Functional redundancy of type I and type II receptors in the regulation of skeletal muscle growth by myostatin and activin A

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Myostatin (MSTN) is a transforming growth factor-β (TGF-β) family member that normally acts to limit muscle growth. The function of MSTN is partially redundant with that of another TGF-β family member, activin A. MSTN and activin A are capable of signaling through a complex of type II and type I receptors. Here, we investigated the roles of two type II receptors (ACVR2 and ACVR2B) and two type I receptors (ALK4 and ALK5) in the regulation of muscle mass by these ligands by genetically targeting these receptors either alone or in combination specifically in myofibers in mice. We show that targeting signaling in myofibers is sufficient to cause significant increases in muscle mass, showing that myofibers are the direct target for signaling by these ligands in the regulation of muscle growth. Moreover, we show that there is functional redundancy between the two type II receptors as well as between the two type I receptors and that all four type II/type I receptor combinations are utilized in vivo. Targeting signaling specifically in myofibers also led to reductions in overall body fat content and improved glucose metabolism in mice fed either regular chow or a high-fat diet, demonstrating that these metabolic effects are the result of enhanced muscling. We observed no effect, however, on either bone density or muscle regeneration in mice in which signaling was targeted in myofibers. The latter finding implies that MSTN likely signals to other cells, such as satellite cells, in addition to myofibers to regulate muscle homeostasis.

myostatin | activin | receptors | skeletal muscle

Myostatin (MSTN) is a secreted signaling molecule that normally acts to limit skeletal muscle growth (for review, see ref. 1). Mice lacking MSTN exhibit dramatic increases in muscle mass throughout the body, with individual muscles growing to about twice the normal size (2). MSTN appears to play two distinct roles in regulating muscle size, one to regulate the number of muscle fibers that are formed during development and a second to regulate the growth of those fibers postnatally. The sequence of MSTN has been highly conserved through evolution, with the mature MSTN peptide being identical in species as divergent as humans and turkeys (3). The function of MSTN has also been conserved, and targeted or naturally occurring mutations in *MSTN* have been shown to cause increased muscling in numerous species, including cattle (3–5), sheep (6), dogs (7), rabbits (8), rats (9), swine (10), goats (11), and humans (12). Numerous pharmaceutical and biotechnology companies have developed biologic agents capable of blocking MSTN activity, and these have been tested in clinical trials for a wide range of indications, including Duchenne and facioscapulohumeral muscular dystrophy, inclusion body myositis, muscle atrophy following falls and hip fracture surgery, age-related sarcopenia, Charcot–Marie–Tooth disease, and cachexia due to chronic obstructive pulmonary disease, end-stage kidney disease, and cancer.

The finding that certain inhibitors of MSTN signaling can increase muscle mass even in $Mstn^{-/-}$ mice revealed that the function of MSTN as a negative regulator of muscle mass is partially redundant with at least one other TGF-β family member (13, 14), and subsequent studies have identified activin A as one of these cooperating ligands (15, 16). MSTN and activin A share many key regulatory and signaling components. For example, the activities of both MSTN and activin A can be modulated extracellularly by naturally occurring inhibitory binding proteins, including follistatin (17, 18) and the follistatin-related protein, FSTL-3 or FLRG (19, 20). Moreover, MSTN and activin A also appear to share receptor components. Based on in vitro studies, MSTN is capable of binding initially to the activin type II receptors, ACVR2 and ACVR2B (also called ActRIIA and ActRIIB) (18) followed by engagement of the type I receptors, ALK4 and ALK5 (21). In previous studies, we presented genetic evidence supporting a role for both ACVR2 and ACVR2B in mediating MSTN signaling and regulating muscle mass in vivo. Specifically, we showed that mice expressing a truncated, dominant-negative form of ACVR2B in skeletal muscle (18) or

Significance

Myostatin and activin A are secreted signaling molecules that act to limit skeletal muscle growth. Here, we show that myostatin and activin A utilize four receptor components to signal directly to muscle fibers. These findings have implications for strategies to target this signaling pathway for clinical applications to treat patients with muscle loss.

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Competing interest statement: The authors are in the process of filing a patent application based on some of the findings reported in this paper.

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carrying deletion mutations in *Acvr2* and/or *Acvr2b* (13) have significantly increased muscle mass. One limitation of the latter study, however, was that we could not examine the consequence of complete loss of both receptors using the deletion alleles, as double homozygous mutants die early during embryogenesis (22). Moreover, the roles that the two type I receptors, ALK4 and ALK5, play in regulating MSTN and activin A signaling in muscle in vivo have not yet been documented using genetic approaches. Here, we present the results of studies in which we used floxed alleles for each of the type II and type I receptor genes in order to target these receptors alone and in combination in muscle fibers. We show that these receptors are functionally redundant and that signaling through each of these receptors contributes to the overall control of muscle mass.

Results

To determine the effect of targeting the two known type II receptors for MSTN specifically in skeletal muscle, we generated mice carrying floxed alleles for both *Acvr2* and *Acvr2b* (23, 24) and then targeted these alleles using a transgene expressing cre recombinase from a myosin light chain promoter/enhancer (*Myl1*-cre) (25), which we showed to be expressed by skeletal muscle fibers but not by satellite cells (23). Quantification of receptor messenger RNA (mRNA) levels in various muscles showed that we were able to reduce expression significantly in each of the floxed lines, with the residual expression likely representing RNA made either by type I fibers or by nonmuscle cells (*SI Appendix*, Fig. S1). Significantly, in mice in which a given receptor was targeted in muscle, expression levels of each of the other receptors were relatively unchanged, indicating that there was not a compensatory up-regulation of expression of any of the other receptors.

As we reported previously (23), targeting *Acvr2b* alone in muscle fibers resulted in increases in muscle mass of about 8 to 12% in females and about 4 to 6% in males depending on the specific muscle examined (Fig. 1*A* and Table 1). Targeting *Acvr2* alone resulted in similar, statistically significant increases in the quadriceps and gastrocnemius but not in the pectoralis or triceps. Targeting both receptors simultaneously resulted in much more substantial increases in all muscles examined, with the greatest effect being seen in the quadriceps (58 and 50% in females and males, respectively) and gastrocnemius (72 and 62% in females and males, respectively). These results show definitively that *Acvr2* and *Acvr2b* are functionally redundant with one another with respect to limiting muscle mass and that targeting signaling just in myofibers is sufficient to induce significant

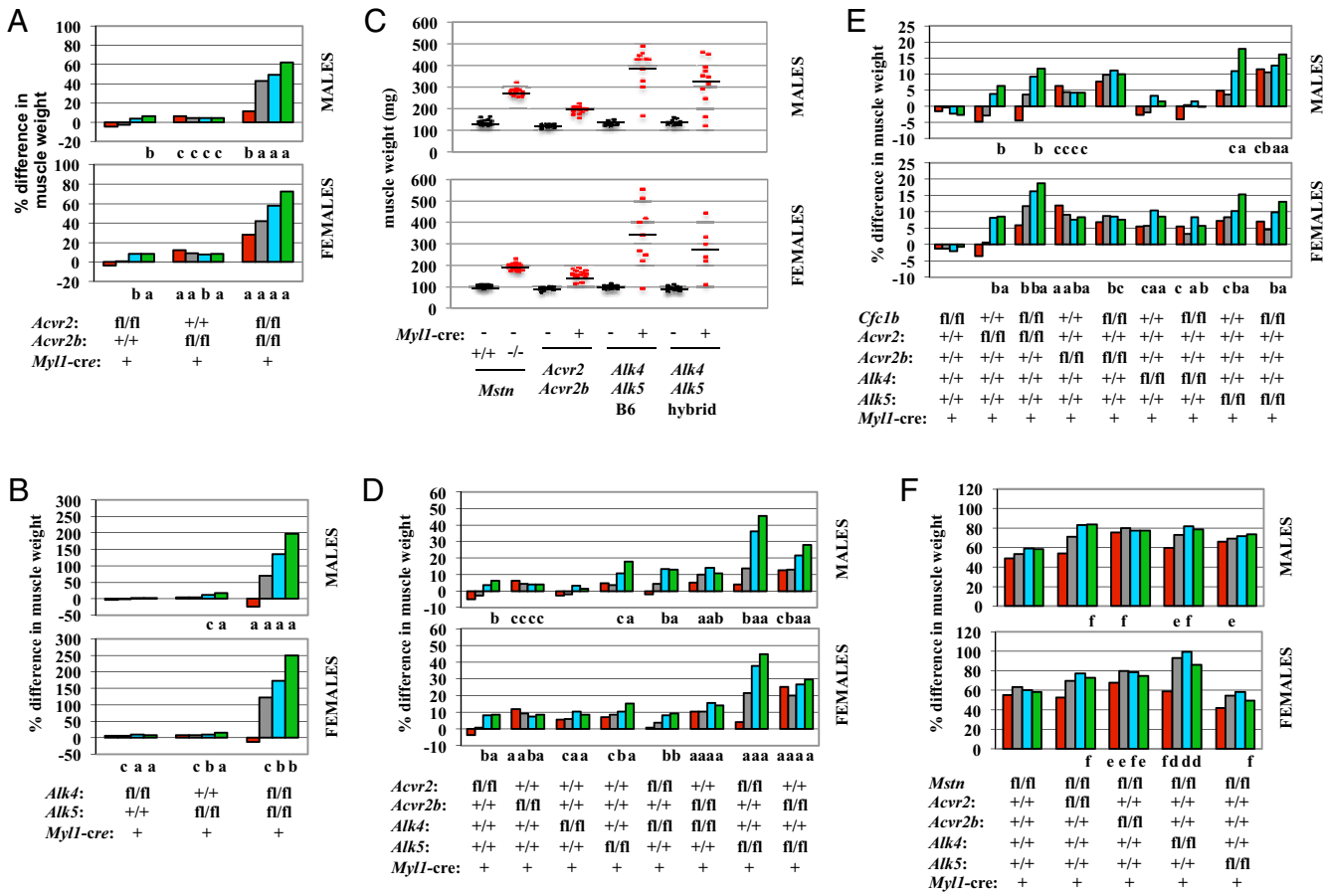

Fig. 1. Effect of targeting type II and type I receptors in myofibers on muscle weights. (*A* and *B*) Relative weights of pectoralis (red), triceps (gray), quadriceps (blue), and gastrocnemius/plantaris (green) muscles in mice in which *Acvr2* and/or *Acvr2b* (*A*) or *Alk4* and/or *Alk5* (*B*) were targeted. Numbers are expressed as percent increase/decrease relative to the same receptor genotypes but in the absence of *Myl1*-cre. (*C*) Gastrocnemius/plantaris muscle weights of individual wild-type C57BL/6 and $Mstn^{-/-}$ mice or individual mice in which *Acvr2*/*Acvr2b* or *Alk4*/*Alk5* were targeted in myofibers. Bars indicates mean values. (*D*) Relative muscle weights of mice in which an individual type II receptor (*Acvr2* or *Acvr2b*) was targeted along with an individual type I receptor (*Alk4* or *Alk5*). (*E* and *F*) Relative muscle weights of mice in which an individual type II or type I receptor was targeted along with *Cfc1b* (*E*) or *Mstn* (*F*). Numbers are expressed as percent increase/decrease relative to the same receptor genotypes but in the absence of *Myl1*-cre, and the color code is the same as in *A* and *B*. The numbers shown in *A*, *B*, *D*, and *F* were calculations based on muscle weights shown in Tables 1–3, which also contain the numbers of mice in each group. [a]$P < 0.001$ vs. cre$^-$; [b]$P < 0.01$ vs. cre$^-$; [c]$P < 0.05$ vs. cre$^-$; [d]$P < 0.001$ vs. *Mstn fl/fl*, cre$^+$; [e]$P < 0.01$ vs. *Mstn fl/fl*, cre$^+$; [f]$P < 0.05$ vs. *Mstn fl/fl*, cre$^+$.

Table 1. Muscle weights

Acvr2	*Acvr2b*	*Alk4*	*Alk5*	*Bmpr2*	cre	*n*	Pectoralis, mg	Triceps, mg	Quadriceps, mg	Gastrocnemius, mg
Males										
fl/fl	+/+	+/+	+/+	+/+	–	17	64.1 ± 1.1	86.4 ± 1.2	170.2 ± 2.0	122.9 ± 1.6
fl/fl	+/+	+/+	+/+	+/+	+	14	61.0 ± 1.9	83.9 ± 1.8	176.6 ± 2.8	130.7 ± 2.2*
+/+	*fl/fl*	+/+	+/+	+/+	–	31	64.9 ± 1.1	90.3 ± 1.2	180.7 ± 2.1	127.9 ± 1.5
+/+	*fl/fl*	+/+	+/+	+/+	+	21	69.0 ± 1.2†	94.2 ± 1.6†	188.1 ± 2.8†	133.2 ± 1.8†
+/+	+/+	*fl/fl*	+/+	+/+	–	19	76.4 ± 1.4	98.1 ± 1.5	192.8 ± 2.6	137.4 ± 2.0
+/+	+/+	*fl/fl*	+/+	+/+	+	11	74.4 ± 2.0	96.3 ± 1.9	199.1 ± 3.5	139.4 ± 3.3
+/+	+/+	+/+	*fl/fl*	+/+	–	16	72.3 ± 2.2	97.4 ± 2.6	196.1 ± 5.6	129.3 ± 3.2
+/+	+/+	+/+	*fl/fl*	+/+	+	18	75.7 ± 2.5	100.9 ± 3.4	217.3 ± 6.9†	152.2 ± 5.1‡
fl/fl	*fl/fl*	+/+	+/+	+/+	–	13	60.4 ± 1.8	83.3 ± 2.1	167.4 ± 3.5	120.8 ± 2.2
fl/fl	*fl/fl*	+/+	+/+	+/+	+	16	67.3 ± 1.6*	118.9 ± 3.9‡	250.3 ± 6.3‡	195.6 ± 4.1‡
+/+	+/+	*fl/fl*	*fl/fl*	+/+	–	13	72.5 ± 1.8	93.8 ± 1.7	183.5 ± 3.6	131.5 ± 2.5
+/+	+/+	*fl/fl*	*fl/fl*	+/+	+	11	54.6 ± 4.1‡	160.0 ± 13.4‡	433.1 ± 25.1‡	390.0 ± 29.1‡
fl/fl	+/+	*fl/fl*	+/+	+/+	–	14	72.4 ± 1.3	95.0 ± 1.6	182.9 ± 3.2	135.4 ± 1.6
fl/fl	+/+	*fl/fl*	+/+	+/+	+	15	70.9 ± 1.7	99.0 ± 2.5	207.1 ± 6.1*	152.9 ± 3.9‡
+/+	*fl/fl*	*fl/fl*	+/+	+/+	–	13	76.3 ± 2.0	98.1 ± 1.9	191.2 ± 3.8	136.4 ± 3.1
+/+	*fl/fl*	*fl/fl*	+/+	+/+	+	13	80.2 ± 1.8	107.8 ± 2.0‡	218.1 ± 4.6‡	150.8 ± 3.3*
fl/fl	+/+	+/+	*fl/fl*	+/+	–	16	69.6 ± 2.3	93.5 ± 2.4	187.8 ± 6.2	129.6 ± 3.6
fl/fl	+/+	+/+	*fl/fl*	+/+	+	10	72.4 ± 2.5	106.2 ± 3.8*	255.9 ± 9.0‡	188.4 ± 9.9‡
+/+	*fl/fl*	+/+	*fl/fl*	+/+	–	18	77.3 ± 2.3	105.7 ± 3.0	210.2 ± 6.1	141.4 ± 3.6
+/+	*fl/fl*	+/+	*fl/fl*	+/+	+	18	87.0 ± 3.0†	119.3 ± 3.2*	255.1 ± 6.1‡	180.7 ± 4.4‡
+/+	+/+	+/+	+/+	*fl/fl*	–	15	83.1 ± 2.0	106.5 ± 2.7	213.9 ± 4.7	148.7 ± 3.2
+/+	+/+	+/+	+/+	*fl/fl*	+	11	79.8 ± 3.0	105.5 ± 4.1	196.4 ± 5.6†	138.4 ± 3.8†
fl/fl	*fl/fl*	+/+	+/+	*fl/fl*	–	5	71.8 ± 2.6	96.4 ± 4.5	178.6 ± 9.3	131.4 ± 6.0
fl/fl	*fl/fl*	+/+	+/+	*fl/fl*	+	6	81.2 ± 4.3	133.2 ± 9.5*	242.7 ± 11.8*	198.0 ± 3.4‡
Females										
fl/fl	+/+	+/+	+/+	+/+	–	17	43.0 ± 1.0	66.1 ± 1.3	132.8 ± 2.7	94.8 ± 1.6
fl/fl	+/+	+/+	+/+	+/+	+	15	41.5 ± 0.9	66.5 ± 1.0	143.7 ± 2.9*	102.8 ± 1.2‡
+/+	*fl/fl*	+/+	+/+	+/+	–	20	45.7 ± 0.9	70.0 ± 1.2	142.9 ± 2.4	99.3 ± 1.7
+/+	*fl/fl*	+/+	+/+	+/+	+	34	51.2 ± 0.8‡	76.4 ± 1.2‡	153.8 ± 2.2*	107.6 ± 1.4‡
+/+	+/+	*fl/fl*	+/+	+/+	–	19	50.8 ± 1.0	73.0 ± 1.0	143.4 ± 2.0	102.7 ± 1.4
+/+	+/+	*fl/fl*	+/+	+/+	+	27	53.7 ± 1.5	77.2 ± 1.8†	158.4 ± 2.9‡	111.4 ± 2.3‡
+/+	+/+	+/+	*fl/fl*	+/+	–	14	43.9 ± 1.5	68.3 ± 1.9	136.3 ± 3.7	91.2 ± 1.7
+/+	+/+	+/+	*fl/fl*	+/+	+	19	47.0 ± 1.1	74.1 ± 1.8†	150.3 ± 3.7*	105.2 ± 2.7‡
fl/fl	*fl/fl*	+/+	+/+	+/+	–	11	41.1 ± 1.2	65.8 ± 1.7	128.2 ± 3.6	90.8 ± 2.4
fl/fl	*fl/fl*	+/+	+/+	+/+	+	15	52.7 ± 1.8‡	93.6 ± 2.5‡	202.6 ± 5.8‡	156.3 ± 5.6‡
+/+	+/+	*fl/fl*	*fl/fl*	+/+	–	14	50.1 ± 1.5	70.4 ± 1.6	142.2 ± 3.0	97.4 ± 1.9
+/+	+/+	*fl/fl*	*fl/fl*	+/+	+	9	43.4 ± 3.5	156.9 ± 27.9†	387.9 ± 60.3*	339.9 ± 52.0*
fl/fl	+/+	*fl/fl*	+/+	+/+	–	10	48.4 ± 0.9	71.2 ± 1.1	144.7 ± 2.4	105.7 ± 1.9
fl/fl	+/+	*fl/fl*	+/+	+/+	+	13	48.8 ± 1.5	73.8 ± 1.1	156.4 ± 3.5*	115.5 ± 2.9*
+/+	*fl/fl*	*fl/fl*	+/+	+/+	–	24	53.5 ± 0.9	75.8 ± 1.3	150.5 ± 2.5	106.8 ± 1.6
+/+	*fl/fl*	*fl/fl*	+/+	+/+	+	20	59.0 ± 1.2‡	83.5 ± 1.5‡	174.1 ± 2.4‡	121.7 ± 1.6‡
fl/fl	+/+	+/+	*fl/fl*	+/+	–	15	45.7 ± 1.2	65.7 ± 1.4	131.7 ± 2.5	89.7 ± 1.8
fl/fl	+/+	+/+	*fl/fl*	+/+	+	17	47.5 ± 1.1	79.6 ± 2.5‡	181.5 ± 5.1‡	129.8 ± 3.3‡
+/+	*fl/fl*	+/+	*fl/fl*	+/+	–	20	44.9 ± 0.8	68.3 ± 1.4	137.9 ± 2.4	93.9 ± 2.0
+/+	*fl/fl*	+/+	*fl/fl*	+/+	+	18	56.3 ± 1.6‡	81.8 ± 1.4‡	174.6 ± 3.6‡	121.7 ± 3.2‡
+/+	+/+	+/+	+/+	*fl/fl*	–	17	50.3 ± 1.3	74.2 ± 1.9	146.9 ± 3.1	101.1 ± 2.2
+/+	+/+	+/+	+/+	*fl/fl*	+	20	46.5 ± 1.1†	68.9 ± 1.3†	133.8 ± 2.6*	87.9 ± 3.5*
fl/fl	*fl/fl*	+/+	+/+	*fl/fl*	–	8	48.6 ± 1.6	71.8 ± 2.0	140.0 ± 4.2	100.9 ± 3.1
fl/fl	*fl/fl*	+/+	+/+	*fl/fl*	+	5	50.4 ± 2.1	100.4 ± 11.1†	184.6 ± 6.3‡	156.4 ± 8.2‡

*$P < 0.01$ vs. cre$^-$.
†$P < 0.05$ vs. cre$^-$.
‡$P < 0.001$ vs. cre$^-$.

muscle growth, confirming the conclusions from our earlier studies that myofibers are the primary direct target for signaling by MSTN in the regulation of muscle growth (23).

In vitro studies have suggested that binding of MSTN to ACVR2 and/or ACVR2B leads to engagement of the type I receptors ALK4 and ALK5 (21). To provide genetic support for roles of these type I receptors in mediating MSTN signaling in vivo, we analyzed the effect of targeting *Alk4* and *Alk5* specifically in myofibers. For *Alk4*, we generated a mouse line in which we flanked exons 2 to 3 with LoxP sites (*SI Appendix*, Fig. S2); removal of these exons by cre recombinase would be predicted to delete the entire ligand-binding and transmembrane domains and result in a null allele. For *Alk5*, we utilized a floxed *Alk5* line that had been described previously (26). As in the case of the type II receptors, the *Myl1*-cre transgene significantly reduced RNA levels for both *Alk4* and *Alk5* in muscle (*SI Appendix*, Fig. S1). Targeting *Alk4* or *Alk5* in myofibers resulted in statistically significant effects on muscle mass, ranging from up to

11% in the case of *Alk4* and up to 18% in the case of *Alk5*, depending on the sex of the mice and the specific muscles examined (Fig. 1*B* and Table 1). As in the case of the type II receptors, targeting both type I receptors simultaneously resulted in much more substantial increases, with the greatest effects being seen in the quadriceps (173 and 136% in females and males, respectively) and gastrocnemius (249 and 197% in females and males, respectively). These increases were even more pronounced than those seen in $Mstn^{-/-}$ mice and more reminiscent of the magnitude of effects seen upon targeting multiple ligands (14). These results demonstrate that both ALK4 and ALK5 play critical roles in limiting skeletal muscle mass in vivo, that these two receptors are functionally redundant in muscle, and that together, they regulate signaling by multiple ligands in myofibers, most likely MSTN and activin A.

The increases seen upon targeting the two type I receptors were much more extensive than those seen upon targeting the two type II receptors. This finding raised the possibility that another type II receptor may be utilized in addition to ACVR2 and ACVR2B. One possible candidate was BMPRII, although we were unable to detect significant binding of MSTN to BMPRII in transfected cells (18). Moreover, a previous study suggested that BMP signaling may actually have the opposite effect of inducing muscle growth (27); specifically, this study reported that expression of a constitutively active type I BMP receptor (ALK3) can induce muscle growth and, conversely, that overexpression of the BMP inhibitor, noggin, can cause muscle atrophy not only in wild-type mice but also in $Mstn^{-/-}$ mice. Nevertheless, we utilized mice carrying a floxed *Bmpr2* allele (28) to examine the possibility that MSTN and/or activin A may signal through BMPRII in muscle. As shown in Table 1, mice in which *Bmpr2* was targeted in myofibers generally had lower muscle weights than cre-negative mice, with the effects being more pronounced in females (7 to 13% depending on the muscle). Mice in which all three type II receptors (*Bmpr2*, *Acvr2*, and *Acvr2b*) were targeted had muscle weights that were comparable to those seen in mice in which just *Acvr2* and *Acvr2b* were targeted. These results imply that BMPRII activity in *Acvr2*/*Acvr2b* mutant mice is unlikely to explain the difference seen between *Acvr2*/*Acvr2b* targeted mice and *Alk4*/*Alk5* targeted mice.

The effects of targeting *Alk4* in combination with *Alk5* were striking not only in terms of their magnitude but also in terms of the variability from mouse to mouse. As shown in Fig. 1*C*, the weight of the gastrocnemius muscle was relatively consistent in wild-type and $Mstn^{-/-}$ mice as well as in mice in which both *Acvr2* and *Acvr2b* were targeted in myofibers. In contrast, the weight of the gastrocnemius muscle was highly variable in mice targeting both *Alk4* and *Alk5* in myofibers, ranging from wild-type levels in some mice to over five times wild-type levels in other mice. This variability, which was also seen in the other muscles that were examined, was unlikely to be due to one or more genetic modifiers segregating independently in these crosses, as we observed similar distributions of effects both on a hybrid C57BL/6-129SvJ and on a pure C57BL/6 background. Clearly, further investigation of the basis for this variability may provide important insights into the control of muscle growth by this signaling pathway.

Given that the results of these genetic studies clearly demonstrated roles for both type II receptors and both type I receptors in mediating muscle growth, we sought to determine whether all four possible combinations of type II and type I receptors (ACVR2/ALK4, ACVR2/ALK5, ACVR2B/ALK4, and ACVR2B/ALK5) are utilized in vivo. Our strategy was to target these receptors pairwise such that we would target one type II receptor with one type I receptor. Our rationale was that by targeting just one receptor of each type, only a single possible type II/type I receptor combination would remain. For example, by targeting both *Acvr2b* and *Alk5*, we examined the role of ACVR2/ALK4, which is the only one of the four possible combinations whose function would be preserved in these mice. As shown in Fig. 1*D* and Table 1, targeting both *Acvr2b* and *Alk5* resulted generally in greater increases in muscle mass than targeting either alone, implying that the ACVR2/ALK4 combination cannot be the sole one that is used in vivo; however, the increases seen in these mice did not approach those seen either in mice lacking both type II receptors or in mice lacking both type I receptors, implying that the ACVR2/ALK4 combination does play some role in signaling. By targeting all four pairwise combinations in this manner, we found that all four type II/type I combinations are functional in vivo and that no one combination is sufficient to maintain wild-type signaling levels in the absence of the other combinations. The most significant effects were observed in mice in which we targeted both *Acvr2* and *Alk5*, which exhibited muscle mass increases of ~40% in some muscles, implying that the ACVR2B/ALK4 combination is the least important of the four combinations in limiting muscle growth; these increases, however, were significantly less than those seen even upon targeting both *Acvr2* and *Acvr2b*, implying that the ACVR2B/ALK4 combination is capable of playing some role in regulating growth of myofibers.

Another receptor component that has been implicated in MSTN signaling is cripto (*Cfc1b*), which is known to serve as a coreceptor for certain ligands and to antagonize the activity of other ligands (for review, see ref. 29). In this regard, one study using C2C12 myoblasts reported that cripto is required for MSTN signaling but inhibits activin A signaling (30); another study, however, showed that during muscle regeneration in vivo, cripto expressed by satellite cells acts to antagonize MSTN signaling (31). To determine whether cripto plays a role in regulating signaling in myofibers, we used the *Myl1*-cre transgene to target *Cfc1b* either alone or in combination with each of the type I or type II receptors. As shown in Fig. 1*E* and Table 2, we observed no effect of targeting *Cfc1b* alone on muscle mass. We did, however, see an effect of targeting *Cfc1b* in combination with *Acvr2*, which resulted in small, but significant, increases in muscle mass compared to targeting *Acvr2* alone. This finding implies that at least some signaling through ACVR2B requires cripto function, although the fact that the effects seen in these mice were substantially lower than those seen in mice in which we targeted both *Acvr2* and *Acvr2b* implies that most signaling through ACVR2B in myofibers does not require cripto. Significantly, we observed no consistent effect of targeting *Cfc1b* in combination with *Alk5*, implying that cripto expressed in myofibers is not required for signaling through ALK4 in myofibers, which contrasts with what has been reported in cell culture studies (30).

In previous studies, we showed that the function of MSTN in limiting muscle growth is redundant with that of at least one other TGF-β family member (13, 14), and several studies have implicated activin A as at least one of the key cooperating ligands (15, 16). To determine whether particular receptor components are used exclusively by particular ligands, we analyzed the effect of targeting each of the type II and type I receptors in combination with *Mstn*. Targeting *Mstn* specifically in myofibers utilizing mice carrying a floxed *Mstn* allele that we had generated previously (32) resulted in a reduction of *Mstn* mRNA in muscle by 95 to 99% and a reduction in circulating levels of MSTN protein by 92 and 85% in females and males, respectively (*SI Appendix*, Fig. S3). We presume that the residual expression represents MSTN made by type I fibers, and although we cannot rule out the possibility of non–muscle-derived MSTN contributing significantly to the circulating pool, these data are consistent with myofibers being at least the predominant (if not sole) source of circulating MSTN protein.

Targeting *Mstn* in myofibers led to increases in muscle mass of 55 to 64% and 49 to 59% in females and males, respectively,

Table 2. Muscle weights in *Cfc1b*-targeted mice

Acvr2	*Acvr2b*	*Alk4*	*Alk5*	*Cfc1b*	cre	*n*	Pectoralis, mg	Triceps, mg	Quadriceps, mg	Gastrocnemius, mg
Males										
+/+	+/+	+/+	+/+	*fl/fl*	–	11	64.6 ± 2.0	90.7 ± 3.5	176.1 ± 6.0	124.6 ± 3.6
+/+	+/+	+/+	+/+	*fl/fl*	+	20	63.7 ± 1.5	90.4 ± 2.1	171.9 ± 3.9	121.3 ± 2.6
fl/fl	+/+	+/+	+/+	*fl/fl*	–	10	71.4 ± 2.4	96.1 ± 2.9	184.6 ± 6.2	134.7 ± 3.5
fl/fl	+/+	+/+	+/+	*fl/fl*	+	18	68.3 ± 2.3	99.6 ± 2.9	201.6 ± 6.8	150.3 ± 4.5*
+/+	*fl/fl*	+/+	+/+	*fl/fl*	–	15	73.0 ± 2.2	97.3 ± 3.4	187.3 ± 7.0	136.5 ± 4.3
+/+	*fl/fl*	+/+	+/+	*fl/fl*	+	8	78.6 ± 4.6	106.9 ± 4.4	208.1 ± 9.5	150.1 ± 5.7
+/+	+/+	*fl/fl*	+/+	*fl/fl*	–	13	76.7 ± 1.5	97.1 ± 1.9	195.1 ± 4.1	140.7 ± 2.4
+/+	+/+	*fl/fl*	+/+	*fl/fl*	+	16	73.5 ± 1.4	97.4 ± 1.8	198.2 ± 4.2	140.3 ± 2.5
+/+	+/+	+/+	*fl/fl*	*fl/fl*	–	17	62.6 ± 1.4	83.5 ± 1.7	164.1 ± 2.5	110.8 ± 1.3
+/+	+/+	+/+	*fl/fl*	*fl/fl*	+	17	69.8 ± 2.3†	92.4 ± 2.6*	184.9 ± 5.1‡	128.6 ± 3.4‡
Females										
+/+	+/+	+/+	+/+	*fl/fl*	–	14	42.1 ± 1.2	66.4 ± 1.3	129.1 ± 2.7	89.5 ± 2.2
+/+	+/+	+/+	+/+	*fl/fl*	+	17	41.6 ± 1.1	65.6 ± 1.7	126.5 ± 3.4	88.9 ± 2.5
fl/fl	+/+	+/+	+/+	*fl/fl*	–	11	43.4 ± 1.8	67.5 ± 2.0	131.9 ± 4.5	95.3 ± 3.0
fl/fl	+/+	+/+	+/+	*fl/fl*	+	11	45.9 ± 1.0	75.5 ± 1.3*	153.4 ± 3.8*	113.1 ± 3.4‡
+/+	*fl/fl*	+/+	+/+	*fl/fl*	–	6	47.5 ± 1.4	70.8 ± 2.4	139.7 ± 2.9	98.5 ± 2.5
+/+	*fl/fl*	+/+	+/+	*fl/fl*	+	5	50.8 ± 2.6	77.0 ± 2.3	151.6 ± 2.6*	106.0 ± 2.0†
+/+	+/+	*fl/fl*	+/+	*fl/fl*	–	20	50.6 ± 0.9	73.6 ± 1.2	147.5 ± 2.1	105.1 ± 1.6
+/+	+/+	*fl/fl*	+/+	*fl/fl*	+	12	53.4 ± 1.0†	75.9 ± 1.5	159.8 ± 2.7‡	111.1 ± 1.0*
+/+	+/+	+/+	*fl/fl*	*fl/fl*	–	26	44.5 ± 0.8	68.2 ± 1.1	128.8 ± 2.2	85.6 ± 1.5
+/+	+/+	+/+	*fl/fl*	*fl/fl*	+	17	47.7 ± 1.5	71.4 ± 1.9	141.4 ± 3.9*	96.9 ± 2.6‡

*$P < 0.01$ vs. cre$^-$.
†$P < 0.05$ vs. cre$^-$.
‡$P < 0.001$ vs. cre$^-$.

depending on the specific muscle (Fig. 1*F* and Table 3). Our strategy was to determine whether targeting a receptor component in addition to *Mstn* would result in further increases in muscle mass. Our rationale was that by simultaneously targeting, for example, both *Mstn* and one type II (or type I) receptor, we would be able to determine the contribution of the other type II (or type I) receptor in mediating activin A signaling. In general, targeting a given receptor in combination with *Mstn* led to greater increases in muscle mass compared to targeting *Mstn* alone. Of the combinations that we tested, the greatest statistically significant effects were seen in females in which *Mstn* was targeted along with *Acvr2b* or *Alk4*. These results imply that

Table 3. Muscle weights in *Mstn*-targeted mice

Acvr2	*Acvr2b*	*Alk4*	*Alk5*	*Mstn*	cre	*n*	Pectoralis, mg	Triceps, mg	Quadriceps, mg	Gastrocnemius, mg
Males										
+/+	+/+	+/+	+/+	*fl/fl*	–	21	69.3 ± 1.8	92.4 ± 1.9	184.3 ± 3.6	132.6 ± 3.0
+/+	+/+	+/+	+/+	*fl/fl*	+	21	103.3 ± 4.2*	141.9 ± 5.1*	293.4 ± 7.7*	209.6 ± 4.8*
fl/fl	+/+	+/+	+/+	*fl/fl*	–	16	68.4 ± 0.7	92.3 ± 1.4	177.0 ± 2.8	129.8 ± 1.4
fl/fl	+/+	+/+	+/+	*fl/fl*	+	15	105.3 ± 6.1*	157.6 ± 8.2*	324.0 ± 15.5*	238.5 ± 11.1*,†
+/+	*fl/fl*	+/+	+/+	*fl/fl*	–	15	65.9 ± 2.8	88.5 ± 4.1	168.3 ± 5.8	122.7 ± 4.2
+/+	*fl/fl*	+/+	+/+	*fl/fl*	+	14	115.6 ± 5.5*	158.9 ± 6.6*,†	298.7 ± 8.3*	217.6 ± 5.8*
+/+	+/+	*fl/fl*	+/+	*fl/fl*	–	18	70.8 ± 1.2	99.4 ± 1.7	185.7 ± 2.9	130.6 ± 1.7
+/+	+/+	*fl/fl*	+/+	*fl/fl*	+	20	113.0 ± 5.4*	171.9 ± 9.0*,‡	337.5 ± 18.6*,†	233.5 ± 10.6*
+/+	+/+	+/+	*fl/fl*	*fl/fl*	–	13	68.9 ± 1.3	95.2 ± 1.6	172.8 ± 2.0	125.6 ± 1.9
+/+	+/+	+/+	*fl/fl*	*fl/fl*	+	19	114.3 ± 3.7*	160.7 ± 4.6*,‡	296.3 ± 6.7*	218.0 ± 3.9*
Females										
+/+	+/+	+/+	+/+	*fl/fl*	–	15	47.9 ± 1.4	71.3 ± 1.6	142.0 ± 3.6	99.2 ± 2.2
+/+	+/+	+/+	+/+	*fl/fl*	+	17	74.4 ± 2.1*	116.5 ± 2.4*	227.6 ± 4.9*	156.8 ± 2.8*
fl/fl	+/+	+/+	+/+	*fl/fl*	–	18	48.1 ± 0.9	72.5 ± 1.3	141.4 ± 2.0	104.3 ± 1.9
fl/fl	+/+	+/+	+/+	*fl/fl*	+	22	73.2 ± 2.8*	123.0 ± 5.7*	251.0 ± 11.4*	180.2 ± 8.2*,†
+/+	*fl/fl*	+/+	+/+	*fl/fl*	–	19	49.5 ± 2.1	71.3 ± 2.0	135.2 ± 3.6	98.2 ± 2.9
+/+	*fl/fl*	+/+	+/+	*fl/fl*	+	18	83.2 ± 2.9*,‡	128.1 ± 3.7*,‡	241.2 ± 5.5*,†	171.5 ± 4.3*,‡
+/+	+/+	*fl/fl*	+/+	*fl/fl*	–	13	51.8 ± 1.1	73.5 ± 1.5	140.2 ± 3.9	101.2 ± 2.7
+/+	+/+	*fl/fl*	+/+	*fl/fl*	+	21	82.4 ± 2.5*,†	141.8 ± 3.1*,§	279.6 ± 6.6*,§	188.7 ± 4.7*,§
+/+	+/+	+/+	*fl/fl*	*fl/fl*	–	12	50.2 ± 2.2	73.5 ± 2.0	136.1 ± 4.5	100.8 ± 2.6
+/+	+/+	+/+	*fl/fl*	*fl/fl*	+	6	71.2 ± 4.4*	113.7 ± 4.6*	215.7 ± 11.4*	150.7 ± 9.0*

*$P < 0.001$ vs. cre$^-$.
†$P < 0.05$ vs. *Mstn fl/fl*, cre$^+$.
‡$P < 0.01$ vs. *Mstn fl/fl*, cre$^+$.
§$P < 0.001$ vs. *Mstn fl/fl*, cre$^+$.

ACVR2B and ALK4 (and perhaps the combination of these two receptors) are likely to be utilized by activin A for signaling in myofibers more than ACVR2 and ALK5; however, these additional increases seen upon targeting a given receptor in combination with *Mstn* were still relatively small compared to the dramatic increases that we have observed in previous studies in which we targeted multiple ligands simultaneously (13, 14), suggesting that the ACVR2B/ALK4 combination is unlikely to be the sole receptor combination that is utilized by activin A in myofibers in vivo.

The findings presented here as well as our prior study (23) demonstrate that myofibers are the primary cellular targets for signaling by MSTN/activin A with respect to the regulation of muscle growth by these ligands. A variety of studies, however, have reported additional effects of genetic and pharmacological targeting of MSTN/activin A on other physiological processes. These findings have raised the question as to whether these other physiological effects reflect loss of MSTN/activin A signaling to other cell types and tissues or whether these are indirect effects resulting from inhibition of signaling to myofibers. In this respect, both MSTN and activin A are known to circulate in the blood, and these receptors are expressed by multiple cell types in multiple tissues. Because we were able to generate significant effects on muscle growth by targeting receptors in myofibers, we investigated other physiological effects in these targeted mice to attempt to address this fundamental question. For these studies, we focused on mice in which we targeted the two type II receptors, ACVR2 and ACVR2B. Although the magnitude of the muscle mass increases was significantly larger in mice in which the two type I receptors were targeted, mice lacking ACVR2 and ACVR2B exhibited a much more consistent phenotype (as discussed above), making these studies easier to interpret.

In one set of studies, we examined the effect of targeting *Acvr2* and *Acvr2b* in myofibers on muscle regeneration. A large number of studies have shown beneficial effects of targeting MSTN signaling on muscle regeneration in the setting of muscle degeneration or following muscle injury (for review, see ref. 1). A key question in this regard is whether these beneficial effects reflect inhibition of direct MSTN signaling to muscle satellite cells or whether inhibition of MSTN signaling to myofibers can enhance satellite cell activation and/or function in the setting of muscle degeneration or injury. To this end, we induced muscle damage and regeneration via injection of barium chloride intramuscularly in *Acvr2 fl/fl-Acvr2b fl/fl* mice carrying the *Myl1*-cre transgene compared to mice lacking *Myl1*-cre. We examined the injured muscles at 5 and 21 d postinjury (DPI) to assess the effect of myofiber-specific knockout of *Acvr2* and *Acvr2b* on muscle regeneration. As shown in Fig. 2 *A* and *B*, we found that fiber cross-sectional area (CSA) was significantly greater in uninjured muscles lacking both *Acvr2* and *Acvr2b*. In contrast, no differences in fiber CSA were observed in injured muscles assessed at 5 and 21 DPI. We next examined the effect of targeting *Acvr2* and *Acvr2b* in myofibers on the expansion and self-renewal of satellite cells during the regenerative process. We found no differences in the number of $Pax7^+$ cells, either at 5 or 21 DPI, between *Acvr2 fl/fl-Acvr2b fl/fl* mice carrying the *Myl1*-cre transgene compared to mice lacking *Myl1*-cre (Fig. 2*C*). These results suggest that loss of *Acvr2* and *Acvr2b* in the muscle fibers results in no significant alteration in satellite cell function during muscle regeneration.

Another physiological consequence of MSTN loss or inhibition in addition to stimulating muscle fiber growth is an overall effect on fat and glucose metabolism. We showed previously that $Mstn^{-/-}$ mice exhibit a significant suppression of fat accumulation and improved glucose metabolism in an otherwise wild-type background as well as in *ob/ob* and *agouti lethal yellow* backgrounds (33). Beneficial metabolic effects have also been described in mice treated with MSTN inhibitors (34–37). A key

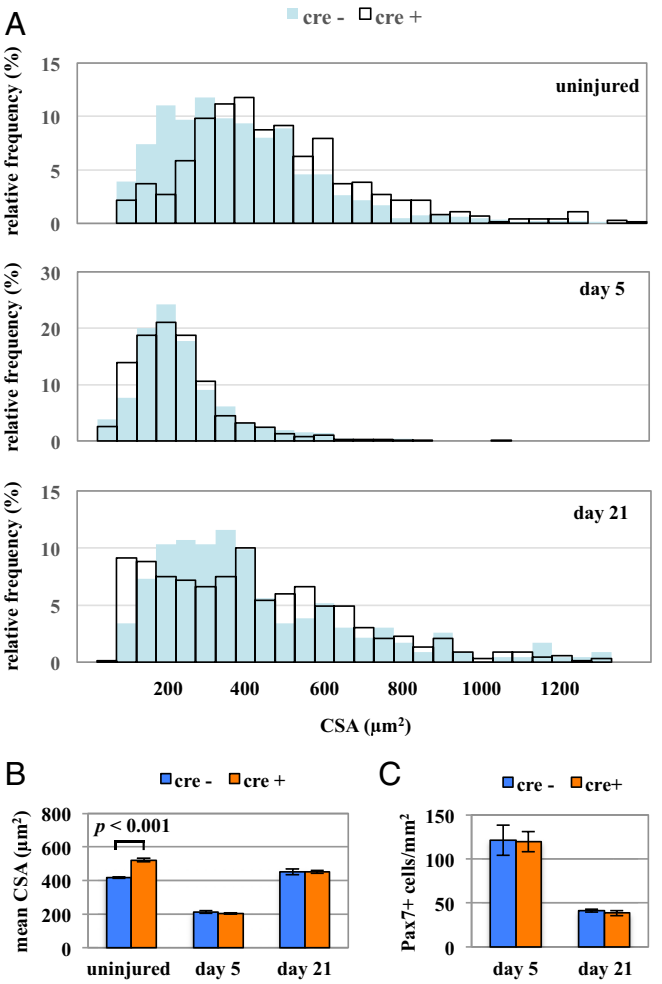

Fig. 2. Lack of effect of targeting *Acvr2* and *Acvr2b* in myofibers on muscle regeneration following chemical injury. (*A*–*C*) Distribution of myofiber CSAs (*A*), mean CSAs (*B*), and number of $Pax7^+$ cells (*C*) in *Acvr2 fl/fl*, *Acvr2b fl/fl* mice with or without *Myl1*-cre either uninjured or 5 or 21 DPI.

question is whether these beneficial effects on fat accumulation and glucose metabolism are the result of inhibition of MSTN signaling to myofibers, leading to muscle growth, or whether they reflect lack of direct MSTN signaling to other cell types and tissues, including adipose tissue. In previous studies, we showed that differences in fat accumulation between $Mstn^{-/-}$ and wild-type C57BL/6 mice become more pronounced as mice age (33). Hence, we focused our initial analysis on 1-y-old mice. By dual-energy X-ray absorptiometry (DXA) analysis, total body fat content in 1-y-old $Mstn^{-/-}$ mice was reduced to less than one-third that of wild-type C57BL/6 mice, with a concomitant reduction in percent body fat (Fig. 3). Similarly, total fat content and percent body fat were reduced in *Acvr2 fl/fl-Acvr2b fl/fl* mice carrying the *Myl1*-cre transgene compared to mice lacking *Myl1*-cre, with the effects being more pronounced in females. Consistent with these differences in body fat content, serum leptin levels were also reduced in $Mstn^{-/-}$ mice as well as in *Acvr2 fl/fl-Acvr2b fl/fl* mice carrying the *Myl1*-cre. Hence, the reduced fat mass seen upon loss of MSTN signaling is likely an indirect effect of increased muscling in these mice. Fasting glucose levels were generally similar among the different genotypes at this age, although we did observe statistically significant lower fasting glucose levels in *Acvr2 fl/fl-Acvr2b fl/fl* female mice carrying the *Myl1*-cre transgene compared to mice lacking *Myl1*-cre. Most

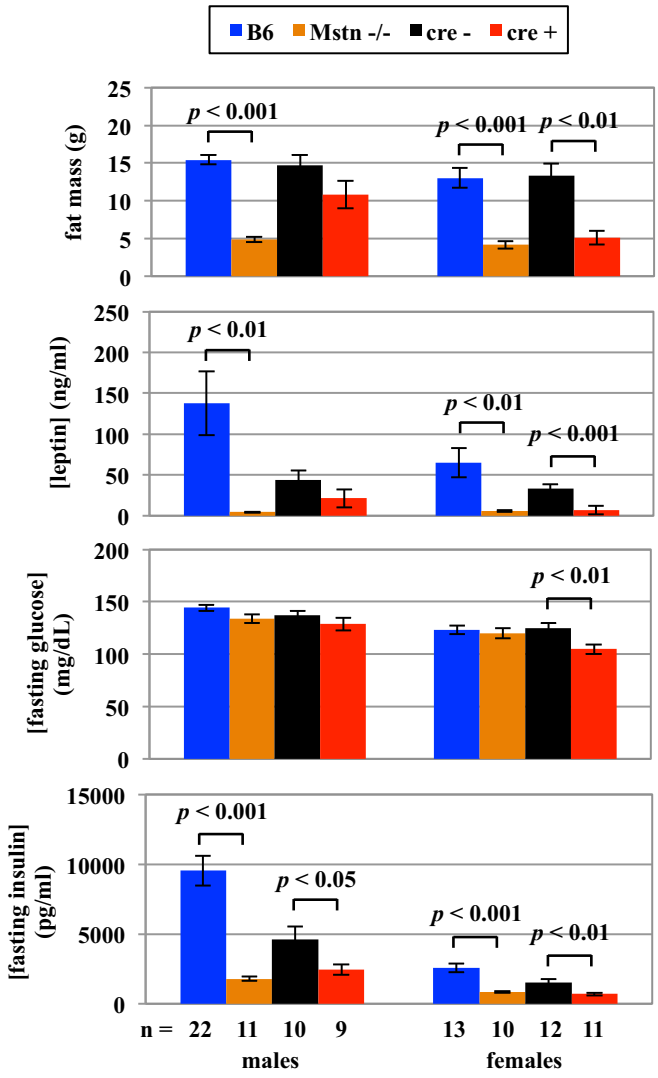

Fig. 3. Total body fat content by DXA analysis, plasma leptin levels, fasting blood glucose levels, and fasting plasma insulin levels in 1-y-old mice lacking MSTN and mice in which both type II receptors were targeted in myofibers. Numbers of mice in each group are shown underneath the bars.

importantly, these normal to lower fasting glucose levels were maintained despite significantly lower fasting insulin levels both in *Mstn*$^{-/-}$ mice compared to wild-type C57BL/6 mice and in *Acvr2 fl/fl-Acvr2b fl/fl* mice carrying the *Myl1*-cre transgene compared to mice lacking *Myl1*-cre, with the effect again being more pronounced in females.

We also examined the effects of placing these mice on high fat diets. For these studies, we analyzed younger mice (12 wk of age) of both sexes. Although we generally observed similar effects in both males and females, the effects were more pronounced in males. As shown in Fig. 4*A*, *Mstn*$^{-/-}$ mice gained much less weight than wild-type mice throughout an 8-wk period on a high-fat diet. Similarly, *Acvr2 fl/fl-Acvr2b fl/fl* mice carrying the *Myl1*-cre transgene gained significantly less weight on a high-fat diet than mice lacking cre. We also observed differences in these mice in terms of glucose metabolism when maintained on a high-fat diet. Fasting glucose levels were lower in *Mstn*$^{-/-}$ mice compared to wild-type mice, and this lowering of fasting glucose levels was also seen in *Acvr2 fl/fl-Acvr2b fl/fl-Myl1*-cre mice compared to mice lacking cre (Fig. 4*B*). In glucose-tolerance tests (GTTs) of mice maintained on standard diets, *Acvr2 fl/fl-*

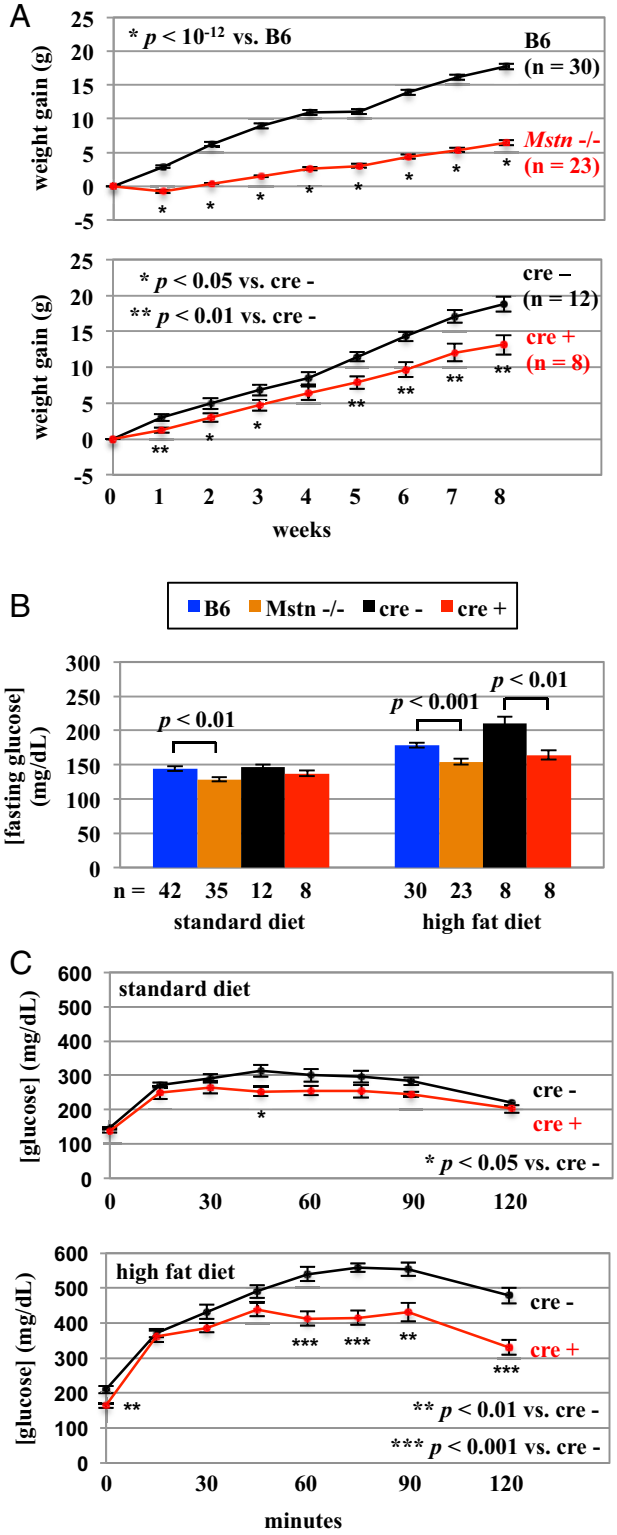

Fig. 4. Effect of a high-fat diet on *Mstn*$^{-/-}$ mice and mice in which *Acvr2* and *Acvr2b* have been targeted in myofibers. (*A*) Weight gain in male mice placed on a high-fat diet starting at 12 wk of age. (*B* and *C*) Fasting blood glucose levels (*B*) and GTTs (*C*) in 12 wk-old male mice on standard diets or after placement on a high-fat diet for 4 wk. In *C*, the numbers of mice in each group are the same as shown in *A*.

Acvr2b fl/fl-Myl1-cre mice had glucose levels that trended lower, although only one time point reached statistical significance (Fig. 4*C*). Following 4 wk on a high-fat diet, however, these differences were accentuated, with *Acvr2 fl/fl-Acvr2b fl/fl-Myl1*-cre mice having significantly lower glucose levels, particularly in the later time points. Taken together, these results clearly demonstrate that targeting this signaling pathway specifically in myofibers can lead to beneficial effects on fat and glucose metabolism.

Finally, we examined effects on bone. Prior studies have identified effects on bone density and structure in $Mstn^{-/-}$ mice (38). In addition, we (13, 24, 39) and others (35, 40) have reported that administration of a soluble form of the activin type IIB receptor (ACVR2B/Fc) systemically to mice can increase not only muscle mass but also bone mineral density. At least part of this effect is due to inhibition of signaling directly to bone (likely by activin A), as targeting *Acvr2* and *Acvr2b* in osteoblasts is sufficient to increase bone density in vivo (24). It is also possible, however, that some of the effects on bone may be indirect as a result of increased mechanical load on the bone due to enhanced muscle mass resulting from inhibition of signaling to myofibers. To test the role of increased muscle mass on bone, we compared bones of mice in which we targeted *Acvr2* and *Acvr2b* in myofibers not only to bones of wild-type and cre-negative mice but also to those of mice receiving the ACVR2B/Fc decoy receptor. We reported previously that systemic administration of the ACVR2B/Fc decoy receptor to mice can induce rapid and significant muscle growth, and at the dose that we used in this study, individual muscle weights increased by about 40 to 50% over the 5-wk treatment period (*SI Appendix*, Fig. S4). Administration of the decoy receptor to wild-type mice also resulted in significant increases in bone density, as assessed by DXA analysis, with bone mineral density being ∼15% higher in treated compared to untreated mice after 5 wk of treatment with ACVR2B/Fc (Fig. 5*A*). This bone anabolic effect was confirmed by micro-computed tomography (microCT) analysis (Fig. 5 *B* and *C* and *SI Appendix*, Tables S1 and S2), which showed dramatic increases in bone volume, bone surface, and trabecular thickness and number in both the femur and L4 and L5 vertebrae in ACVR2B/Fc-treated mice. In contrast, bones of mice in which *Acvr2* and *Acvr2b* were targeted in myofibers exhibited no statistically significant differences in any of these parameters. These results demonstrate that increasing muscle mass per se by targeting these receptors specifically in myofibers does not lead to corresponding increases in bone density and that the effect of ACVR2B/Fc in increasing bone density is almost certainly due to inhibition of direct signaling to bone, likely by activin A.

Discussion

Like other TGF-β family members, MSTN signals by utilizing a two-component receptor mechanism. Using biochemical approaches, we previously showed that MSTN is capable of binding initially to either of the two activin type II receptors, ACVR2 (also called ACVR2A or ActRIIA) and ACVR2B (also called ActRIIB) (18). Because MSTN appeared to bind ACVR2B with higher affinity than ACVR2 and because overexpression of a truncated form of ACVR2B (lacking the cytoplasmic kinase domain) in skeletal muscle could phenocopy the *Mstn* loss-of-function mutation in terms of increased muscle mass, it was presumed that ACVR2B is the primary type II receptor utilized by MSTN in vivo. In a follow-up genetic study, however, we showed that mice null for either *Acvr2* or *Acvr2b* exhibit increased muscling and that these two receptors are partially functionally redundant (13). One limitation of that study was that examining the consequence of complete loss of both receptors was not possible using the deletion alleles for these genes because mice completely lacking both receptors are not viable (22). In order to circumvent this lethality issue, we sought to target these receptors specifically in myofibers, as we had shown that myofibers are direct targets for MSTN signaling and that blocking signaling in myofibers is sufficient to induce muscle growth (23). In the studies presented here, we targeted these receptors by crossing in a myosin light chain-cre (*Myl1*-cre) transgene (25), which is expressed specifically by myofibers, into mice that we had generated carrying floxed alleles for *Acvr2* and *Acvr2b*. We show that targeting *Acvr2* and *Acvr2b* individually results in small, although significant, increases in muscle mass but that simultaneously targeting both type II receptors leads to much more dramatic effects, demonstrating conclusively that both receptors function to suppress muscle growth and that the two receptors are functionally redundant in this regard.

The identification of ACVR2 and ACVR2B as MSTN receptors led to two strategies to develop therapeutics targeting MSTN signaling to treat patients with muscle loss or degeneration. One approach was to generate a soluble form of ACVR2B in which the ligand-binding domain was fused to an immunoglobulin Fc domain (13, 41). Indeed, this decoy receptor (ACVR2B/Fc) is still the most potent agent described to date in terms of its ability to promote muscle growth; in fact, just two injections of this decoy receptor at high doses to mice can cause greater than 50% muscle growth throughout the body in just 2 wk (13). Although this effect of the decoy receptor has been widely cited in the literature as evidence that ACVR2B is the primary receptor utilized by MSTN in vivo, it is important to note that because this decoy receptor is a ligand trap, it is capable of blocking signaling of ligands through other receptors as well. A second approach, which was taken by Novartis, was to develop a monoclonal antibody targeting ACVR2B directly (42–46). Although the initial publication of this monoclonal antibody (bimagrumab, or BYM338) reported that it had over 200-fold higher affinity for ACVR2B compared to ACVR2, subsequent studies showed that by X-ray crystallography analysis, BYM338 is capable of blocking the ligand-binding domain of both receptors (47). They further showed that highly specific monoclonal antibodies directed against each receptor (with no cross-reactivity to the other receptor) had an additive effect in terms of stimulating muscle growth when given to mice. Hence, these pharmacologic studies taken together with our genetic studies presented here demonstrate conclusively that both ACVR2 and ACVR2B play critical roles in regulating muscle growth.

Binding of MSTN to the type II receptors then leads to engagement of the two type I receptors, ALK4 and ALK5 (18, 21). Although a number of studies have examined the roles of these receptors in cell culture systems, very few studies have addressed the functions of these receptors in vivo with respect to control of muscle growth. Some of these in vivo studies reached somewhat contradictory conclusions. In particular, whereas small-molecule inhibitors of ALK4/5 were shown to induce muscle fiber hypertrophy in both wild-type and dystrophic mice (48) as well as to preserve muscle mass in a cancer cachexia model (49), delivery of an antisense oligonucleotide directed against *Alk4* had the opposite effect, leading to a reduction in muscle mass (50). Here, we took a genetic approach in which we used the *Myl1*-cre transgene to target floxed *Alk4* and *Alk5* alleles in myofibers. We show that targeting *Alk4* alone has no effect on muscle mass and that targeting *Alk5* alone has a small, although significant, effect. Targeting both type I receptors simultaneously, however, leads to dramatic increases in muscle size. These increases are substantially higher than those seen upon targeting ACVR2 and ACVR2B, raising the possibility that yet another type II receptor may also be involved in regulating muscle mass. We showed that targeting BMPR2 along with ACVR2 and ACVR2B does not cause further increases in muscle mass, leaving TGFBR2 as the only remaining candidate, although no binding of either MSTN or activin A to TGFBR2 has been reported.

These increases seen upon targeting both *Alk4* and *Alk5* even surpassed the doubling of muscle mass seen in mice completely

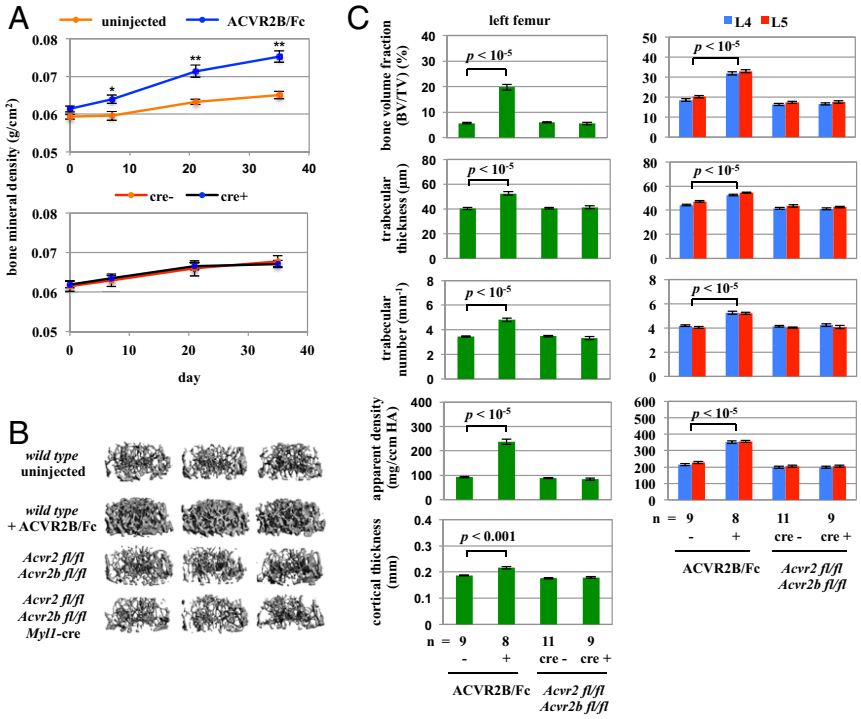

Fig. 5. Lack of bone effects of targeting *Acvr2* and *Acvr2b* in myofibers. (*A*, *Top*) DXA analysis of wild-type C57BL/6 mice either uninjected ($n = 9$) or injected weekly with the ACVR2B/Fc decoy receptor at a dose of 10 mg/kg ($n = 8$) starting at 10 wk of age. (*A*, *Bottom*) DXA analysis of Acvr2 flox/flox, *Acvr2b flox/flox* mice with ($n = 8$) and without ($n = 12$) the *Myl1*-cre transgene at the same ages as in *A*, *Top*. $^{*}P < 0.05$, $^{**}P < 0.001$. (*B*) MicroCT images of femurs taken from these same mice at 16 wk of age. (*C*) Bone volume/total volume fraction, trabecular thickness, trabecular number, apparent density, and cortical thickness of femurs and L4 and L5 vertebrae determined by microCT analysis in these mice at 16 wk of age. Numbers of mice in each group are shown underneath the bars.

lacking MSTN and are more reminiscent of the quadrupling of muscle mass seen in *Mstn*$^{-/-}$ mice that also carry a transgene overexpressing the MSTN inhibitor, follistatin, in skeletal muscle (14). The ability of follistatin as well as the ACVR2B/Fc decoy receptor (13) to induce further muscle growth even in *Mstn*$^{-/-}$ mice showed that at least one other TGF-β family member besides MSTN must also play an important role in suppressing muscle growth, and subsequent studies have identified activin A as the key cooperating ligand (15, 16). The dramatic increases in muscle growth that we obtained by targeting both *Alk4* and *Alk5* in myofibers suggests that these receptors must be involved in mediating signaling by both MSTN and activin A to myofibers. Furthermore, by simultaneously targeting different combinations of type I and type II receptors and by simultaneously targeting *Mstn* with each type I and type II receptor, we were able to investigate whether specific receptor combinations are utilized by specific ligands. Although we detected some quantitative differences targeting different combinations, the overall conclusion from all of these studies is that muscle mass is regulated by at least two ligands (MSTN, activin A) signaling directly to myofibers through two type II (ACVR2, ACVR2B) and two type I (ALK4, ALK5) receptors, with all ligand and receptor combinations being utilized in a highly redundant manner. These findings suggest that in order to generate maximal effects on muscle growth, it is essential that any therapeutic strategy be capable of targeting either multiple receptor components simultaneously, such as with BYM338, or multiple ligands simultaneously, such as with the ACVR2B/Fc decoy receptor.

These studies also demonstrate conclusively that myofibers are the direct targets for MSTN and activin A signaling with respect to control of muscle growth. Given that we were able to generate substantial effects on muscle mass by blocking MSTN/activin signaling just in myofibers, we used this experimental approach to investigate other physiological effects in these mice. In particular, previous studies using *Mstn*$^{-/-}$ mice have identified effects of MSTN loss on other tissues besides skeletal muscle, raising the question as to whether MSTN is capable of signaling directly to these other tissues in vivo or whether these are indirect effects of lack of MSTN signaling to skeletal muscle. In the studies presented here, we were able to distinguish direct versus indirect effects by blocking MSTN and activin A signaling just in myofibers. Although targeting the two type I receptors generated the greatest effects on muscle mass, the phenotype was highly variable in these mice, and we therefore decided to focus instead on mice in which we targeted the two type II receptors, ACVR2 and ACVR2B, for which the phenotype was much more consistent.

One tissue other than skeletal muscle known to be affected in *Mstn*$^{-/-}$ mice is adipose tissue. We showed previously that *Mstn*$^{-/-}$ mice have a reduction in fat accumulation, particularly as a function of age, not only in a wild-type background but also in *ob*/*ob* and *agouti lethal yellow* backgrounds, as well as beneficial effects on glucose metabolism (33). Subsequent studies showed that loss or inhibition of MSTN can increase skeletal muscle glucose uptake and energy expenditure and protect against high-fat diet-induced weight gain as well as glucose intolerance (34–37). A key question is whether all of these effects on adipose tissue and glucose metabolism reflect loss of MSTN signaling to skeletal muscle or whether some of these effects reflect loss of MSTN signaling to other tissues. In this regard, a previous study showed that mice overexpressing a truncated form of ACVR2B in skeletal muscle (18) also exhibit some of the metabolic effects seen in *Mstn*$^{-/-}$ mice (51); however, because this truncated receptor could act as a ligand trap, one could not rule out the possibility that one mode of action of this truncated

receptor may be to act as a sink by binding MSTN produced by skeletal muscle and thereby leading to inhibition of MSTN signaling not only to muscle but also to other tissues. Indeed, among the cell types known to be responsive to MSTN in cell culture are adipocytes (52). Moreover, although *Mstn* is expressed at low levels in adipose tissue in wild-type mice (2), *Mstn* expression is significantly up-regulated in both subcutaneous and visceral fat in *ob*/*ob* mice (53). To address whether the effects on adipose tissue and glucose metabolism seen in $Mstn^{-/-}$ mice reflect direct or indirect effects of MSTN loss, we analyzed the metabolic effects of targeting *Acvr2* and *Acvr2b* in myofibers. We show that these mice, like $Mstn^{-/-}$ mice, have reduced overall body fat, lower serum leptin levels, and reduced weight gain on a high-fat diet. These receptor-targeted mice also have lower fasting blood glucose despite having lower fasting insulin levels and are able to maintain lower glucose levels in GTTs. These findings demonstrate that these metabolic effects as well as the suppression of fat accumulation can be achieved by inhibition of signaling solely in myofibers.

Another tissue known to be affected in $Mstn^{-/-}$ mice is bone. In particular, $Mstn^{-/-}$ mice have been reported to have a generalized increase in bone mineral density at many sites, including femurs (38, 39). A key question raised by these findings is whether this increased bone mineral density results from increased mechanical load on the bones due to the hypermuscularity in these mice or rather from loss of MSTN signaling directly to bone. In this regard, MSTN has been reported to be capable of acting directly on bone-progenitor cells in vitro to regulate cell differentiation (54). It is also known that MSTN inhibitors, like follistatin and the ACVR2B/Fc decoy receptor, can have significant effects on bone repair and bone density in vivo (39, 40, 55, 56), but because these inhibitors can also block activin signaling, the identities of the key ligands being blocked in these studies is not clear. Similarly, it is known that targeting activin type II receptors in osteoblasts in vivo can also increase bone density (24), but this effect likely reflects inhibition of signaling by activin rather than by MSTN. In order to determine the contribution of increased skeletal muscle mass on bone density, we analyzed the bones of mice in which we targeted *Acvr2* and *Acvr2b* in myofibers. Here, we show that whereas systemic administration of the ACVR2B/Fc decoy receptor to wild-type mice can induce dramatic increases in both muscle mass and bone mineral density, increasing muscle mass by targeting these receptors in myofibers has no effect on bone mineral density either in the femurs or in the lumbar vertebrae. These findings demonstrate that the effect of the ACVR2B/Fc decoy receptor on increasing bone mass is almost certainly due to inhibition of signaling by TGF-β–related ligands directly to bone and, furthermore, that increasing muscle mass by up to even 60 to 70% in the hindlimbs was not sufficient to increase bone density either through increased mechanical load on the bones or through the release of hypothetical secondary mediators by muscle.

Finally, we showed that blocking signaling specifically in myofibers also has no effect on muscle regeneration, implying that the effects of MSTN loss or inhibition observed in prior studies likely reflect inhibition of signaling directly to satellite cells. Although a formal demonstration of the role of signaling in satellite cells will require targeting these receptors specifically in that cell population, these findings suggest that MSTN and/or activin A signal to multiple cell types within skeletal muscle and play at least two distinct roles in regulating muscle homeostasis, one to regulate myofiber growth and another to regulate muscle regeneration following injury.

Materials and Methods

All animal experiments were carried out in accordance with protocols that were approved by the Institutional Animal Care and Use Committees at The Jackson Laboratory, University of Connecticut School of Medicine, and Johns Hopkins University School of Medicine. Mice carrying floxed alleles for *Acvr2* (24), *Acvr2b* (23), *Alk5* (26), *Bmpr2* (28), *Cfc1b* (57), and *Mstn* (32) have been described previously. To generate *Alk4* conditional knockout mice, we generated a targeting construct in which we flanked exons 2 to 3 with LoxP sites (*SI Appendix*, Fig. S2). Following electroporation of the targeting construct into embryonic stem (ES) cells, ES cell colonies carrying the homologously targeted allele were injected into blastocysts, and mice generated from these blastocysts were bred to identify those exhibiting germline transmission of the targeted allele. Offspring from these matings were then bred with *EIIa-Cre* transgenic mice (58) in order to delete the neomycin resistance cassette in the germline. From these crosses, we obtained mice carrying an *Alk4 flox* allele lacking the NEO cassette.

For measurement of muscle weights, individual muscles were dissected from both sides of 10-wk-old mice, and the average weight was used for each muscle. Circulating MSTN levels were determined on acid-activated serum samples by enzyme-linked immunosorbent assay using the R&D Systems DGDF80 kit. To induce muscle damage and regeneration, 50 μL of 1.2% barium chloride (wt/vol) (Sigma) was delivered to the right tibialis anterior (TA) muscle over 10 intramuscular punctures. The left TA served as the uninjured control. TA muscles were harvested either 5 or 21 DPI, mounted in optimal cutting temperature compound, and frozen in thawing isopentane. Serial sections (8 μm) were cut transversely through the belly of the TA muscle using a refrigerated cryostat. TA sections were immunoreacted to laminin (Sigma L9393) and Pax7 (DSHB) applied with the M.O.M Basic Kit (Vector Laboratories). Sections were then counterstained with DAPI to visualize nuclei and imaged with a Zeiss Observer Z1 microscope with a color camera (Hammatsu Orca-ER camera) controlled by Volocity software (PerkinElmer). Images were then quantified using ImageJ software.

Live animal imaging was performed using a Piximus dual-energy X-ray absorptiometer. GTTs were performed by giving mice an intraperitoneal injection of 1 g glucose/kg body weight following a 6-h fast. Mice were then placed on a 60 kcal% fat diet (D12492; Research Diets, Inc.) for 8 wk, with a repeat GTT being performed after 4 wk. The ACVR2B/Fc decoy receptor was expressed in Chinese hamster ovary cells, purified from the conditioned medium using a protein A Sepharose column, and administered intraperitoneally at a dose of 175 μg per injection. For microCT analysis, the left femur and lumbar vertebrae were placed in 70% ethanol. MicroCT was performed in a Scanco MicroCT40 at 8-μm^3 resolution. Samples were scanned in 70% ethanol 55 kVp, 145-μA intensity, 300 ms. The instrument is calibrated weekly using Scanco phantoms, and all scans passed routine quality-control verification. Analysis of femurs and vertebrae was conducted using standard protocols, with a lower threshold of 2,485 Hounsfield units (HU) for femoral trabeculae, 4,932 HU for femoral cortex, and 3,078 HU for vertebral trabeculae (59). Surface renderings were generated corresponding to each of these thresholds.

Data Availability. All study data are included in the article and *SI Appendix*.

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