell size in myocytes (22), unbiased gene expression profiling in adult KLF15-deficient mice reveals that KLF15 is not required for GC-mediated induction of canonical atrogenes and GC-mediated muscle wasting in vivo (Fig. 1). Furthermore, skeletal muscle-specific overexpression of KLF15 at levels within the physiological range does not induce atrogenes or produce muscle atrophy (Fig. 2). These data establish the principle that a pathway mediating ergogenic metabolic effects of GCs can be dissociated from the molecular pathways that cause GC-mediated muscle wasting.

We show that KLF15 expression is reduced in dystrophic human and murine muscle and that increasing or decreasing KLF15 levels affects disease phenotype in the *mdx* mouse model of DMD. Although KLF15-deficient mice have been documented to have abnormalities in adaptive substrate metabolism (23, 52), this mouse strain has no evidence of abnormal myogenesis, myocyte fragility, or histologic muscle damage in a nondystrophic background (23), findings that were confirmed in the present study. However, when introduced into the *mdx* background, KLF15 deficiency leads to exaggerated muscle damage, suggesting a causal role for decreased muscle KLF15 in DMD disease pathogenesis. One caveat of our murine loss-of-function experiments is that

KLF15 is systemically targeted, raising the possibility that KLF15 deficiency in nonmuscle tissues may contribute to the observed phenotypes. However, our transgenic mouse studies support a skeletal muscle-specific role for KLF15 in vivo. Muscle-specific KLF15 overexpression at levels that approximate the GC effect activates a metabolic gene program, drives ergogenic physiology, and ameliorates dystrophic severity in *mdx* mice. Future studies using conditional deletion of KLF15 in the *mdx* background will be useful to further annotate the muscle-specific contribution of this metabolic transcription factor in muscular dystrophy.

Our published work detailing the consequences of KLF15 deficiency on muscle physiology (23) and the current study of the GC–KLF15 axis in vivo provide important insight into the putative downstream mechanisms by which activation of this GR target can ameliorate DMD. Collectively, these studies show that KLF15 does not directly regulate a transcriptional program governing myogenesis, myofiber specification, mitochondrial biogenesis, autophagic flux, or muscle mass. Rather, our physiological and transcriptomic studies in mice with KLF15 gain- or loss-of-function reveal a dominant role in regulation of metabolic substrate catabolism, particularly that of critical amino acid and lipid species. Deficiency of KLF15 may lead to accumulation of metabolic intermediates that are toxic to muscle, produce excess reactive oxygen species (ROS), and render myocytes more susceptible to dysfunction and death when combined with the "second hit" of genetic dystrophinopathy. Our finding that KLF15 and a significant number of its downstream targets are deficient in dystrophic muscle is consistent with several studies that have documented abnormalities in lipid and amino acid metabolism in DMD (36–47). The observation that muscle-specific overexpression of KLF15 improves aspects of dystrophic pathology suggests that restoration of the KLF15-dependent metabolic program plays a role in ameliorating DMD. Although the clear common denominator between our gain- and loss-of-function models is regulation of a metabolic gene program, we recognize that KLF15-dependent regulation of other processes such as ROS homeostasis, neuromuscular junction plasticity, or sarcolemmal integrity may also contribute to the phenotypes that are observed in this study.

Previous studies have used locus-specific ChIP to demonstrate the principle that key metabolic genes are direct targets of KLF15 (22, 23, 26, 27). We note that a number of KLF15-regulated targets are also known to be direct targets of the GR (23, 27, 53, 54), suggesting that these two transcription factors can cooperate. Indeed, a recent transcriptomic study from our group in airway cells has established that GR and KLF15 participate in robust and dynamic feed-forward transcriptional signaling at metabolic targets (27), supporting our current finding that KLF15 is a major molecular effector of the GR in vivo. We note that currently available antibodies for KLF15 are not adequate for ChIP-Seq analysis in skeletal muscle (23), precluding the genome-wide definition of the muscle KLF15 cistrome. Based on the importance of the GC–KLF15 axis, we postulate that GR and KLF15 may enrich at common loci genome-wide and functionally interact to fine tune GC-dependent transcriptional responses.

In light of our current observations pertaining to GR, we note that several other members of the nuclear receptor superfamily and associated coregulators have been shown to be potent effectors of adaptive muscle metabolism and ergogenic physiology (55–58). Activation of some of these transcriptional regulators, such as ERRγ (59), the androgen receptor (60), PPARδ (61), and the PGC-1 coactivators (62, 63), have also been shown to improve pathology in animal models of DMD. These observations support the contention that gene regulatory pathways such as the GR–KLF15 axis ameliorate DMD via modulation of muscle metabolic programming and add to an emerging body of evidence that defines intimate functional relationships between KLF family members and nuclear receptors (23, 24, 64–66).

Our data also provide potentially important insights into one of the most commonly prescribed classes of drugs. GCs have been classically associated with muscle wasting and weakness, a side effect of excessive or sustained exposure to pharmacologic GR agonists that is mechanistically linked to direct transactivation of atrogenes (e.g., Myostatin, Foxo3a, Atrogin1, Trim63/Murf1) (2). This has fueled the pervasive view that the beneficial therapeutic effects of GCs largely arise from their transrepressive function (e.g., inhibition of NFκB signaling), whereas GR-dependent gene transactivation is principally responsible for side effects (1, 2), a view that has been the basis for large-scale drug development programs to improve GC-based pharmacotherapy. The current study, however, establishes that transient or moderate exposure to GCs improves muscle performance and ameliorates DMD via direct transactivation of a potent downstream metabolic effector (KLF15), establishing that GR-dependent transactivation can mediate important therapeutic effects in certain settings. Furthermore, our transgenic experiments provide proof-of-principle that muscle-specific activation of KLF15 can ameliorate DMD in a GC-independent manner, suggesting a previously unidentified strategy for development of steroid-sparing therapeutics for DMD and other myopathic diseases.

Materials and Methods

Mouse Models. All protocols concerning animal use were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University and conducted in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (67). Mice were housed in a temperature and humidity-controlled barrier facility with a 12-h light/dark cycle and ad libitum access to water and standard laboratory rodent chow. Dex studies in congenic WT mice were performed with age- and sex-matched littermate controls (12 wk old, male, pure C57BL/6 background). *Klf15*$^{-/-}$ mice in the C57BL/6 background have been previously described (25). KLF15 MTg mice were generated by cloning a full-length mouse KLF15 cDNA downstream of the MCK (–4.8 kb) promoter/enhancer with a 3′ hGH-polyA sequence (68). The 3.2 kb backbone (pBS II SK+) was released via digestion with *XhoI* and *NotI*. The linearized transgene was injected into pure C57BL/6 ES cells via the Case Western Reserve University Transgenic and Targeting Core. F0 offspring were screened for transgene expression, and germ-line transmission was established in several lines. After screening several lines, we focused studies on a line with fivefold KLF15 overexpression that was highly skeletal muscle-specific and lacking any elevation of total KLF15 in the heart. The *mdx* mice in the C57BL/10-ScSn background and C57BL/10-ScSn controls were purchased from Jackson Laboratory (cat. no. 001801 and 000476). To generate KLF15-deficient mice harboring the mutant *mdx* allele, we first crossed *Klf15*$^{-/-}$ male mice with homozygous *mdx* female mice to generate the F1 generation (i.e., male *Klf15*$^{+/-}$ mice homozygous for the *mdx* allele and female *Klf15*$^{+/-}$ mice heterozygous for the *mdx* allele). F1 intercrosses produced both male and female mice that were *Klf15*$^{+/-}$ and homozygous for the *mdx* allele (F2 generation). All subsequent study mice were generated via intercrosses of littermate males and females that were *Klf15*$^{+/-}$ and homozygous for the *mdx* allele. All data were generated using F3–F6 generations and strict littermate controls to control for the mixed background of the parental strains. To generate KLF15 MTg mice harboring the *mdx* allele, male MTg mice harboring a single copy of the KLF15 transgene were mated to female mice homozygous for the *mdx* allele. We studied male offspring of this cross, which were homozygous for the *mdx* allele and either carried the muscle transgene or were nontransgenic littermate controls. Each cohort of study mice were bred from the original parental strains, and all studies were performed using strict littermate controls. Genotyping for the *mdx* allele was performed using endpoint PCR as detailed on the Jackson Laboratories website (jaxmice.jax.org/strain/001801.html).

Statistical Analysis. All pooled results are expressed as means, and error bars depict SEM. Statistical analysis to detect for genotype effect on energy flux during exercise was performed using ANOVA (69). Statistical analyses to probe for effects of GC administration or genotype were performed using two-way ANOVA followed by a Bonferroni posttest. Survival analysis of KLF15-deficient mice in the *mdx* background was performed on a total of 157 mice, and statistical analysis was performed using a χ^2 test with the expected number of mice determined from predicted Mendelian inheritance ratios of the targeted *Klf15* allele and the X-linked mutant *mdx* allele. For experiments

comparing means of two independent and normally distributed datasets, two-tailed Student's t tests for unpaired data were used. Statistical significance was defined as $P < 0.05$. Statistical analysis of the human dex study, microarrays, and GSEA are described in *SI Materials and Methods*.

Human Skeletal Muscle Biopsies. Skeletal muscle samples were obtained from five healthy male subjects as previously described (70) in accordance with the Declaration of Helsinki and was approved by the Ethics Committee and Institutional Review Board of Copenhagen and Frederiksberg communities. For further experimental details of human tissue studies, see *SI Materials and Methods*.

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