Denervation atrophy is independent from Akt and mTOR activation and is not rescued by myostatin inhibition

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ABSTRACT

The purpose of our study was to compare two acquired muscle atrophies and the use of myostatin inhibition for their treatment. Myostatin naturally inhibits skeletal muscle growth by binding to ActRIIB, a receptor on the cell surface of myofibers. Because blocking myostatin in an adult wild-type mouse induces profound muscle hypertrophy, we applied a soluble ActRIIB receptor to models of disuse (limb immobilization) and denervation (sciatic nerve resection) atrophy. We found that treatment of immobilized mice with ActRIIB prevented the loss of muscle mass observed in placebo-treated mice. Our results suggest that protection from disuse atrophy is regulated by serum and glucocorticoid-induced kinase (SGK) rather than by Akt. Denervation atrophy, however, was not protected by ActRIIB treatment, yet resulted in an upregulation of the pro-growth factors Akt, SGK and components of the mTOR pathway. We then treated the denervated mice with the mTOR inhibitor rapamycin and found that, despite a reduction in mTOR activation, there is no alteration of the atrophy phenotype. Additionally, rapamycin prevented the denervation-induced upregulation of the mTORC2 substrates Akt and SGK. Thus, our studies show that denervation atrophy is not only independent from Akt, SGK and mTOR activation but also has a different underlying pathophysiological mechanism than disuse atrophy.

KEY WORDS: Skeletal muscle, Muscle atrophy pathophysiology, TGF-β signaling, Myostatin, Denervation atrophy

INTRODUCTION

Healthy skeletal muscle maintains a balance between protein synthesis and degradation, which will lead to an increase in protein translation (Fenton and Gout, 2011; Ma and Blenis, 2009; Richter and Sonenberg, 2005). The phosphorylation and activation of p70S6k, specifically in skeletal muscle, has been shown to be dependent upon a second scaffold protein mammalian lethal with Sec13 protein 8 (mLST8; also called GβL) (Inoki et al., 2005; Laplante and Sabatini, 2012). The phosphorylation of p70S6k, both of which will lead to an increase in protein translation (Fenton and Gout, 2011; Ma and Blenis, 2009; Richter and Sonenberg, 2005). The phosphorylation and activation of p70S6k, specifically in skeletal muscle, has been shown to be dependent upon a second scaffold protein eIF4E-binding protein (4E-BP1) and p70 ribosomal S6 kinase (p70S6k), both of the phosphorylated form of FoxO3a is excluded from the nucleus and therefore unable to activate the muscle-specific E3 ubiquitin ligases atrogin-1 and MuRF1, collectively referred to as atrogens (Bodine et al., 2001a; Gomes et al., 2001; Mammucari et al., 2007; Zhao et al., 2008). Atrogens have been shown to mediate the loss of muscle mass in multiple pathological conditions (Lecker and Goldberg, 2002; Glass, 2010; Rommel et al., 2007). The Akt signaling cascade can regulate muscle mass by both inhibiting protein degradation and promoting protein synthesis; overexpression of Akt can lead to both muscle hypertrophy and the prevention of atrophy (Bodine et al., 2001b; Glass, 2010; Rommel et al., 2007).

Akt is also capable of inducing hypertrophy by promoting protein synthesis through the activation of the mammalian target of rapamycin (mTOR) pathway (Bodine et al., 2001b; Glass, 2010; Laplante and Sabatini, 2012; Ma and Blenis, 2009). The mTOR pathway consists of two complexes, mTORC1 and mTORC2 (Laplante and Sabatini, 2012; Ma and Blenis, 2009). Both complexes, when activated, contain the phosphorylated form of mTOR and the shared scaffold protein mammalian lethal with Sec13 protein 8 (mLST8; also called GβL) (Inoki et al., 2005; Laplante and Sabatini, 2012). The Akt signaling cascade can regulate muscle mass by both inhibiting protein degradation and promoting protein synthesis; overexpression of Akt can lead to both muscle hypertrophy and the prevention of atrophy (Bodine et al., 2001b; Glass, 2010; Rommel et al., 2007).

Activated phosphorylated Akt (pAkt) blocks atrophy by phosphorylating and thus inactivating the transcription factor FoxO3a (Mammucari et al., 2007; Sandri et al., 2004; Zhao et al., 2008). The phosphorylated form of FoxO3a is excluded from the nucleus and therefore unable to activate the muscle-specific E3 ubiquitin ligases atrogin-1 and MuRF1, collectively referred to as atrogens (Bodine et al., 2001a; Gomes et al., 2001; Mammucari et al., 2007; Zhao et al., 2008). Atrogens have been shown to mediate the loss of muscle mass in multiple pathological conditions (Lecker and Goldberg, 2002; Glass, 2010; Rommel et al., 2007). Akt signaling can activate the mTOR pathway (Bodine et al., 2001b; Glass, 2010; Laplante and Sabatini, 2012; Ma and Blenis, 2009). The Akt signaling cascade can regulate muscle mass by both inhibiting protein degradation and promoting protein synthesis; overexpression of Akt can lead to both muscle hypertrophy and the prevention of atrophy (Bodine et al., 2001b; Glass, 2010; Rommel et al., 2007).
naturally occurring potent negative regulator of skeletal muscle mass
(Lee, 2004; Lee et al., 2005; McPherron and Lee, 1997). Mice deficient in myostatin and wild-type mice given a myostatin inhibitor both exhibit a profound hypertrophic muscle phenotype (Lee et al., 2005; McPherron and Lee, 1997). Myostatin is a member of the transforming growth factor-β (TGFβ) family of growth and differentiation factors that naturally inhibits skeletal muscle growth. Consequently, the effects of myostatin inhibitors are being explored in animal models of inherited and acquired neuromuscular disorders and of age-related loss of muscle mass. The results from these studies have been mixed, with most benefits of myostatin inhibition being observed in the dystrophin-deficient mdx mouse, a model of inherited human muscular dystrophy. Although clinical trials of myostatin inhibition are being considered for individuals with muscular dystrophy, such patients would have to be treated throughout their lives and the risks associated with chronic treatment are currently unknown.

**RESULTS**

Myostatin inhibitor ActRIIB protects muscle from disuse, but not denervation, atrophy

To assess whether myostatin inhibition would protect muscle from atrophy, we used two separate mouse models. We either attached a surgical staple to immobilize one hindlimb of our mice or denervated them by surgical removal of the sciatic nerve from one hindlimb, and then treated both groups with 10 mg/kg ActRIIB for 3 weeks. Owing to the enlargement of all non-challenged muscle, ActRIIB treatment resulted in a substantial increase in total body mass in both atrophy models (Fig. 1A,C, left graphs).

The immobilized placebo-treated group had 19.5% less tibialis anterior (TA) muscle mass compared with controls (Fig. 1A, center graph). However, the ActRIIB-treated immobilized mice did not show loss of TA muscle mass compared with untreated controls (Fig. 1A, center graph). When compared with controls, the measurement of the minimum feret diameter (MFD) showed a similar pattern of loss of muscle fiber diameter in the immobilized placebo group (13.3% reduction), but not in the ActRIIB-treated mice (Fig. 1A, right graph, 1B).

In contrast to the immobilization experiment, both the placebo- and ActRIIB-treated denervated mice lost a significant amount of TA muscle mass compared with sham-operated controls (49.7% and 44.8%, respectively; Fig. 1C, center graph). Measurement of the MFD also showed that the denervated groups had 39.6% and 45.2% smaller muscle fibers (placebo- and ActRIIB-treated, respectively) compared with sham-operated controls (Fig. 1C, right graph, 1D).

Based on muscle-mass and fiber-size measurements, we found that myostatin inhibition protects against disuse, but not denervation, atrophy.

**Non-canonical TGF-β signaling markers are targeted by ActRIIB treatment**

To understand the molecular basis for the differences in ActRIIB treatment outcome in the immobilization and denervation models, we next performed western blot analysis of TA muscle protein lysates. We found that immobilization alone does not change the activation of the canonical TGF-β signaling markers Smad2 and...
Smad3. In addition, ActRIIB treatment of immobilized mice also did not alter the activation of Smad2 or Smad3 (Fig. 2A). Denervation alone induced a threefold upregulation in total Smad2 and a fivefold increase in active pSmad3, but ActRIIB treatment did not attenuate either of these (Fig. 2A). We subsequently examined the expression of the non-canonical TGF-β signaling markers extracellular-signal-regulated kinases 1 and 2 (ERK1/2). Both models of atrophy demonstrated an increase in active pERK1/2 compared with their respective controls (Fig. 2B). ActRIIB treatment prevented the activation of ERK in the immobilized model but not in the denervated model. Denervation also resulted in a significant upregulation of total ERK1/2 protein expression, something not observed in the immobilized model (supplementary material Fig. S1).

We found that muscle disuse results in an upregulation of the non-canonical TGF-β signaling marker pERK1/2, which was prevented by ActRIIB treatment. In addition, another marker of myostatin signaling, myogenin, is sensitive to immobilization but not in ActRIIB-treated mice. ActRIIB treatment did not inhibit the upregulation of canonical or non-canonical TGF-β signaling markers or any other marker of myostatin signaling in denervated muscle.

Akt and SGK are dysregulated in disuse and denervation atrophy

Next we examined the expression levels of Akt in both atrophy models. We found that immobilization with or without ActRIIB treatment did not alter the amount of active pAkt (Fig. 3). An upregulation in total Akt, however, was observed in immobilized mice treated with ActRIIB compared with controls. Conversely, in both the placebo- and ActRIIB-treated denervated models showed a threefold upregulation in phosphorylated and total Akt (Fig. 3).

Because the changes observed in Akt activation and expression cannot explain either the denervation or immobilization phenotypes, we also examined an additional regulator of muscle mass maintenance, SGK. We found that SGK expression was reduced almost twofold in immobilized mice, but not in immobilized mice treated with ActRIIB (Fig. 3). In contrast, both the placebo- and ActRIIB-treated denervated models showed a threefold upregulation.

**Fig. 1. Myostatin inhibition prevents disuse, but not denervation, atrophy.** (A,C) ActRIIB treatment leads to an increase in body mass for both the immobilization (‘I+A’) (P<5.0×10⁻⁸) and denervation (‘D+A’) (P=1.0×10⁻⁴) models (panels A and C, respectively, left graph). (A) The TA mass of immobilized (‘I’) mice is significantly lower than controls (‘C’) (P<1.0×10⁻⁸); however, no loss of muscle mass is seen in ActRIIB-treated immobilized mice (center graph). MFD quantification of the fiber size of immobilized TA muscle (A, right graph) confirmed visual analysis by laminin-γ1 staining (B) that the immobilized mice lose fiber size (P<1.0×10⁻²) but not when treated with ActRIIB. (C) The denervated TA muscle (‘D’) is significantly smaller than sham-operated controls (‘S’) (P<5.0×10⁻¹⁵) and this is not prevented by ActRIIB treatment (center graph). MFD quantification (C, right graph) and visual analysis (D) showed that both the denervated and denervated with ActRIIB treatment lose the same amount of muscle fiber diameter over the course of the treatment (P<1.0×10⁻⁴). Data are represented as mean ± s.e.m. *P-values indicate significant differences with respect to controls. Scale bars: 100 μm.
of SGK expression when compared with sham-operated controls (Fig. 3).

We next examined the expression and activation levels of FoxO3a, a downstream target of SGK and Akt. Both SGK and Akt have been shown to have equal affinity to phosphorylate FoxO3a at T32, but Akt preferentially phosphorylates S253, the site that will inactivate the protein (Brunet et al., 2001). In both atrophy models, with or without ActRIIB treatment, we found no significant difference in phosphorylation at either the T32 or S253 site or in total expression levels of FoxO3a (Fig. 4A). Expression levels of the FoxO3a target atrogin-1 further substantiated these results. We found no difference in expression in either immobilization model, but both placebo- and ActRIIB-treated denervated muscle had reduced atrogin-1 levels (Fig. 4A).

The Akt-mTOR-FoxO3a pathway is also known to play an important part in the regulation of autophagy in skeletal muscle (Schiaffino and Mammucari, 2011; Zhao et al., 2008). Examination of autophagy markers in immobilized mice showed no change between the control, placebo-treated and ActRIIB-treated groups (Fig. 4B). Denervation, however, induced a significant increase in several markers of autophagy, including Lamp2, LC3b, ATG7 and p62 (Fig. 4B). Treatment with ActRIIB did not further change the expression of autophagy markers in denervated muscle.

Our results showed that SGK, not Akt, is lost as a result of immobilization, but not when the muscle is protected by treatment with ActRIIB. Levels of both SGK and Akt were significantly upregulated in denervated muscle with or without ActRIIB treatment. Moreover, a significant upregulation in autophagy markers was observed in denervation, but not disuse, atrophy.

The mTOR signaling pathway is upregulated in denervation atrophy

The mTOR signaling cascade plays an important role in skeletal muscle maintenance by promoting protein synthesis (Glass, 2010; Ma and Blenis, 2009). We therefore examined the activation of both the mTORC1 and mTORC2 complexes in our models of atrophy and ActRIIB treatment.

We found no change in the mTOR complex components p-mTOR, total mTOR, raptor and rictor in immobilized mice with or without ActRIIB treatment (Fig. 5A). However, a scaffold protein common between both mTOR complexes, GBL, was downregulated with immobilization, but was maintained at control levels in ActRIIB-treated immobilized mice (supplementary material Fig. S2). A substrate of mTORC1, p70S6k, and a second scaffold protein, elf3f, which facilitates the interaction between mTORC1 and p70S6k, also showed reduced expression in immobilized muscle (Fig. 5B and supplementary material Fig. S2). The loss of p70S6k and elf3f expression was not seen in immobilized muscle treated with ActRIIB (Fig. 5B). A second mTORC1 substrate, 4E-BP1, did not show any change in phosphorylation or total abundance in placebo- or ActRIIB-treated immobilized mice compared with controls (supplementary material Fig. S2).

The denervated mice demonstrated a significant upregulation in several components of both the mTORC1 and mTORC2 complexes (Fig. 5A). The three- to fivefold upregulation of p-mTOR, total mTOR, raptor and rictor was unchanged by ActRIIB treatment of denervated mice (Fig. 5A). The scaffold proteins GBL and elf3f were also upregulated in both denervation models compared with sham-operated controls (supplementary material Fig. S2). Downstream of the mTORC1 complex, however, we found that denervation results in a significant, almost threefold, drop in phosphorylation at the T389 activation site of p70S6k, despite no change in total p70S6k expression (Fig. 5B). ActRIIB treatment of denervated mice did not prevent the loss of p70S6k activation. Both placebo and ActRIIB treatment of denervated mice led to an upregulation in total and phosphorylated 4E-BP1 (supplementary material Fig. S2).

Active p-mTOR can also regulate autophagy by the phosphorylation and inactivation of ULK1 (the mammalian homolog of Caenorhabditis elegans ATG1), a protein known to be involved in autophagy.
negatively regulate p70S6k (Egan et al., 2011; Lee et al., 2007). We found no change in total ULK1 expression or phosphorylation at the mTOR-specific inhibitory S757 site in immobilized mice, with or without ActRIIB treatment, compared with controls (supplementary material Fig. S3). Similarly, sham-operated, denervated and ActRIIB-treated denervated muscle did not show any difference in total ULK1 expression levels. However, both placebo- and ActRIIB-treated denervated mice did produce a significant drop in phosphorylation at the S757 inhibitory site compared with sham-operated controls (supplementary material Fig. S3).

We found that, compared with controls, muscle disuse did not change the levels of the main mTOR-complex components. However, immobilization did lead to reduced expression of p70S6k and several mTOR-associated scaffold proteins. Immobilized mice were protected from the loss of these proteins by treatment with ActRIIB. In contrast, denervation atrophy resulted in a significant upregulation in nearly all components of the mTOR pathway except for p70S6k – changes that were not prevented by ActRIIB treatment. Although total p70S6k expression did not change, denervation led to the loss of active p-p70S6k, which might be due to an increase in active ULK1.

Rapamycin treatment does not alter the denervation atrophy phenotype

The upregulation of mTOR signaling observed in denervated muscle could be a compensatory mechanism employed to prevent further atrophy or could be contributing to the pathogenic phenotype (Ramos et al., 2012). In order to clarify this difference in our model, we next treated the denervated mice with the mTOR inhibitor rapamycin (2 mg/kg body weight) for 3 weeks.

We found that, compared with sham-operated controls, denervation resulted in a 50.2% loss of TA muscle mass that was not prevented by treatment with rapamycin (Fig. 6A, top graph). MFD measurements also showed that muscle fiber size was reduced by 39.8% and 41.1% in denervated muscle with or without rapamycin treatment, respectively, compared with sham-operated controls (Fig. 6A, bottom graph, 6B).

We then looked at the mTOR signaling pathway in TA muscle protein lysate. As previously demonstrated, muscle denervation led to an increase in p-mTOR, total mTOR, raptor, rictor, GβL and eIF3f compared with sham-operated controls (Fig. 6C and supplementary material Fig. S4). However, compared with the placebo group, rapamycin treatment of denervated mice resulted in a nearly twofold reduction in active p-mTOR. A trend towards reduced raport expression was also seen in rapamycin compared with placebo-treated denervated mice, but it did not reach significance (Fig. 6B). We observed that, of the scaffold proteins, rapamycin treatment of denervated mice reduced the expression of GβL, but not eIF3f (supplementary material Fig. S4). No change was seen between the denervated and denervated with rapamycin treatment in the mTORC1 substrates p70S6k and 4E-BP1 (Fig. 6D and supplementary material Fig. S4). We also noted that the expression of rictor was not upregulated in denervated mice given rapamycin treatment, compared with sham-operated controls (Fig. 6B). A complete knock down of p-mTOR was achieved by treating denervated mice with a higher dose of rapamycin (10 mg/kg body weight); however, because the phenotype remained the same as with the low-dose treatment, we continued our analysis using the more physiological dose of 2 mg/kg body weight (supplementary material Fig. S5).

Because both Akt and SGK are substrates of the mTORC2 complex, we also examined the expression levels of these proteins in our rapamycin-treated denervation model. Denervation alone resulted in an upregulation of pAkt, total Akt, and SGK (Fig. 7A). However, we found that rapamycin treatment prevented the increase in active pAkt and SGK expression, but not the increase in total Akt expression, caused by denervation alone (Fig. 7A). We once again observed no difference in expression or activation of FoxO3a in denervated muscle, with or without rapamycin treatment. In addition, rapamycin treatment did not prevent the loss of atrogin-1 expression observed with denervation alone (Fig. 7B).

Our data demonstrate that rapamycin treatment does not rescue denervation atrophy despite inhibiting the activation of mTOR in the atrophic muscle. In addition, rapamycin treatment prevented the upregulation of pAkt and SGK – but not the downregulation of atrogin-1 – that is normally seen in denervation atrophy.

DISCUSSION

The ability to prevent or treat acquired forms of skeletal muscle atrophy has the potential for wide-reaching benefits to millions of patients. Immobilization alone is a natural complication from many primary conditions, including limb casting, reduced movement when ill or bed rest, all of which can lead to atrophy of the skeletal muscles. The loss of muscle mass can prolong recovery from the primary condition of the patient and increase rehabilitation time. Myostatin inhibitors are excellent candidates for the treatment of acquired muscle atrophies owing to their dramatic and immediate
effect on muscle. Indeed, numerous pharmaceutical companies are, in fact, currently working on developing myostatin inhibitors for the treatment of a variety of muscle disorders. A careful analysis of how these inhibitors influence muscle mass under various and unique pathological conditions is an essential step towards bringing them to the clinic.

Our studies show that myostatin inhibition has the potential for clinical application in the prevention of disuse atrophy. This protection was demonstrated by the preservation of both muscle mass and fiber diameter in immobilized mice treated with ActRIIB (Fig. 1A). However, when this type of treatment does advance to the clinic, it will have to be taken into consideration that currently available myostatin inhibitors cause an increase in mass of all skeletal muscles in the body. Of equal importance are our findings that myostatin inhibition is not effective against atrophy when the neuromuscular connection has been lost (Fig. 1C). This result was somewhat surprising given that the mouse model for amyotrophic lateral sclerosis (ALS), a disease that results in loss of muscle innervation, showed improvement with myostatin inhibition (Morrison et al., 2009). In light of our results, however, this phenomenon is probably due to the heterogeneity of the innervated and denervated muscle fibers that are a consequence of this disease. The innervated muscles would benefit and become larger as a result of myostatin inhibition and be able to compensate for the unaffected, non-innervated fibers. In addition, despite previous research suggesting that denervation leads to an increase in myostatin transcript and protein expression, our data indicate that this might not be the main reason for the loss of muscle mass (Baumann et al., 2003; Liu et al., 2007; Shao et al., 2007). Owing to the extensive post-translational processing and modification that occurs to myostatin before it is a mature protein, it is possible that the previous studies have measured non-functional protein or untranslated mRNA (Lee, 2004).

Our subsequent analyses of the TGF-β signaling pathway in skeletal muscle from the immobilized and denervated groups showed that myostatin inhibition did not reduce the canonical TGF-β signaling markers, pSmad2 and pSmad3, in either model (Fig. 2A). This is in contrast to previously published work demonstrating that myostatin itself will increase Smad activation in skeletal muscle and one report showing that ActRIIB will lower pSmad2 levels in a non-wild-type mouse (Langley et al., 2002; Rebbapragada et al., 2003; Sartori et al., 2009; Zhou et al., 2010). Our data might instead suggest that it is the downregulation of non-canonical TGF-β signaling markers that is of greater importance in understanding the effect that ActRIIB has on the muscle. However, it is possible that, owing to the long duration of our experiment, we have missed the ActRIIB-induced downregulation of Smad activation in our models.
We also explored the Akt-FoxO3a-mTOR pathway because myostatin itself can inhibit its activation and it is another commonly investigated atrophy regulation pathway (Glass, 2010; Trendelenburg et al., 2009). Neither of our atrophy mouse models, however, demonstrated the expected increase in activation and phosphorylation of Akt when treated with ActRIIB (Fig. 3) (Morissette et al., 2009; Trendelenburg et al., 2009). This complements a previous study showing that loss of Akt expression does not attenuate the muscle hypertrophy response to ActRIIB treatment (Goncalves et al., 2010). In addition, in our immobilization model, we found that SGK, not Akt, exhibited the expected pattern of loss of expression with atrophy but not in mice protected by ActRIIB treatment (Fig. 3). Our results suggest that loss of SGK could mediate long-term disuse atrophy and further supports our previous work showing that overexpression of SGK preserves muscle (Andres-Mateos et al., 2013). One speculation, which would reconcile our study with the previously mentioned reports of Akt-mediated regulation of disuse atrophy, is the idea of a temporal switch from Akt to SGK as the atrophy progresses. Our denervated model, on the other hand, produced the initially puzzling result that active pAkt, total Akt and SGK were all significantly upregulated in the atrophic muscle (Fig. 3). These results would have been expected in a hypertrophic muscle rather than in a severely diseased muscle phenotype. We initially hypothesized that the increase of phosphorylated Akt is of compensatory nature to prevent further exaggeration of muscle atrophy in response to denervation. However, the significant decrease of phosphorylated Akt signaling in denervated mice treated with rapamycin, despite no exacerbation of muscle atrophy, makes this less likely. We are currently exploring a number of different experiments to analyze this interesting observation.

We then proceeded to examine the downstream markers of Akt and SGK signaling to further clarify our results. We first examined the expression and activation of FoxO3a, the main transcription factor needed for the activation of atrogenes, a set of E3 ubiquitin ligases known to be highly involved in muscle atrophy (Lecker and Goldberg, 2002; Zhao et al., 2008). Despite loss or overexpression of either Akt or SGK, we found no difference in the phosphorylation of FoxO3a in either atrophy model (Fig. 4A). A FoxO3a atrogene target, atrogin-1, is also unchanged in the immobilized mice and is actually decreased in denervated muscle. We conclude that, at 3 weeks, neither FoxO3a nor atrogin-1 are mediating either form of atrophy or providing protection from disuse atrophy with ActRIIB treatment. It is possible that the involvement of FoxO3a and atrogin-1 are more immediate and occur at an earlier time point than we analyzed. A temporal involvement of atrogin-1 is indeed likely because an atrogin-1 knockout mouse is partially protected from denervation atrophy (Bodine et al., 2001a).

We also examined markers of another type of protein degradation regulated by the Akt-mTOR-FoxO3a pathway, autophagy. Because immobilization does not change any of the autophagy markers studied, we propose that autophagy is not a regulator of long-term disuse atrophy. Conversely, denervation led to a significant increase in all pro-autophagy markers examined, as has been previous described in the literature (Fig. 4B) (O’Leary and Hood, 2009; Zhao et al., 2007). It is reasonable to speculate that this pronounced...
increase in autophagy has such a detrimental effect on the muscle that it is interfering with the ability of ActRIIB to prevent denervation atrophy.

Because Akt and SGK can regulate the pro-growth mTOR pathway, we also examined whether this pathway was dysregulated in our models of atrophy. We found that, although denervation does cause a substantial increase in most components of the mTOR complexes, levels of these proteins were all unchanged in immobilized muscle compared with controls (Fig. 5A). We did observe, however, that both atrophies bring about a loss of activity of the mTORC1 substrate p70S6k. Although that is suggestive that both immobilization and denervation lead to decreased protein synthesis, this is brought about through different mechanisms in the two atrophies. Immobilization leads to a loss p70S6k expression, possibly mediated by the loss of the scaffold protein eIF3f (Csibi et al., 2009; Csibi et al., 2008), but denervation resulted in a loss of p70S6k activation – possibly due to an increase in ULK1 activity (Fig. 5B and supplementary material Fig. S3) (Egan et al., 2011; Lee et al., 2007).

A recent report demonstrated that treating a laminin-deficient mouse model with an mTOR inhibitor reduced autophagy and significantly improved the aberrant muscle phenotype of the mice (Ramos et al., 2012). Similarly, we also treated our denervated mouse model with the mTOR inhibitor rapamycin to test whether the upregulation of mTOR that we observed is a compensatory mechanism or is contributing to the atrophy phenotype. We found that, compared with placebo-treated mice, rapamycin had no effect on the denervation atrophy phenotype despite downregulating mTORC1 signaling in the atrophic muscle (Fig. 6A,B).

Of great interest were our findings that rictor, more so than raptor, is sensitive to rapamycin treatment in our denervation model (Fig. 6C). This complements several reports suggesting that examination of both the mTORC1 and mTORC2 complexes is necessary to fully understand the in vivo effects of rapamycin (Lamming et al., 2012; Sarbassov et al., 2006; Ye et al., 2012). We found this to be the case in our models, because rapamycin completely prevented the denervation-induced upregulation of rictor. In addition, the mTORC2 substrates Akt and SGK also exhibited reduced activation with rapamycin treatment compared with placebo-treated denervated muscle. In fact, when compared with sham-operated controls, rapamycin-treated denervated mice demonstrated no significant upregulation in active pAkt and SGK (Fig. 7A). Why there is an upregulation of so many pro-growth proteins even when they are not actually beneficial is still unclear, although finding the reason behind that would likely aid our understanding of how to effectively treat denervation atrophy.

Previous research has indicated that several different pathways can contribute to atrophy pathogenesis. For example, upregulation of PGC-1α has been shown to be important for preventing sarcopenic and ALS-induced muscle atrophy, and activation of the IL-6–STAT3 pathway has been linked to the muscle wasting seen in cancer cachexia (Bonetto et al., 2012; Bonetto et al., 2011; Da Cruz...
et al., 2012; Wenz et al., 2009). However, it should be noted that the vast majority of papers indicate that it is the IGF-Akt-FoxO3a pathway that is the most important for the progression of muscle atrophy (Lecker and Goldberg, 2002; Lecker et al., 2004; Sacheck et al., 2007). Although it is well-established that Akt can play a substantial role in muscle hypertrophy and atrophy under certain circumstances, it is becoming clear that denervation is an exception to this rule. Even though some evidence has indicated that increasing Akt activation might be beneficial to the muscle during early stages of denervation atrophy (Bodine et al., 2001b), our results nonetheless outline an important point for future treatment approaches – Akt, mTOR and SGK do not represent promising therapeutic targets for individuals suffering from prolonged denervation. A detailed timecourse assessment of the molecular events that occur as denervation atrophy progresses could clarify how certain proteins and pathways are involved in the pathogenesis. Indeed, we are currently in the process of conducting a cohesive study looking at these various signaling pathways at multiple time points after denervation.

In conclusion, our studies bring two very significant contributions to the field of skeletal muscle atrophy. First, disuse atrophy, but not denervation atrophy, can be prevented by the application of a myostatin inhibitor. Myostatin inhibitors are currently being tested in clinical trials and will soon be available for use by physicians. The knowledge that myostatin inhibition is not a ubiquitous treatment option for all forms of atrophy will aid in providing targeted therapies to individual patients. Secondly, denervation atrophy is independent from Akt, mTOR and SGK activation, and therefore does not follow the usual molecular pathway paradigm for muscle wasting. Our study highlights the fundamental differences in the pathophysiologies of disuse and denervation atrophy, and emphasizes the importance of developing unique strategies for their treatment.

MATERIALS AND METHODS
Animal models
Mice were housed in facilities in accordance with the Animal Care and Use Committee of Johns Hopkins University School of Medicine safety protocols. All animals used for experiments were 2-month-old male, C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA). Mice were immobilized by use of a surgical staple (Autosuture Royal 35W stapler, Mansfield, MA, USA) attached to the hind foot held against the tibia as previously described (Caron et al., 2009). Mice were denervated by surgical removal of ~6 mm of sciatic nerve from one hindlimb. Control mice were sham operated in the same way, but without the removal of the nerve. Wounds were closed with surgical staples. The mice were monitored for infection, then anesthetized with isoflurane and euthanized after 3 weeks. Skeletal muscles were dissected and either flash-frozen in liquid nitrogen or imbedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Torrance, CA, USA) for cryostat sectioning. Experiments were repeated at least twice with a minimum of five mice per treatment group. Data shown are averages across all experimental sets. All TA mass measurements are normalized to tibia length.
Drug delivery
Mice were given intraperitoneal injections of soluble ActivinB at a dosage of 10 mg/kg body weight on days 1, 4, 8 and 15 in a volume of 500 μl PBS. Control and placebo mice were injected with 500 μl PBS on the same days. Soluble ActivinB was obtained from Dr Se-Jin Lee and was produced as previously described (Lee et al., 2005). Rapamycin (LC Laboratories, Woburn, MA, USA) was dissolved in DMSO and mice were given daily intraperitoneal injections at a dosage of 2 mg/kg or 10 mg/kg body weight diluted to 200 μl in saline solution (saline with 0.2% carboxymethylcellulose and 0.25% Tween80). Control and placebo mice received an equal volume of DMSO diluted to 200 μl in saline solution.

Sectioning and staining
Tissue imbedded in OCT was sectioned with a cryostat (Microm HM 550) into 10-mm sections. Slides were stained by blocking in 5% BSA/PBS then incubated overnight with primary antibody against laminin-γ1 (Chemicon, Temecula, CA, USA) in 1% BSA/PBS at 4°C. The slides were washed three times for 5 minutes with 1% BSA/PBS, incubated 90 minutes at room temperature with appropriate secondary antibody (Alexa Fluor 488, Invitrogen, Grand Island, NY, USA), washed again three times for 5 minutes with 1% BSA/PBS, and finally mounted with hard set mounting media (Vector Laboratories, Burlingame, CA, USA). Immunofluorescent pictures were taken with an Eclipse i80 microscope (Nikon, Melville, NY, USA) at 10× magnification for myofiber measurement analysis. Myofiber size was determined by measuring the minimum ferrat diameter (MFD) of 700-1000 fibers for three to five mice per treatment group using Nikon NS elements 2.0 software. Representative pictures of myofiber size were taken at 20× magnification.

Western blots
Flash-frozen tissue was homogenized using a polytron in Total Protein Extraction protease inhibitors (Millipore, Billerica, MA, USA) and supplemented with phosphatase inhibitors (Roche, San Francisco, CA, USA). Protein concentration was obtained using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL, USA). 20 μg of total protein lysates were separated on 4-12% gradient Bis-Tris midi or 18% Tris-Glycine mini gels (Invitrogen, Grand Island, NY, USA) then wet transferred to nitrocellulose membrane. Nitrocellulose membranes were blocked for 30 minutes at room temperature in either 5% BSA/PBST or 5% milk/PBST then incubated with primary antibodies overnight at 4°C. Membranes were then washed twice for 20 minutes in PBST, incubated for 1 hour at room temperature with appropriate secondary antibody, and then washed twice for 20 minutes using PBST. Membranes were then developed with SuperSignal West Femto or Dura secondary antibody, and then washed twice for 20 minutes using PBST. Membranes to X-ray films. Quantification of all western blots was performed by scanning the films and using NIH Image J. Western blots were done using R statistical software to generate a one-way ANOVA followed by Tukey’s HSD pairwise comparison for P-values. P-value ≤ 0.5×10^-2 was considered significant.

Acknowledgements
We thank Aimee Clark for technical aid in fiber measurements, and the laboratories of Drs Tao Wong and Hai Dietz for equipment support for some of our experiments.

Competing interests
The authors declare no competing financial interests.

Author contributions
E.M.M. performed all experiments, analysis, and writing of the manuscript. E.A.-M., R. Mejias, J.L.S. assisted in experimental design, data analysis, and writing of the manuscript. E.M.M., R. Mi and J.S.-P. performed the denervation surgeries. S.Y. aided in fiber size measurements. S.-J.L., A.H. and E.M.M. contributed to experiment design and writing of the manuscript. R.D.C. designed and developed the original experimental plan.

Funding
R.D.C. is supported by the National Institutes of Health Director’s New Innovator Award DP2 OD004515, and the Dana and Albert R. Broccoli Charitable Foundation for Research. S.-J.L. is supported by the National Institutes of Health grant RO1AR060636. R. Mi and A.H. are supported by Dr Miriam and Sheldon Adelson Medical Research Foundation.

Supplementary material
Supplementary material available online at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.014126/-/DC1

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