Regulation of Muscle Mass by Follistatin and Activins

Se-Jin Lee, Yun-Sil Lee, Teresa A. Zimmers, Arshia Soleimani, Martin M. Matzuk, Kunihiro Tsuchida, Ronald D. Cohn, and Elisabeth R. Barton

Department of Molecular Biology and Genetics (S.-J.L., Y.-S.L.) and Departments of Pediatrics and Neurology (A.S., R.D.C.), McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; Departments of Surgery and Cell Biology and Anatomy (T.A.Z.), University of Miami Miller School of Medicine, Miami, Florida 33136; Departments of Pathology and Immunology (M.M.M.), Molecular and Human Genetics, and Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030; Division for Therapies against Intractable Diseases (K.T.), Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan; and Department of Anatomy and Cell Biology (E.R.B.), University of Pennsylvania School of Dental Medicine, Philadelphia, Pennsylvania 19104

Myostatin is a TGF-β family member that normally acts to limit skeletal muscle mass. Follistatin is a myostatin-binding protein that can inhibit myostatin activity *in vitro* and promote muscle growth *in vivo*. Mice homozygous for a mutation in the *Fst* gene have been shown to die immediately after birth but have a reduced amount of muscle tissue, consistent with a role for follistatin in regulating myogenesis. Here, we show that *Fst* mutant mice exhibit haploinsufficiency, with muscles of *Fst* heterozygotes having significantly reduced size, a shift toward more oxidative fiber types, an impairment of muscle remodeling in response to cardiotoxin-induced injury, and a reduction in tetanic force production yet a maintenance of specific force. We show that the effect of heterozygous loss of *Fst* is at least partially retained in a *Mstn*-null background, implying that follistatin normally acts to inhibit other TGF-β family members in addition to myostatin to regulate muscle size. Finally, we present genetic evidence suggesting that activin A may be one of the ligands that is regulated by follistatin and that functions with myostatin to limit muscle mass. These findings potentially have important implications with respect to the development of therapeutics targeting this signaling pathway to preserve muscle mass and prevent muscle atrophy in a variety of inherited and acquired forms of muscle degeneration. (*Molecular Endocrinology* 24: 1998–2008, 2010)

Myostatin is a TGF-β family member that acts as a negative regulator of skeletal muscle mass (1). *Mstn* mRNA is first detectable in midgestation embryos in cells of the myotome compartment of developing somites and continues to be expressed in muscle throughout embryogenesis as well as in adult mice. Mice homozygous for a deletion of the *Mstn* gene exhibit dramatic and widespread increases in skeletal muscle mass, with individual muscles of *Mstn*-knockout mice weighing about twice as much as those of *wild-type* mice as a result of a combination of increased fiber number and muscle fiber hypertrophy. These findings suggested that myostatin plays two distinct roles to regulate

muscle mass, one to regulate the number of muscle fibers that are formed during development and a second to regulate growth of those fibers. In this respect, selective postnatal loss of myostatin signaling as a result of either deletion of the *Mstn* gene (2, 3) or pharmacological inhibition of myostatin activity (4–7) can cause significant muscle fiber hypertrophy, demonstrating that myostatin plays an important role in regulating muscle homeostasis in adult mice. Moreover, genetic studies in cattle (8–11), sheep (12), dogs (13), and humans (14) have all shown that the function of myostatin as a negative regulator of muscle mass is highly conserved across species.

Abbreviations: EDL, Extensor digitorum longus; FSTL-3, follistatin-like 3; GDF, growth differentiation factor; MHC, myosin heavy chain.

The identification of myostatin and its biological function has raised the possibility that inhibition of myostatin activity may be an effective strategy for increasing muscle mass and strength in patients with inherited and acquired clinical conditions associated with debilitating muscle loss (for reviews, see Refs. 15–17). Indeed, studies employing mouse models of muscle diseases have suggested that loss of myostatin signaling has beneficial effects in a wide range of disease settings, including muscular dystrophy, spinal muscular atrophy, cachexia, steroid-induced myopathy, and age-related sarcopenia. Moreover, loss of myostatin signaling has been shown to decrease fat accumulation and improve glucose metabolism in models of metabolic diseases, raising the possibility that targeting myostatin may also have applications for diseases such as obesity and type II diabetes. As a result, there has been an extensive effort directed at understanding the mechanisms by which myostatin activity is normally regulated and on identifying the components of the myostatin-signaling pathway with the long-term goal of developing the most effective therapeutic strategies for targeting its actions.

In this regard, considerable progress has been made in terms of understanding how myostatin activity is regulated extracellularly by binding proteins (for review, see Ref. 15). One of these regulatory proteins is follistatin (FST), which is capable of acting as a potent myostatin antagonist. Follistatin has been shown to be capable of binding directly to myostatin and inhibiting its activity in receptor binding and reporter gene assays in vitro (18–20). Moreover, follistatin also appears to be capable of blocking endogenous myostatin activity in vivo, as transgenic mice overexpressing follistatin specifically in skeletal muscle have been shown to exhibit dramatic increases in muscle growth comparable to those seen in Mstn-knockout mice (18, 21, 22). Finally, mice homozygous for a targeted mutation in the Fst gene have reduced muscle mass at birth (23), consistent with a role for follistatin in inhibiting myostatin activity during embryonic development. The fact that $Fst^{-/-}$ mice die immediately after birth, however, has hampered a more detailed analysis of the role of follistatin in regulating muscle homeostasis. Here, we show that Fst mutant mice exhibit haploinsufficiency, with $Fst^{+/-}$ mice having significant reductions in muscle mass accompanied by corresponding decreases in muscle function and impaired muscle regeneration. Furthermore, we show that this muscle phenotype reflects a normal role for follistatin in regulating not only myostatin but also other TGF-β family members that cooperate with myostatin to limit muscle growth, and we present genetic evidence that activin A may be one of these key cooperating ligands.

Results

Because mice homozygous for a deletion of Fst gene die immediately after birth (23) and because many components of the myostatin-regulatory system have shown dose-dependent effects when manipulated in vivo, we investigated the possibility that Fst mutant mice might exhibit haploinsufficiency with respect to muscle growth and function. We backcrossed the Fst loss-of-function mutation at least 10 times onto a C57BL/6 background and then analyzed muscle weights in $Fst^{+/-}$ mice at 10 wk of age. As shown in Table 1 and Fig. 1 (bottom panel), $Fst^{+/-}$ mice exhibited a clear muscle phenotype, with muscle weights in $Fst^{+/-}$ mice being lower by about 15–20% compared with those of wild-type mice. These reductions in muscle weights were highly statistically significant (P values ranged from 10^{-8} to 10^{-12}), were seen in all four muscles that were analyzed (pectoralis, triceps, quadriceps, and gastrocnemius) as well as in both males and females, and were also apparent after normalizing for total body weights (Supplemental Table 1 and Supplemental Fig. 1 published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

These effects on muscle mass were the converse of what has been observed in mice with mutations in the Mstn gene and were therefore consistent with a normal role for follistatin in inhibiting myostatin activity in vivo. We showed previously that the higher muscle mass seen in $Mstn^{-/-}$ mice results from effects on both fiber numbers and fiber sizes (1). To determine whether both fiber numbers and fiber sizes are also affected by the Fst mutation, we carried out morphometric analysis of sections of the gastrocnemius muscle. As shown in Table 2, total fiber number in the gastrocnemius appeared to be unaffected in $Fst^{+/-}$ mice compared with wild-type controls. One difference clearly evident in hematoxylin and eosin-stained sections, however, was the increased proportion of smaller, more darkly stained fibers in muscles of $Fst^{+/-}$ mice (Fig. 2A), raising the possibility that heterozygous loss of Fst might affect fiber type distribution. In this respect, previous studies have shown that loss of myostatin affects the relative proportions of the different fiber types, with $Mstn^{-/-}$ mice having a decreased number of type I fibers and an increased number of type II fibers in the soleus as well as a shift in the distribution of type II fibers toward more of the glycolytic type IIb fibers in the extensor digitorum longus (EDL) (24–27). Fiber type analysis of the gastrocnemius muscle of $Fst^{+/-}$ mice revealed an opposite shift toward more oxidative fibers. In particular, the number of oxidative type I fibers was increased significantly in the gastrocnemius muscle of $Fst^{+/-}$ mice (Table 2), and most of the small darkly

TABLE 1.   Muscle weights of mutant mice

		Body weight (g)	Muscle weights (mg)			
			Pectoralis	Triceps	Quadriceps	Gastrocnemius
Males						
Wild type	(n=36)	24.0 ± 0.3	74.5 ± 1.0	94.8 ± 1.2	193.3 ± 2.5	137.5 ± 1.7
$Fst^{+/-}$	(n=15)	23.3 ± 0.7	60.7 ± 1.0[a]	78.3 ± 1.4[a]	157.4 ± 3.0[a]	118.2 ± 2.0[a]
$Fstl3^{-/-}$	(n=23)	25.3 ± 0.6	77.2 ± 1.7	97.9 ± 1.6	200.7 ± 3.8	138.2 ± 2.1
$Mstn^{+/-}$	(n=17)	27.6 ± 0.4	97.4 ± 1.6	123.2 ± 2.4	252.6 ± 4.5	181.2 ± 3.9
$Mstn^{+/-}$, $Fst^{+/-}$	(n=12)	26.0 ± 0.4[b]	79.3 ± 1.5[c]	100.0 ± 2.0[c]	197.8 ± 5.3[c]	147.4 ± 3.2[c]
$Mstn^{-/-}$	(n= 8)	33.8 ± 0.9	215.1 ± 7.9	231.8 ± 7.3	407.1 ± 12.1	302.0 ± 10.3
$Mstn^{-/-}$, $Fst^{+/-}$	(n=10)	31.0 ± 1.3	174.3 ± 10.0[d]	206.7 ± 10.4	352.6 ± 17.3[e]	274.4 ± 13.4
$inh\beta A^{+/-}$	(n=21)	25.0 ± 0.4[f]	80.8 ± 1.2[a]	104.0 ± 1.6[a]	215.0 ± 3.8[a]	152.3 ± 2.2[a]
$inh\beta B^{+/-}$	(n=13)	24.3 ± 0.5	73.5 ± 1.7	95.8 ± 2.5	190.1 ± 3.9	134.6 ± 2.2
$inh\beta B^{-/-}$	(n=15)	24.7 ± 0.4	78.9 ± 1.7[f]	100.4 ± 2.5[f]	201.3 ± 5.0	139.1 ± 2.9
$inh\beta C^{+/-}$, $\beta E^{+/-}$	(n=12)	24.8 ± 1.2	76.2 ± 3.3	95.8 ± 2.6	192.2 ± 8.3	133.9 ± 5.7
$inh\beta C^{-/-}$, $\beta E^{-/-}$	(n=18)	25.0 ± 0.4[f]	76.9 ± 1.3	96.8 ± 1.9	196.9 ± 3.3	138.6 ± 2.0
Females						
Wild type	(n=22)	19.0 ± 0.2	48.3 ± 0.8	69.5 ± 0.9	146.2 ± 1.8	102.5 ± 1.2
$Fst^{+/-}$	(n=14)	17.2 ± 0.4[a]	39.1 ± 0.9[a]	56.6 ± 1.0[a]	115.2 ± 2.0[a]	84.7 ± 1.7[a]
$Fstl3^{-/-}$	(n=29)	19.1 ± 0.3	50.6 ± 0.8[f]	72.5 ± 0.9[f]	149.4 ± 1.8	100.7 ± 1.1
$Fst^{+/-}$, $Fstl3^{+/-}$	(n= 9)	16.3 ± 0.5[a]	37.3 ± 1.6[a]	57.0 ± 2.2[a]	113.9 ± 4.6[a]	82.9 ± 2.8[a]
$Fst^{+/-}$, $Fstl3^{-/-}$	(n= 7)	16.9 ± 0.6[g]	36.9 ± 1.4[a]	57.3 ± 1.5[a]	116.1 ± 4.0[a]	80.3 ± 2.6[a]
$Mstn^{+/-}$	(n=13)	22.0 ± 0.4	64.2 ± 1.0	90.5 ± 1.1	185.1 ± 2.3	130.5 ± 1.8
$Mstn^{+/-}$, $Fst^{+/-}$	(n=10)	20.6 ± 0.4[h]	52.3 ± 1.3[c]	74.6 ± 2.0[c]	152.6 ± 3.5[c]	111.7 ± 2.2[c]
$Mstn^{-/-}$	(n=13)	24.5 ± 0.4	111.0 ± 2.3	148.5 ± 1.8	276.8 ± 4.2	195.8 ± 2.4
$Mstn^{-/-}$, $Fst^{+/-}$	(n=17)	24.3 ± 0.3	94.4 ± 1.6[i]	139.2 ± 1.6[i]	251.2 ± 3.4[i]	186.9 ± 2.4[e]
$inh\beta A^{+/-}$	(n=20)	19.4 ± 0.2	52.4 ± 0.9[a]	73.3 ± 1.3[f]	155.3 ± 2.2[g]	109.3 ± 1.7[g]
$inh\beta B^{+/-}$	(n=15)	19.8 ± 0.4	50.5 ± 0.8[f]	70.0 ± 1.2	147.5 ± 2.7	100.8 ± 1.8
$inh\beta B^{-/-}$	(n=12)	20.0 ± 0.6	51.8 ± 1.4[f]	69.9 ± 1.2	144.3 ± 3.3	100.8 ± 2.2
$inh\beta C^{+/-}$, $\beta E^{+/-}$	(n=15)	18.5 ± 0.7	49.2 ± 1.5	69.4 ± 2.3	141.0 ± 5.5	97.5 ± 4.5
$inh\beta C^{-/-}$, $\beta E^{-/-}$	(n=25)	19.1 ± 0.2	49.9 ± 0.9	70.5 ± 1.0	150.4 ± 2.1	103.0 ± 1.4

[a] $P < 0.001$ *vs. wild type*; [b] $P < 0.01$ *vs. Mstn$^{+/-}$*; [c] $P < 0.001$ *vs. Mstn$^{+/-}$*; [d] $P < 0.01$ *vs. Mstn$^{-/-}$*; [e] $P < 0.05$ *vs. Mstn$^{-/-}$*; [f] $P < 0.05$ *vs. wild-type*; [g] $P < 0.01$ *vs. wild type*; [h] $P < 0.05$ *vs. Mstn$^{+/-}$*; [i] $P < 0.001$ *vs. Mstn$^{-/-}$*.

stained fibers that appeared to be increased in number in $Fst^{+/-}$ mice corresponded to mixed glycolytic/oxidative type IIa fibers, representing a further shift away from glycolytic type IIb fibers (Fig. 2A). We observed similar trends toward more oxidative fibers in other muscles as well, with the appearance of a significant percentage of type I fibers in the EDL ($P < 0.001$) (Fig. 2B) and an approximately 5% shift from type IIa fibers to type I fibers in the soleus, although these latter data did not reach statistical significance.

To determine whether differences in fiber sizes could account for the differences in muscle weights between $Fst^{+/-}$ and *wild-type* mice, we measured fiber diameters in representative sections of the gastrocnemius muscle. As shown in Fig. 2C and Table 2, the distribution of fiber diameters was shifted toward smaller fibers in the gastrocnemius muscle of $Fst^{+/-}$ mice compared with that of *wild-type* mice. Significantly, the shift in the distribution toward smaller fibers was observed not only in type II fibers but also in type I fibers. Hence, the overall decrease in the weight of the gastrocnemius muscle of $Fst^{+/-}$ mice appeared to result from a combination of an increase in the proportion of fiber types that are generally smaller in size and a decrease in mean fiber diameter for each fiber type.

We also analyzed the effects of heterozygous loss of *Fst* on muscle function. Previous studies have shown that inhibition of myostatin activity in mice results in increased muscle force (4). To determine whether the lower muscle mass seen in $Fst^{+/-}$ mice results in lower muscle force production, we carried out force measurements on isolated muscles. As shown in Fig. 3, twitch and tetanic force were lower in $Fst^{+/-}$ mice by 27% and 26%, respectively, in the soleus and by 28% and 17%, respectively, in the EDL, which was commensurate with the reduction in cross-sectional area. Hence, the lower muscle weights seen in $Fst^{+/-}$ mice appear to result in corresponding decreases in tetanic force production, with no statistically significant changes in specific force.

Loss of myostatin has been shown to affect not only muscle mass and strength but also the ability of the muscle to regenerate. In particular, loss of myostatin activity has been shown to result in an enhanced regenerative response to both chronic (for reviews, see Refs. 15–17) and acute (28–30) injury. We investigated the possibility that heterozygous loss of *Fst* might have the opposite effect on muscle regeneration by examining the response of the gastrocnemius muscle to cardiotoxin-induced injury. Indeed, 21 d after induction of injury, *Fst* heterozygous mice
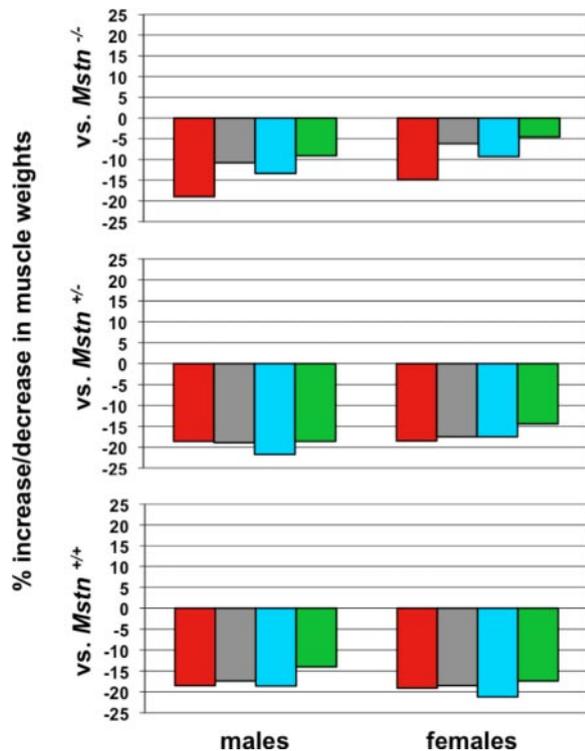
FIG. 1. Effect of heterozygous loss of *Fst* on muscle mass. *Bottom panel* shows percent decrease in muscle weights in $Fst^{+/-}$ mice compared with *wild-type* mice. *Middle panel* shows percent decrease in muscle weights in $Fst^{+/-}$, $Mstn^{+/-}$ mice compared with $Fst^{+/+}$, $Mstn^{+/-}$ mice. *Top panel* shows percent decrease in muscle weights in $Fst^{+/-}$, $Mstn^{-/-}$ mice compared with $Fst^{+/+}$, $Mstn^{-/-}$ mice. All calculations were made from the data shown in Table 1. Muscles analyzed were: pectoralis (*red*), triceps (*gray*), quadriceps (*blue*), and gastrocnemius (*green*).

showed clear deficits in muscle remodeling (Fig. 4A) with an almost 4-fold increase in the amount of muscle fibrosis as compared with *wild-type* mice (Fig. 4B). Interestingly, at 4 d after cardiotoxin-induced injury, *Fst* heterozygous mice showed no obvious difference in neonatal myosin-positive fibers compared with *wild-type* mice (Fig. 4A), suggesting that the defects in muscle remodeling may re-

TABLE 2. Analysis of gastrocnemius/plantaris muscles

|	*Wild type* *(n = 3)*	$Fst^{+/-}$ *(n = 3)*	% **Difference**
Total fiber number	8340 ± 323	8260 ± 521	−1.0
Type I fiber number	124 ± 30	206 ± 21	+66.1
Percentage type I fibers	1.47 ± 0.3	2.50 ± 0.2[a]	+69.7
Mean fiber diameters (μm)			
Type I fibers	32.8 ± 1.0	28.5 ± 1.2[a]	−13.0
Type IIa fibers	35.6 ± 2.4	29.9 ± 1.2[b]	−16.1
Type IIb fibers	41.9 ± 0.1	37.3 ± 1.3[a]	−11.0
All fiber types	41.0 ± 0.6	36.0 ± 1.5[a]	−12.2

[a] $P < 0.05$ vs. wild type; [b] $P = 0.08$ vs. wild type.

sult from failed muscle maturation rather than impaired satellite cell function.

The fact that we could observe reductions in muscle mass in $Fst^{+/-}$ mice opened up the possibility of looking at genetic interactions between *Fst* and other genes encoding components of this regulatory system. We first looked at genetic interactions between *Fst* and *Fstl3*, which is another member of the follistatin gene family. Previous studies had shown that like follistatin, FSTL-3 (follistatin-like 3; also called FLRG) is capable of blocking myostatin activity *in vitro* (31) and promoting muscle growth when overexpressed *in vivo* (21, 22). What role FSTL-3 normally plays in regulating myostatin activity *in vivo* is unclear, however, as homozygous *Fstl3* mutant mice have been reported to have normal muscle mass (32). We investigated the possibility that the lack of a clear muscle phenotype in *Fstl3* mutant mice might reflect functional redundancy between FSTL-3 and follistatin. For these studies, we used a line of mice that we independently generated carrying a targeted deletion of *Fstl3*. As shown in Fig. 5A, we generated mice in which we deleted exons 3–5, which contains most of the protein-coding region, including both of the follistatin domains of FSTL-3. We then backcrossed this deletion allele at least seven times onto a C57BL/6 background for analysis.

Consistent with findings previously reported by others (32), mice homozygous for a deletion of *Fstl3* were viable and had relatively normal muscle weights (Table 1). To investigate possible functional redundancy, we analyzed the effect of crossing the *Fst* loss-of-function mutation onto an *Fstl3* mutant background. As shown in Table 1 and Fig. 5B, we observed no additive effects of the *Fst* and *Fstl3* mutations in terms of reducing muscle mass; that is, neither $Fst^{+/-}$, $Fstl3^{+/-}$ nor $Fst^{+/-}$, $Fstl3^{-/-}$ mice showed further reductions in muscle weights compared with $Fst^{+/-}$, $Fstl3^{+/+}$ mice. Although we have not ruled out the possibility that we might see effects of FSTL-3 loss in mice completely lacking follistatin, our data suggest that these two proteins are not functionally redundant in terms of regulating muscle growth. Hence, despite all of the evidence implicating FSTL-3 as a key regulator of myostatin activity *in vivo*, we were unable to uncover any effects of genetic loss of *Fstl3* on muscle mass in these studies.

We also took advantage of the muscle phenotype in $Fst^{+/-}$ mice to investigate genetic interactions between *Fst* and *Mstn*. Our rationale for these studies was that two lines of investigation had demonstrated that other members of the TGF-β family, in addition to myostatin, seem to play important roles in limiting muscle growth. In particular, both overexpression of follistatin as a muscle-specific transgene and systemic administration of a soluble form of one of the known myostatin receptors
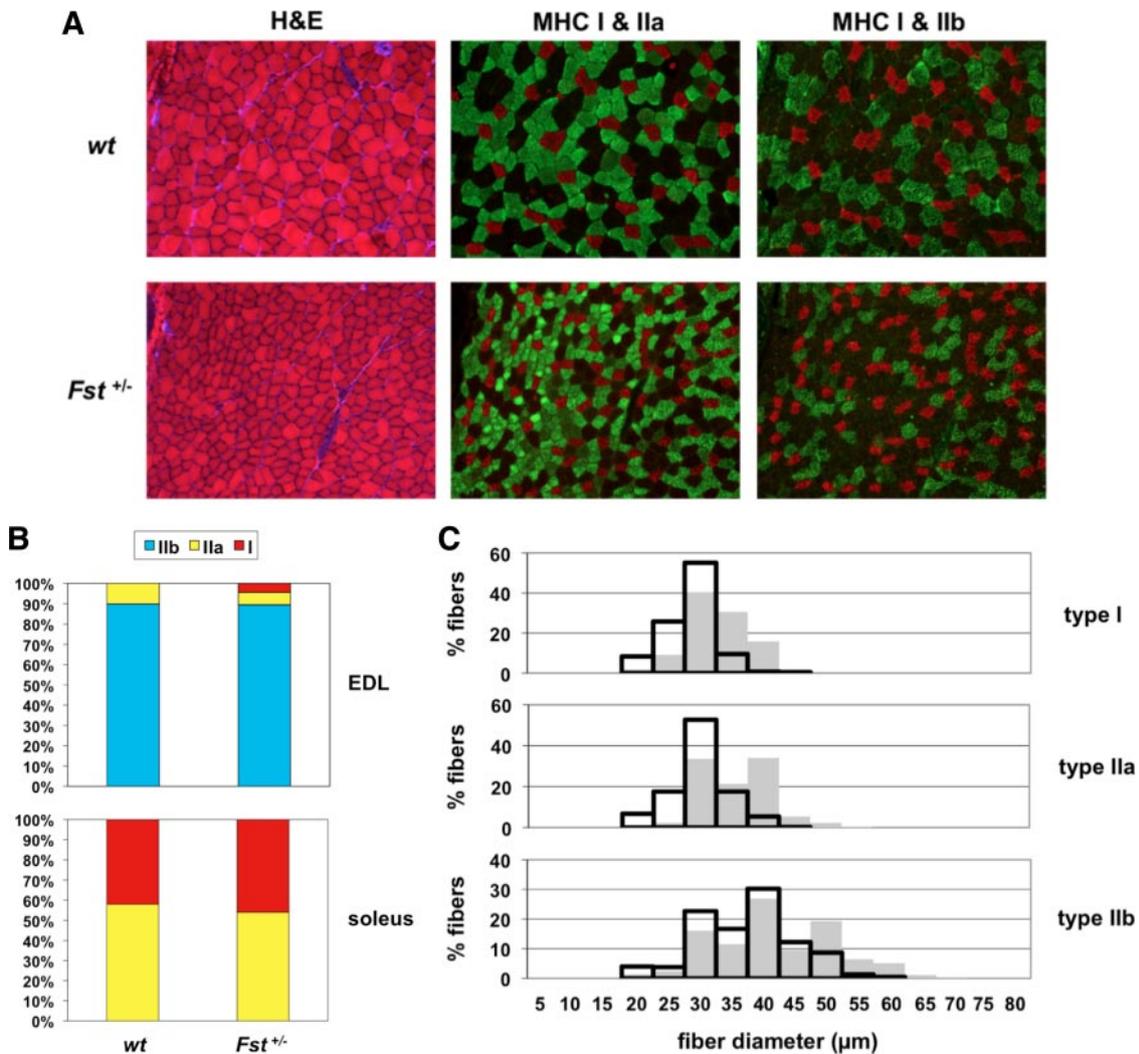
FIG. 2. Fiber type analysis. A, Sections of gastrocnemius muscles either stained with hematoxylin and eosin or incubated with antibodies against type I (*red*), type IIa (*green*), or type IIb (*green*) MHC isoforms. Note that muscles of $Fst^{+/-}$ mice had increased numbers of small, darkly stained fibers, which corresponded to type IIa fibers, as well as increased numbers of type I fibers. B, Fiber type distributions in the EDL and soleus muscles. Note the appearance of type I fibers and the decrease in proportion of type IIa fibers in $Fst^{+/-}$ EDL muscle. C, Distribution of type I, IIa, and IIb fiber diameters in the gastrocnemius muscle. *Solid gray bars* represent muscle fibers from *wild-type* mice, and *open black bars* represent muscle fibers from $Fst^{+/-}$ mice. Note the shift in the distributions toward fibers with smaller diameters in muscles of $Fst^{+/-}$ mice. H&E, hematoxylin and eosin; wt, wild type.

(ACVR2B) had been shown to cause increases in muscle mass not only in *wild-type* mice but also in $Mstn^{-/-}$ mice, implying that these inhibitors were exerting their effects by targeting other TGF-β family members in addition to myostatin (7, 18, 21). Hence, we sought to determine whether the reductions in muscle weights seen in $Fst^{+/-}$ mice result entirely from increased levels of myostatin signaling.

Our approach was to look for genetic interactions between *Fst* and *Mstn* by examining the effect of introducing the *Fst* mutation onto a *Mstn* mutant background. If the sole role for follistatin in regulating muscle mass *in vivo* is to block myostatin signaling, then the *Fst* mutation would be predicted to have no effect in the complete absence of myostatin. If, on the other hand, follistatin normally acts to block multiple ligands to regulate muscle

mass, then the *Fst* mutation might be expected to have at least some effect on muscle mass even in a *Mstn* mutant background. As shown in Table 1 and Fig. 1, we found the latter to be the case. Specifically, heterozygous loss of *Fst* caused reductions in muscle mass in both $Mstn^{+/-}$ and $Mstn^{-/-}$ mutant backgrounds in both male and female mice. The effects of the *Fst* mutation were somewhat attenuated in the complete *Mstn*-null background, implying that part of the effect of follistatin loss in $Mstn^{+/+}$ mice likely results from loss of inhibition of myostatin signaling. Nevertheless, the fact that the *Fst* mutation had at least some effect on muscle weights even in the *Mstn*-null background implies that this residual effect resulted from loss of inhibition of other TGF-β family members in these mutant mice. Hence, these studies suggest that follistatin normally acts *in vivo* to inhibit multiple TGF-β family
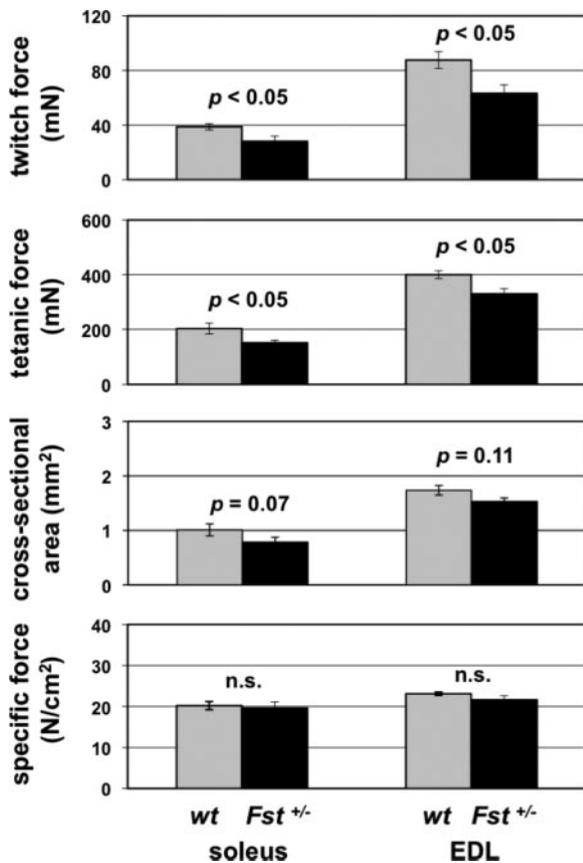
FIG. 3. Force measurements in the soleus and EDL muscles of *wild-type* and *Fst*$^{+/-}$ mice. Note the decreased twitch and tetanic force with no change in specific force in muscles of *Fst*$^{+/-}$ mice. n.s., Nonsignificant; $P > 0.20$ wt, wild type.

members, including myostatin, that function to limit muscle mass.

In the final set of experiments, we used genetic approaches to attempt to determine the identity of the ligand (or ligands) that cooperates with myostatin to suppress muscle growth. The TGF-β superfamily consists of almost 40 proteins (for reviews, see Refs 34 and 35), and many could be eliminated as possible candidates based on their known binding properties, because the key ligand can be blocked both by follistatin and by the soluble ACVR2B receptor. The most obvious candidate was growth/differentiation factor (GDF)-11, which is highly related to myostatin and is also expressed in skeletal muscle; genetic studies to date, however, have not revealed any role for GDF-11 in regulating muscle (26). As a result, we decided to extend our genetic analysis to other candidate ligands. The activins, which are dimers of inhibin-β subunits, were attractive as candidates because they had been shown to have *in vitro* activities on muscle cells (36–39). Moreover, a recent study showed that activin A is capable of inducing atrophy when overexpressed in muscle (40).

We decided to focus our initial analysis on mice carrying mutations in genes encoding the inhibin-β subunits. In

mice, four genes encoding inhibin-β subunits have been identified, *InhβA*, *InhβB*, *InhβC*, and *InhβE* (for review, see Ref. 34). Mice carrying targeted mutations in each of these genes have been generated and characterized previously (41–44), and for *InhβA* and *InhβB*, we analyzed the existing mutant mouse lines. For *InhβC* and *InhβE*, however, we analyzed a double-mutant mouse line that we generated independently in which the exon encoding the C-terminal domain of *InhβC* and the entire coding sequence of *InhβE* were deleted in the same mutant allele (Fig. 5C). All of these *Inhβ* mutant alleles were backcrossed at least six times onto a C57BL/6 genetic background before analysis.

For the *InhβB* and *InhβC/βE* mutations, we were able to analyze the effect of complete loss of function, as the homozygous mutants are viable as adults. In the case of *InhβA*, however, homozygous loss has been shown to lead to embryonic lethality (43); therefore, we were only able to analyze the effect of heterozygous loss of *InhβA*. As shown in Table 1 and Fig. 5D, the most significant effect that we observed was, in fact, in mice heterozygous for the *InhβA* loss-of-function mutation, which exhibited statistically significant increases in weights of all four muscles that were examined. The effects seen in *InhβA*$^{+/-}$ mice were most pronounced in males, which had increases ranging from about 8–11%, with P values ranging from 2×10^{-4} to 2×10^{-6} depending on the specific muscle. The effects in females were generally lower, with increases ranging from about 5–8%. These trends were also present after normalizing muscle weights to total body weights (Supplemental Table 1 and Supplemental Fig. 1). Mutations in each of the other genes had little or no effect, except in the case of *InhβB* homozygous mutants, in which two muscles (pectoralis and triceps) also showed statistically significant increases, although the magnitude of the effects was lower than that seen in *InhβA*$^{+/-}$ mice. These data provide the first loss-of-function genetic evidence that activin A may be one of the key ligands that functions with myostatin to limit muscle mass.

Discussion

Follistatin is a potent myostatin inhibitor that can cause dramatic increases in muscle mass when overexpressed as a transgene in mice (18, 21, 22). Follistatin is known to play an important role in regulating muscle development, because newborn *Fst* mutant mice have a reduced amount of muscle tissue, which is readily discernible by histological analysis (23). Because homozygous *Fst* mutants die immediately after birth, however, little is known about the role that follistatin normally plays in regulating mus-
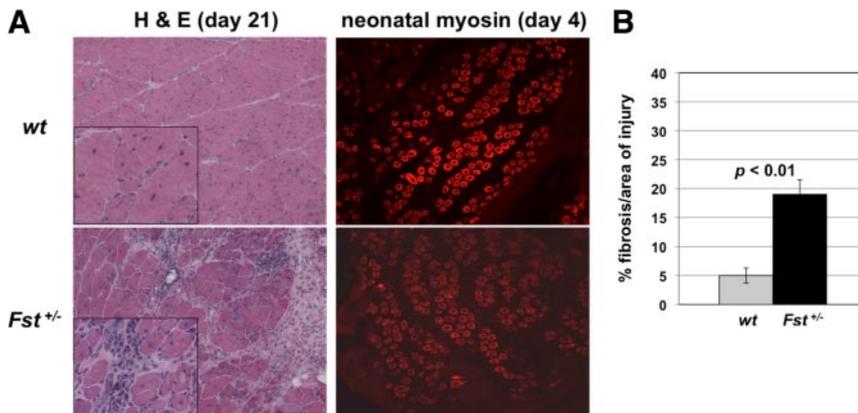
FIG. 4. Impaired muscle regeneration in $Fst^{+/-}$ mice. A, Sections of gastrocnemius muscles of *wild-type* and $Fst^{+/-}$ mice after cardiotoxin-induced injury. At 21 d after injury, note the centrally located nuclei characteristic of regenerating fibers in the *wild-type* injured muscle (see *inset*) and the significantly increased extent of fibrosis in the $Fst^{+/-}$ muscle. At 4 d after injury, note the presence of neonatal myosin in both *wild type* and $Fst^{+/-}$ muscle. B, Quantification of amount of fibrosis at 21 d after cardiotoxin-induced injury as assessed by measurement of percent fibrotic area relative to total injured area. H&E, Hematoxylin and eosin; wt, wild type.

cle homeostasis. Here, we have shown that the *Fst* loss-of-function mutation exhibits haploinsufficiency, with $Fst^{+/-}$ mice having lower overall muscle mass by about 15–20%. These reductions in muscle mass were highly statistically significant and resulted from a shift toward smaller diameter fibers with little or no apparent effect on total fiber number. This shift toward smaller fibers could be attributed to two distinct effects of the *Fst* mutation. First, there was a shift in the distribution of fiber types resulting in an increased proportion of smaller, more oxidative fibers in muscles of $Fst^{+/-}$ mice compared with those of *wild-type* mice. Second, for each fiber type that was examined, there was a shift toward fibers with smaller diameters in muscles of $Fst^{+/-}$ mice compared with those of *wild-type* mice. All of these effects are the opposite of what has been described in mice with absent or reduced myostatin activity, which exhibit increased muscle mass, a shift in fiber types toward more glycolytic fibers, and hypertrophy of both type I and type II fibers (1, 24–27, 45). We also found that $Fst^{+/-}$ mice exhibit an impaired muscle remodeling response to chemical injury, which also contrasts with the enhanced muscle regeneration seen in $Mstn^{-/-}$ mice (for reviews, see Refs. 15–17).

All of these findings demonstrate that follistatin normally functions to suppress activity of this signaling pathway in muscle. In this respect, an important point is that we were able to document significant effects of follistatin loss even though these mice still retained one normal copy of the *Fst* gene. Hence, the effect of follistatin is almost certainly dose dependent, as has been shown for many other components of this regulatory system, and we presume that complete loss of follistatin activity in muscle would lead to much more dramatic effects. We also in-

vestigated the possibility that the effects of follistatin loss might have been attenuated by functional compensation by the related protein, FSTL-3. FSTL-3 (also called FLRG) contains two follistatin domains (*vs.* three for follistatin itself), and like follistatin, FSTL-3 is capable of binding and inhibiting both activin and myostatin *in vitro* (31, 46–48) and increasing muscle mass when overexpressed *in vivo* (21, 22). FSTL-3 has been further implicated in the regulation of myostatin based on the fact that FSTL-3 could be detected in a complex with myostatin in both mouse and human blood samples (31). Gene-targeting studies, however, demonstrated that complete loss of FSTL-3 had no effects on muscle mass (32). Using an independently generated *Fstl3*-knockout line, we also observed no effect of homozygous loss of *Fstl3* on muscle mass, and furthermore, we were unable to detect any additive effects of the *Fst* and *Fstl3* loss-of-function mutations. Hence, we were unable to detect any evidence that follistatin and FSTL-3 are functionally redundant with respect to the regulation of muscle mass by myostatin and related proteins.

We also examined genetic interactions between *Fst* and *Mstn*. In particular, we showed that the effects of follistatin loss are seen even in mice null for *Mstn*, implying that myostatin cannot be the sole target for follistatin and that follistatin normally acts to block the activities of multiple TGF-β family members that function to limit muscle mass. These findings are consistent with the results of two prior studies. One set of experiments was the analysis of mice treated with a soluble form of ACVR2B, which has been shown to be one of the activin type II receptors involved in mediating myostatin signaling (7, 18, 49). The soluble form of ACVR2B (ACVR2B/Fc) was shown to be capable of blocking myostatin activity *in vitro*, and administration of ACVR2B/Fc to adult mice was shown to cause dramatic muscle growth (up to 40–60% in 2 wk). Significantly, this effect was attenuated, but not eliminated, in $Mstn^{-/-}$ mice, implying that ACVR2B/Fc was targeting at least one additional ligand that also functions to block muscle growth (7). A second set of experiments was the analysis of transgenic mice overexpressing follistatin in muscle (18). As expected, based on the ability of follistatin to inhibit myostatin, these transgenic mice exhibited significant increases in muscle mass. As in the studies with the soluble ACVR2B receptor, however, the follistatin transgene could also cause increases in muscle
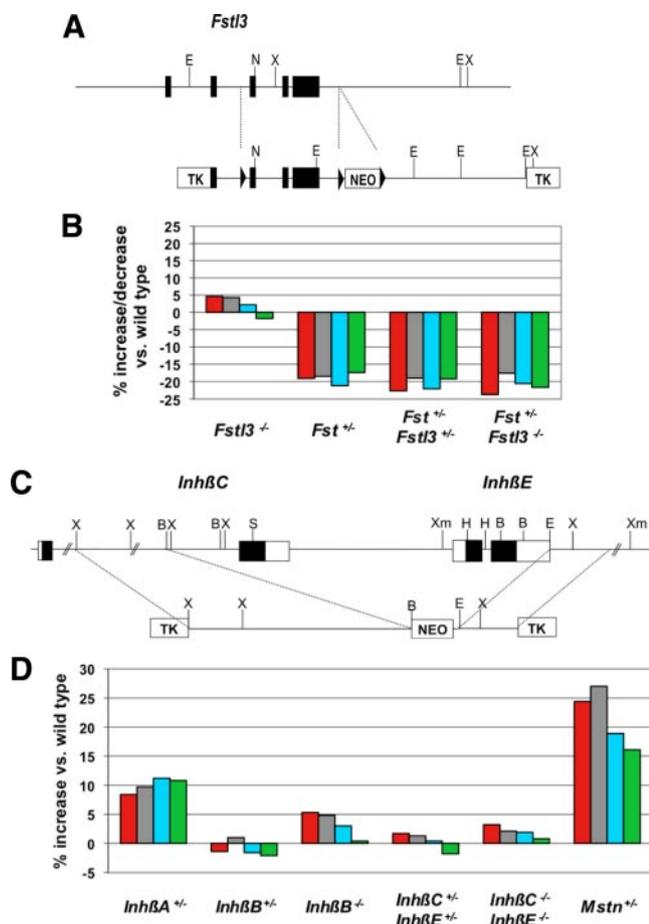
FIG. 5. Effect of a mutation in *Fstl3* and in genes encoding inhibin β-subunits on muscle mass. A, Diagram of *Fstl3*-targeting strategy. Mice carrying the targeted allele were crossed to Ella-cre transgenic mice (33) to generate mice in which recombination had occurred between the outside LoxP sites (denoted by *triangles*), thereby resulting in a mutant allele in which exons 3–5 were completely deleted in the germline. B, Effects of the deletion mutation in *Fstl3* either alone or in combination with heterozygous loss of *Fst*. C, Diagram of *InhβC/InhβE*-targeting strategy. D, Effect of *Inhβ* mutations in male mice. In the *bar graphs* shown in Panels b and d, *numbers* represent percent increase or decrease in muscle mass relative to *wild-type* mice and were calculated from the data shown in Table 1. Data from *Mstn*^{+/−} mice (21) are shown for comparison. Muscles analyzed were: pectoralis (*red*), triceps (*gray*), quadriceps (*blue*), and gastrocnemius (*green*). TK, Thymidine kinase.

growth even in mice lacking myostatin (21); in fact, the follistatin transgene could cause yet another doubling of muscle mass on top of the doubling seen in the absence of myostatin (*i.e.* an overall quadrupling).

All of these studies demonstrate that at least one other TGF-β family member, in addition to myostatin, also functions to limit muscle mass *in vivo*. Thus, the capacity for increasing muscle growth by targeting this signaling pathway is much more substantial than previously appreciated. We have been using a genetic approach to determining the identity of this other ligand. An obvious candidate was GDF-11, which is highly related to myostatin (1, 50). Initial gene-knockout studies demonstrated that

mice completely lacking GDF-11 exhibit multiple developmental defects and die during the perinatal period (51), which precluded a detailed analysis of the role of GDF-11 in muscle. Subsequent studies utilizing a floxed *Gdf11* allele, however, revealed no effect of *Gdf11* deletion specifically in skeletal muscle either alone or in combination with a *Mstn*-knockout mutation (26).

Perhaps the next most likely candidates were the activins, which have been shown to be capable of regulating differentiation of muscle cells in culture (36–39) and share a common receptor with myostatin (for reviews, see Refs. 15 and 34). Moreover, a recent study also suggested the possibility that activins may be involved in regulating muscle mass based on their ability to induce muscle atrophy when overexpressed *in vivo* and based on differential effects seen *in vivo* between follistatin and a follistatin variant with reduced affinity for activin (40). Finally, another recent study implicated activins as well as a number of other ligands, including GDF-11, BMP-9, and BMP-10, as possible candidates based on the fact that these could be affinity purified from serum using the ACVR2B/Fc ligand trap (38). Indeed, by analyzing mouse strains carrying targeted deletions in each of the genes encoding the inhibin β-subunits, we observed increases in muscle mass in mice heterozygous for the *InhβA* mutation, consistent with an important role for activin A in regulating muscle mass. Although further characterization of the muscles of these mice will be required to demonstrate that these effects on muscle mass result from muscle fiber hypertrophy, these data provide the first genetic loss-of-function evidence that activin A may be one of the key ligands that function with myostatin to limit muscle mass.

Although the increases in muscle mass that we observed in *InhβA* mutants were relatively modest, we believe that the overall role that activin A may play is potentially much more substantial for several reasons. First, this phenotype was observed in mice that still retained one functional copy of the *InhβA* gene. By comparison, male mice heterozygous for a mutation in *Mstn* exhibit increases in muscle weights ranging from 16–27% (21); hence, the magnitude of the effects seen in male *InhβA*^{+/−} mice was approximately half that seen in *Mstn*^{+/−} mice. Homozygous loss of *Mstn* results in increases in muscle weights of 100–150%, and we presume that greater loss of activin A signaling would similarly result in a significantly enhanced effect. Second, the existence of multiple *Inhβ* genes raises the possibility of functional redundancy, and in this respect, we did see some effect, albeit quite small, in *InhβB* homozygous mutants. Third, a mutation in the *InhβA* gene affects the production of both activin A (as well as activin AB) and inhibin A, which

share the βA-subunit. Given that activins and inhibins generally have counteracting activities, it is perhaps fortuitous that we were able to see any phenotype at all in $Inh\beta A^{+/-}$ mice, because the mutation would lead to decreases in both activin A and an inhibitor of activin signaling. We believe that the likely explanation is that whereas activins are believed to act mostly via a paracrine mechanism, inhibins appear to be capable of regulating signaling in an endocrine manner (for reviews, see Refs. 52–54), and the predominant circulating form of inhibin is known to be inhibin B (for reviews, see Refs. 55 and 56). Hence, the $Inh\beta A$ mutation would be predicted to reduce levels of activin A but have only a minimal effect on circulating inhibin levels.

Clearly, additional studies will be required to elucidate the precise roles that all of the activin isoforms may play in regulating muscle growth and function in different physiological states. It is interesting to note, however, that circulating levels of activin A in humans have been shown to increase during aging, and conversely, circulating levels of inhibin B have been shown to decrease during aging (57–61), raising the intriguing possibility that enhanced activin signaling during aging may be a key contributing factor in the etiology of age-related sarcopenia. Understanding how muscle homeostasis is coordinately regulated by myostatin and by activins under both normal and pathological conditions will be essential for developing the most optimal strategies to tap the full potential of targeting this general signaling pathway to preserve muscle mass and prevent muscle atrophy in a variety of clinical settings associated with debilitating loss of muscle function.

Materials and Methods

Targeting constructs were generated from 129 SvJ genomic clones and used to transfect R1 embryonic stem cells (kindly provided by A. Nagy, R. Nagy, and W. Abramow-Newerly). Blastocyst injections of targeted clones were carried out by the Johns Hopkins Transgenic Core Facility. All mice were back-crossed at least six times onto a C57BL/6 background before analysis. All analysis was carried out on 10-wk-old mice, except for the force measurements and cardiotoxin studies, which were carried out on 14-wk-old mice. All animal experiments were carried out in accordance with protocols that were approved by the Institutional Animal Care and Use Committees at the Johns Hopkins University School of Medicine and the University of Pennsylvania School of Dental Medicine.

For measurement of muscle weights, muscles were dissected from both sides of the animal and weighed, and the average weight was used. For morphometric analysis, the gastrocnemius muscle was sectioned to its widest point using a cryostat, and fiber diameters were measured as the shortest width passing through the center of the fiber. Measurements were carried out on 84 type I fibers, 150 type IIa fibers, and 150 type IIb fibers per muscle, and mean fiber diameters for each type were calculated for each animal. For plotting the distribution of fiber sizes, data from all three mice in each group were pooled. Measurements were also carried out on 250 fibers of mixed types randomly selected from five representative areas of each section (every attempt was made to analyze the same five regions from muscle to muscle) to estimate overall mean fiber diameters.

For isolated muscle mechanics, mice were anesthetized with ketamine/xylazine. Muscles were removed and placed in a bath of Ringers solution gas equilibrated with 95% O_2/5% CO_2. Sutures were attached to the distal and proximal tendons of the EDL and soleus muscles. Muscles were subjected to isolated mechanical measurements using a previously described apparatus (Aurora Scientific, Ontario, Canada) (62). After optimal length (Lo) was determined by supramaximal twitch stimulation, maximal isometric tetanus was measured in the muscles during a 500-msec stimulation. For histological analysis, samples were rinsed in PBS, blotted, weighed, covered in mounting medium before freezing in melting isopentane, and then stored at -80 C. Muscle cross-sectional areas were determined using the following formula: cross-sectional area = m/(Lo $\times$ L/Lo $\times$ 1.06 g/cm^3), where m is muscle mass, Lo is muscle length, L/Lo is the ratio of fiber length to muscle length, and 1.06 is the density of muscle (63). L/Lo was 0.45 for EDL and 0.69 for soleus.

For muscle fiber typing, 10-μm frozen cross-sections taken from the midbelly of each muscle were subjected to immunohistochemistry for laminin (rabbit antilaminin Ab-1; Neomarkers, Fremont, CA) to outline the muscle fibers. Fiber typing was performed with antibodies recognizing myosin heavy chain (MHC)2a (SC-71), MHC 2b (BF-F3), and MHC 1 (BAF-8) as previously described (64). Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Images were acquired on an epifluorescence microscope (Leica, Deerfield, IL) and analyzed for the proportion of myosin-positive fibers using image analysis software (OpenLab, Improvision; Coventry, UK).

For skeletal muscle injury studies, 250 μl of cardiotoxin (10 μM *Naja nigricollis*; Calbiochem, La Jolla, CA) were injected into the gastrocnemius, and muscles were harvested 4 d or 21 d after induction of injury. Quantification of areas of fibrosis per area of muscle injury was performed using Nikon's NIS elements BR3.0 software (Laboratory Imaging; Nikon, Melville, NY).

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