

The TWEAK–Fn14 system is a critical regulator of denervation-induced skeletal muscle atrophy in mice

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Skeletal muscle atrophy occurs in a variety of clinical settings, including cachexia, disuse, and denervation. Inflammatory cytokines have been shown to be mediators of cancer cachexia; however, the role of cytokines in denervation- and immobilization-induced skeletal muscle loss remains unknown. In this study, we demonstrate that a single cytokine, TNF-like weak inducer of apoptosis (TWEAK), mediates skeletal muscle atrophy that occurs under denervation conditions. Transgenic expression of TWEAK induces atrophy, fibrosis, fiber-type switching, and the degradation of muscle proteins. Importantly, genetic ablation of TWEAK decreases the loss of muscle proteins and spared fiber

cross-sectional area, muscle mass, and strength after denervation. Expression of the TWEAK receptor Fn14 (fibroblast growth factor-inducible receptor 14) and not the cytokine is significantly increased in muscle upon denervation, demonstrating an unexpected inside-out signaling pathway; the receptor up-regulation allows for TWEAK activation of nuclear factor κ B, causing an increase in the expression of the E3 ubiquitin ligase MuRF1. This study reveals a novel mediator of skeletal muscle atrophy and indicates that the TWEAK–Fn14 system is an important target for preventing skeletal muscle wasting.

Introduction

Skeletal muscle undergoes atrophy, or wasting, characterized by a reduction in fiber cross-sectional area (CSA), protein content, and strength, in several chronic conditions, including cancer, chronic heart failure, cystic fibrosis, AIDS, and after high dose treatment with glucocorticoids (Glass, 2005; Sandri, 2008). Furthermore, skeletal muscle also undergoes atrophy when its level of neuromuscular activity is reduced, including in the conditions of denervation, unloading, or immobilization (Jackman and Kandarian, 2004), and also in settings of functional denervation as in the elderly patients with sarcopenia (Macaluso and De Vito, 2004).

Inflammation plays a pivotal role in skeletal muscle wasting, especially in chronic disease states (Späte and Schulze, 2004; Argilés et al., 2005). Elevated levels of proinflammatory cytokines precede the onset of muscle wasting in sepsis-induced cachexia, AIDS, chronic heart failure, and cancer (Späte and Schulze, 2004; Argilés et al., 2005; Li et al., 2008). Consistent with its recognized role as a mediator of inflammation, we have recently reported that TNF-like weak inducer of apoptosis (TWEAK) is a powerful skeletal muscle–wasting cytokine (Dogra et al., 2007a). The addition of TWEAK to cultured myotubes or chronic administration in mice causes significant loss of skeletal muscle mass (Dogra et al., 2007a). However, the physiological significance of TWEAK in vivo and the conditions in which TWEAK acts as a mediator of muscle-wasting remain largely unknown.

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Abbreviations used in this paper: CK, creatine kinase; CSA, cross-sectional area; EDL, extensor digitorum longus; EMSA, electrophoretic mobility shift assay; GA, gastrocnemius; mTOR, mammalian target of rapamycin; MyHC, myosin heavy chain; NF- κ B, nuclear factor κ B; nNOS, neuronal nitric oxide synthase; QRT-PCR, quantitative real-time PCR; TA, tibialis anterior; TWEAK, TNF-like weak inducer of apoptosis; TWEAK-KO, TWEAK knockout; TWEAK-Tg, TWEAK transgenic.

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In recent years, remarkable progress has been made toward understanding the intracellular mechanisms responsible for loss of skeletal muscle mass in various atrophying conditions. Nuclear factor κ B (NF- κ B) is a major proinflammatory transcription factor that is strongly linked to skeletal muscle wasting not only in chronic diseases but also in disuse conditions (Li et al., 2008). Specific inhibition of NF- κ B activity has been found to rescue muscle atrophy in response to tumor growth, denervation, and unloading (Cai et al., 2004; Hunter and Kandarian, 2004; Mourkioti et al., 2006). Activation of NF- κ B is sufficient to induce a particular E3 ubiquitin ligase, MuRF1 (Cai et al., 2004), which is required for skeletal muscle atrophy (Bodine et al., 2001a). MuRF1 activation causes the breakdown of myosin heavy chain (MyHC) and other components of the thick filament of the sarcomere during atrophy (Clarke et al., 2007; Cohen et al., 2009). In addition to MuRF1, a second E3 ligase called MAFbx or Atrogin-1 is also induced (Bodine et al., 2001a; Gomes et al., 2001), although not via NF- κ B (Cao et al., 2005).

Although the potential role of proinflammatory cytokines in muscle-wasting in chronic diseases is now recognized, little is known about the triggers and/or the molecular events leading to loss of skeletal muscle mass in disuse conditions. In this study, we demonstrate that constitutive overexpression of TWEAK causes significant muscular abnormalities reminiscent of skeletal muscle wasting in chronic diseases. More importantly, we demonstrate that TWEAK is also an important mediator of skeletal muscle atrophy in response to denervation. Transgenic overexpression of TWEAK in skeletal muscle exacerbates atrophy, whereas genetic ablation of TWEAK rescues the loss of skeletal muscle mass and strength after denervation. Our results also show that TWEAK functions through the activation of NF- κ B and by stimulating the expression of the E3 ubiquitin ligase MuRF1 in denervated skeletal muscle.

Results

Characterization of TWEAK transgenic (TWEAK-Tg) and TWEAK knockout (TWEAK-KO) mice

To examine the contribution of TWEAK in skeletal muscle physiology and pathophysiology, transgenic mice were generated overexpressing wild-type TWEAK using the muscle-specific creatine kinase (CK) promoter (Kronqvist et al., 2002). Previously, founder TWEAK-Tg mice were produced that expressed high levels (>14-fold) of TWEAK protein; these mice were significantly smaller in size and died at perinatal or neonatal stages because of excessive muscle loss (Dogra et al., 2007a). However, by performing additional pronuclear injections of transgenic DNA, we have now established two additional TWEAK-Tg lines (Fig. 1 A). Because TWEAK-Tg mice were generated in B6D2F1 background, these mice were crossed with C57BL/6 mice for seven generations before using them for this study. Both of the transgenic lines expressed similar elevated levels of TWEAK in skeletal muscle and showed no major variation in any of the phenotypes reported in this study. To validate that TWEAK is predominantly expressed in the

skeletal muscle of TWEAK-Tg mice, mRNA levels of TWEAK were assessed in different tissues of 3-mo-old TWEAK-Tg and control mice by quantitative real-time PCR (QRT-PCR) assays. An approximately four- to sixfold increase in the level of TWEAK mRNA was consistently observed in skeletal muscle and an \sim 1.5-fold increase in the cardiac muscle of the TWEAK-Tg compared with control mice (Fig. 1 A). No significant difference in mRNA levels was observed in liver or spleen (Fig. 1 A). We also measured the protein levels of TWEAK in tissue extracts and serum using an ELISA assay kit. As shown in Fig. 1 B, the levels of TWEAK protein were approximately three- to fourfold higher in the skeletal muscle of TWEAK-Tg mice compared with littermate control mice (TWEAK-Tg, 2.88 ± 0.05 ng/mg vs. littermate control, 0.72 ± 0.03 ng/mg protein in tibialis anterior [TA] muscle extracts). In addition to transgenic mice, we also used TWEAK-KO mice (Maecker et al., 2005). TWEAK-KO mice did not show any overt phenotype. Although body and different organ weights (e.g., brain, liver, kidney, heart, lung, and thymus) were comparable between control and TWEAK-KO mice, a significant increase in the number of natural killer cells in secondary lymphoid organs of TWEAK-KO mice compared with wild-type mice has been previously reported (Maecker et al., 2005).

TWEAK functions by binding to Fn14 (fibroblast growth factor-inducible receptor 14) in skeletal muscle (Girgenrath et al., 2006; Dogra et al., 2007b). We examined whether overexpression or genetic ablation of TWEAK in 3-mo-old mice affects the expression of Fn14 in skeletal muscle. The transcript levels of Fn14 in skeletal muscle of TWEAK-Tg mice were comparable with control mice (Fig. 1 C). However, mRNA levels of Fn14 were found to be increased by \sim 1.6-fold in skeletal muscle of TWEAK-KO mice compared with controls (Fig. 1 C), suggesting the possibility of a compensatory mechanism that might be activated in the absence of the Fn14 ligand TWEAK. Transcript levels of TNF, IL-1 β , and IL-6 in TWEAK-Tg mice were comparable with controls, indicating the absence of any nonspecific inflammation in TWEAK-Tg mice (Fig. 1 D). In addition, serum levels of CK in TWEAK-Tg and TWEAK-KO mice were also comparable with control mice, suggesting that overexpression or genetic ablation of TWEAK does not cause any overt muscle pathology in mice (Fig. 1 E).

Transgenic overexpression of TWEAK causes muscle atrophy in vivo

We next studied the in vivo effects of TWEAK on skeletal muscle. Hematoxylin and eosin (H&E) staining did not show any major structural differences in skeletal muscle between control, TWEAK-Tg, and TWEAK-KO mice at 1 or 3 mo of age (unpublished data). However, differences in skeletal muscle structure became apparent after 4–5 mo, especially in soleus muscle. Soleus muscle of 6-mo-old TWEAK-Tg mice showed reduced fiber size (Fig. 2 A). Masson's trichrome staining of muscle sections revealed increased levels of collagen fibers in soleus muscle of 6-mo-old TWEAK-Tg mice compared with control mice (Fig. 2 A, middle). Immunohistochemical analysis and Western blotting confirmed increased levels of collagen I and III (but not collagen IV) in soleus muscle of 6-mo-old TWEAK-Tg

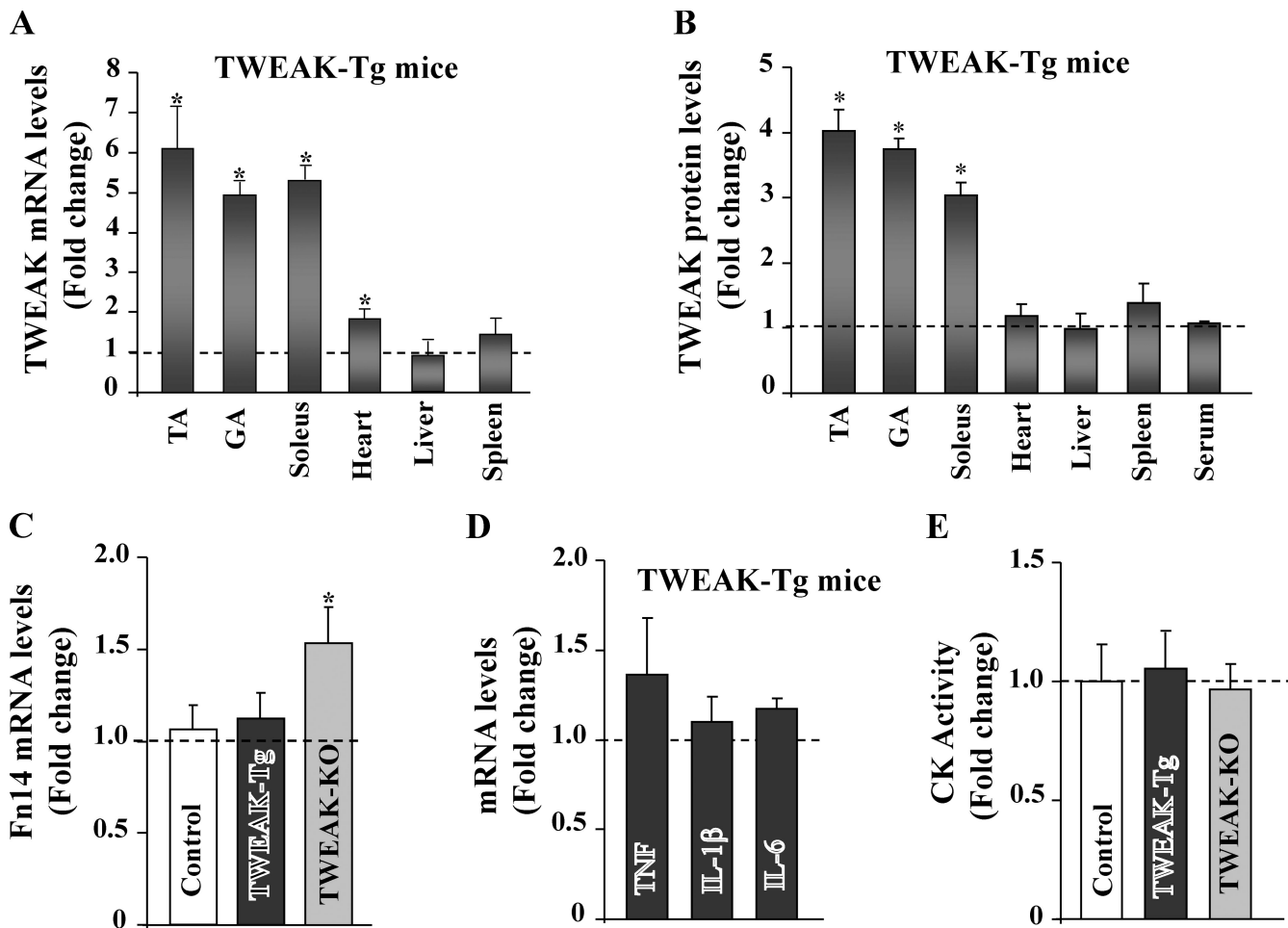


Figure 1. Characterization of TWEAK-Tg and TWEAK-KO mice. (A) Fold change in mRNA levels of TWEAK in skeletal muscle and other organs of TWEAK-Tg mice ($n = 6$) compared with littermate control ($n = 6$) mice. (B) Fold change in protein levels of TWEAK in skeletal muscle, other organs, and serum of TWEAK-Tg ($n = 3$) mice compared with control ($n = 3$) mice. (A and B) *, $P < 0.01$ (values significantly different from littermate control mice). (C) Fold change in the mRNA levels of TWEAK receptor Fn14 in TA muscle of TWEAK-Tg ($n = 5$) and TWEAK-KO ($n = 6$) mice compared with control ($n = 6$) mice. *, $P < 0.01$ (values significantly different from age-matched wild-type mice). (D) Fold change in mRNA levels of TNF, IL-1 β , and IL-6 in TA muscle of TWEAK-Tg ($n = 3$) mice compared with control mice ($n = 3$). (E) Serum CK levels in TWEAK-Tg or TWEAK-KO mice compared with control mice measured using a CK assay kit (Stanbio Laboratory). (A–E) Error bars represent the SD. Dashed lines represent the basal levels in control mice.

mice compared with control mice (Fig. S1, A and B). However, we did not find any significant difference in the protein level of TGF- β , which is a predominant mediator of the development of interstitial fibrosis in skeletal muscle, between control and TWEAK-Tg (Fig. S1 B). To evaluate whether increased fibrosis in soleus muscle of TWEAK-Tg mice was caused by increased infiltration of immune cells, especially macrophages, we studied the levels of macrophages in skeletal muscle tissues. However, there was no difference in the number of macrophages in soleus muscle of control, TWEAK-Tg, and TWEAK-KO mice, which was also confirmed by measuring transcript levels of CD68 and Mac-1, which are the major cell surface markers for macrophages, by QRT-PCR (Fig. S2). In contrast to TWEAK-Tg mice, TWEAK-KO mice showed normal soleus muscle structure and no indication of fibrosis (Fig. 2 A). The changes in fiber size were quantified after immunostaining soleus muscle sections (Fig. 2 A, bottom) for laminin and measuring fiber CSA. The mean fiber CSA of soleus muscle was reduced by $\sim 33\%$ in 6-mo-old TWEAK-Tg mice. Furthermore, the mean fiber

CSA in soleus muscle of TWEAK-KO mice was $\sim 12\%$ higher compared with age-matched control mice, demonstrating that TWEAK modulates normal muscle size (Fig. 2 B).

Results from previous studies favor the idea that exercise intolerance in chronic diseases is attributable to a shift from the slow-twitch, fatigue-resistant oxidative fibers to the more fatigable, fast-twitch glycolytic fibers (Drexler et al., 1992; Pette and Staron, 2001). To evaluate the role of TWEAK in skeletal muscle fiber-type remodeling, double immunostaining was performed on soleus muscle sections using antibody against laminin and an antibody that recognizes all three fast-type fibers, IIa, IIx, and IIb. Compared with controls ($48.3 \pm 1.3\%$), the number of fast-type fibers was increased in TWEAK-Tg ($63.6 \pm 3.6\%$) and reduced in TWEAK-KO ($42.8 \pm 1.2\%$) mice (Fig. 2, C and D), suggesting that TWEAK favors a transition from slow- to fast-type fibers. To further confirm the role of TWEAK in fiber-type switching, we also performed immunostaining on extensor digitorum longus (EDL) muscle sections using an antibody that recognizes only type I fibers along with laminin antibody.

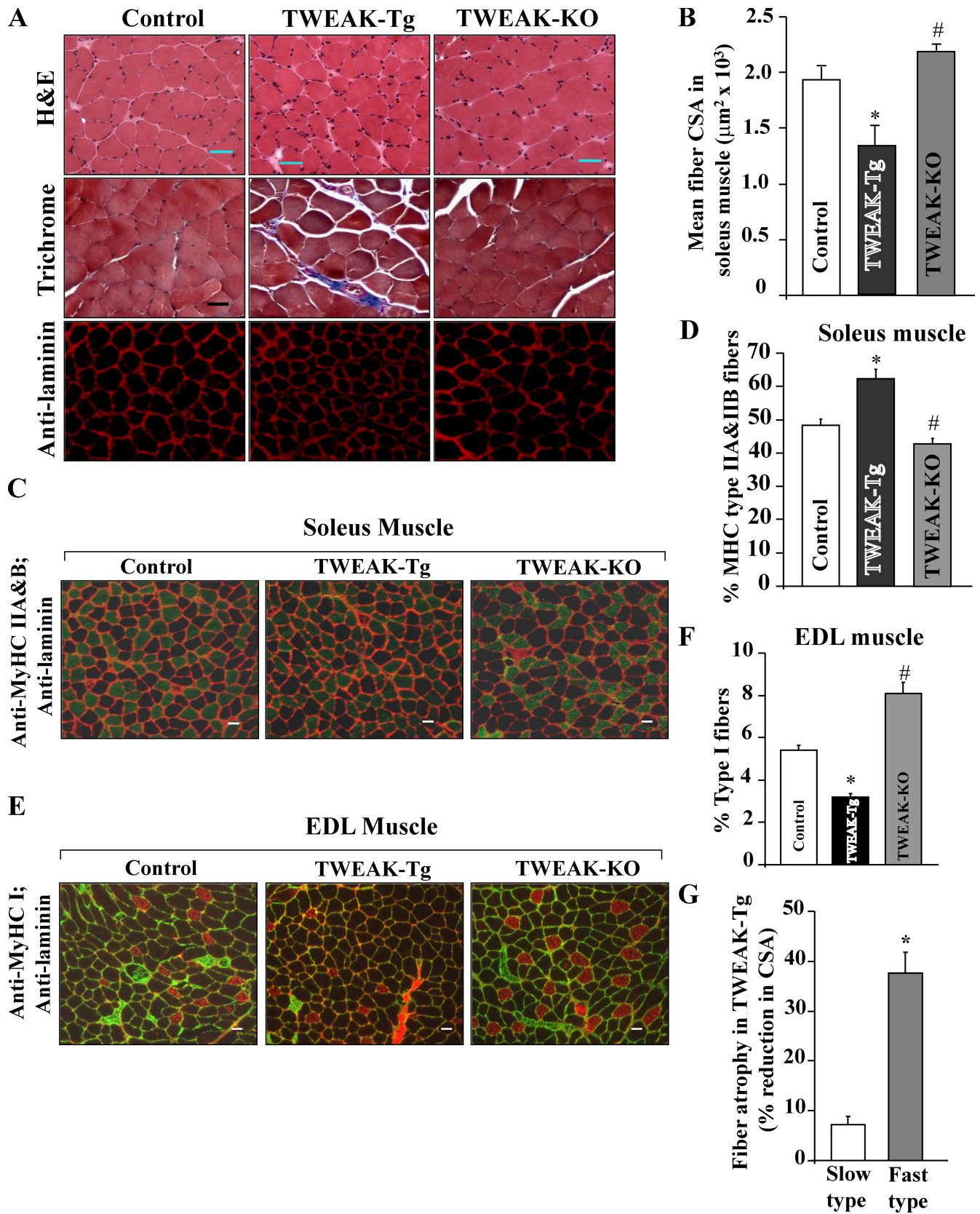


Figure 2. **Effect of TWEAK on skeletal muscle phenotypes in vivo.** (A) Soleus muscle from 6-mo-old control ($n = 6$), TWEAK-Tg ($n = 8$), and TWEAK-KO ($n = 5$) mice was analyzed after H&E or Masson's trichrome staining or after immunostaining with laminin antibody. (B) Quantification of mean fiber CSA in soleus muscle of control, TWEAK-Tg, and TWEAK-KO mice. *, $P < 0.01$; and #, $P < 0.05$ (value significantly different from control mice). (C) Representative photomicrographs of soleus muscle sections from 6-mo-old control ($n = 6$), TWEAK-Tg ($n = 7$), and TWEAK-KO ($n = 6$) mice taken after immunostaining with anti-MyHC fast type and antilaminin. (D) Quantification of MyHC fast type-positive (filled with green color in C) and -negative fibers (empty) in

Again, the number of slow-type fibers was found to be significantly reduced in TWEAK-Tg mice and increased in TWEAK-KO mice as compared with control mice (Fig. 2, E and F). Comparison of CSA of slow- and fast-type fibers in soleus muscle of control and TWEAK-Tg mice revealed an ~36% reduction in fast-type fibers and only an ~7.2% reduction in slow-type fibers, suggesting that TWEAK-induced atrophy is predominantly restricted to fast-type fibers (Fig. 2 G).

TWEAK triggers activation of proteolytic pathways in vivo

What is the mechanism of action by which TWEAK induces skeletal muscle atrophy in vivo? It was of interest to know whether TWEAK induces atrophy by perturbing protein synthesis or proteolysis and if the latter was activated, whether particular proteins were subjected to degradation in a TWEAK-dependent manner. A marked reduction in the levels of MyHC, tropomyosin, and neuronal nitric oxide synthase (nNOS) was observed in the soleus muscle of TWEAK-Tg mice, whereas TWEAK-KO mice showed an increase in the levels of both MyHC and nNOS proteins compared with control mice (Fig. 3, A–C). The levels of cytoplasmic proteins troponin and sarcomeric α -actin and cytoskeletal proteins dystrophin and laminin showed no noticeable changes (Fig. 3, A and B).

QRT-PCR analysis revealed no significant changes in mRNA levels of MyHC or nNOS in soleus muscle of TWEAK-Tg and TWEAK-KO mice compared with control mice (Fig. 3 D), suggesting that the reduction in the levels of proteins in the TWEAK-Tg mice was not the result of reduced transcription but rather was a result of a perturbation in protein levels. An increase in proteolysis was suggested downstream of TWEAK activation by the observation that the activation of NF- κ B (but not activator protein AP-1) and level of MuRF1, a muscle-specific E3 ligase which is required for muscle atrophy (Bodine et al., 2001a), were significantly higher (1.6 ± 0.2 -fold; $P < 0.05$) in soleus muscle of 6-mo-old TWEAK-Tg mice compared with control mice (Fig. 3, E and F). In contrast, there was no significant difference in the mRNA levels of MAFbx (another muscle-specific E3 ligase) between control and TWEAK-Tg mice (Fig. 3 F). It was recently shown that MuRF1 is the NF- κ B-regulated E3 ligase for MyHC (Cai et al., 2004; Clarke et al., 2007); therefore, the coincident demonstration of a TWEAK-induced increase in NF- κ B and MuRF1 and a decrease in MyHC suggests that TWEAK promotes atrophy by inducing the degradation of specific muscle proteins in vivo.

Expression of TWEAK receptor Fn14 is increased in denervated skeletal muscle

Although it was of interest to learn that increased TWEAK levels were sufficient to induce muscle atrophy, it was important

to determine whether the TWEAK–Fn14 pathway perturbed physiological atrophy. To address this issue, first, it was investigated whether the expression of TWEAK or its receptor Fn14 is affected in skeletal muscle in conditions of atrophy and hypertrophy in vivo, as determined by Affymetrix microarray analysis. To induce atrophy, 12-wk-old mice were treated with a cast on a lower limb for 3–7 d or denervated for 3–7 d (meaning the sciatic nerve was transected), as previously described (Bodine et al., 2001a). To study hypertrophy, the casted muscles were allowed to recover for 1 wk after the casts were removed. Also, as an additional model of hypertrophy, animals were treated with clenbuterol for 1 wk, as described previously (Hinkle et al., 2002). Although TWEAK expression did not change significantly either in settings of atrophy or hypertrophy (not depicted), the expression of TWEAK receptor Fn14 was found to be up-regulated upon casting or denervation (Fig. 4 A). Fn14 was not universally up-regulated in atrophy conditions because the glucocorticoid dexamethasone did not cause its up-regulation (Fig. 4 A). In contrast, the expression of Fn14 was found to be somewhat reduced in conditions of hypertrophy such as clenbuterol and casting recovery (Fig. 4 A).

We also measured the expression of Fn14 in denervated skeletal muscle using real-time PCR and Western blot methods. Once again, it was observed that the mRNA level of Fn14 but not TWEAK was increased approximately six- to sevenfold in denervated gastrocnemius (GA) muscle compared with contralateral sham-operated control muscle (Fig. 4 B). To determine whether denervation-induced elevation in mRNA level was specific to the TWEAK receptor Fn14, in the same experiment, we also measured the levels of TNF receptors 1 and 2 (TNFR1 and -2; Fig. 4 B), which belong to the same TNF receptor super family of which Fn14 is also a member (Winkles, 2008). However, no significant increase in the mRNA levels of TNFR1 or -2 was observed between denervated and sham-operated control GA muscle (Fig. 4 B). Similar to mRNA, the protein level of Fn14 in GA muscle was also drastically increased, and it remained elevated even after 10 d of denervation, suggesting a potential role of the TWEAK–Fn14 pathway in skeletal muscle atrophy (Fig. 4 C). Furthermore, we observed that the expression of Fn14 was equally increased in soleus (slow/mixed) and GA, TA, and EDL muscles (fast type) upon denervation (Fig. 4, D and E).

Denervation-induced skeletal muscle atrophy is rescued in TWEAK-KO mice

We next determined whether the loss of skeletal muscle mass in response to denervation is modulated in TWEAK-Tg or TWEAK-KO mice. Because at 3 mo TWEAK-Tg or TWEAK-KO mice did not show any apparent skeletal muscle phenotype and mice at this age are fully developed, we used 3-mo-old mice to evaluate the role of TWEAK in denervation-induced loss

control, TWEAK-Tg, and TWEAK-KO mice. (E) Representative photomicrographs of EDL muscle sections from 6-mo-old control ($n = 4$), TWEAK-Tg ($n = 4$), and TWEAK-KO ($n = 4$) mice taken after immunostaining with anti-type I and antilaminin. (F) Percentage of type I fibers (filled with red color in E) in control, TWEAK-Tg, and TWEAK-KO mice. (D and F) */#, $P < 0.05$ (values significantly different from control mice). (G) Measurement of mean fiber CSA of fast- and slow-type fibers in control ($n = 5$) and TWEAK-Tg ($n = 5$) mice. *, $P < 0.01$ (value significantly different from slow-type fibers). (B, D, F, and G) Error bars represent the SD. Bars, 50 μ m.

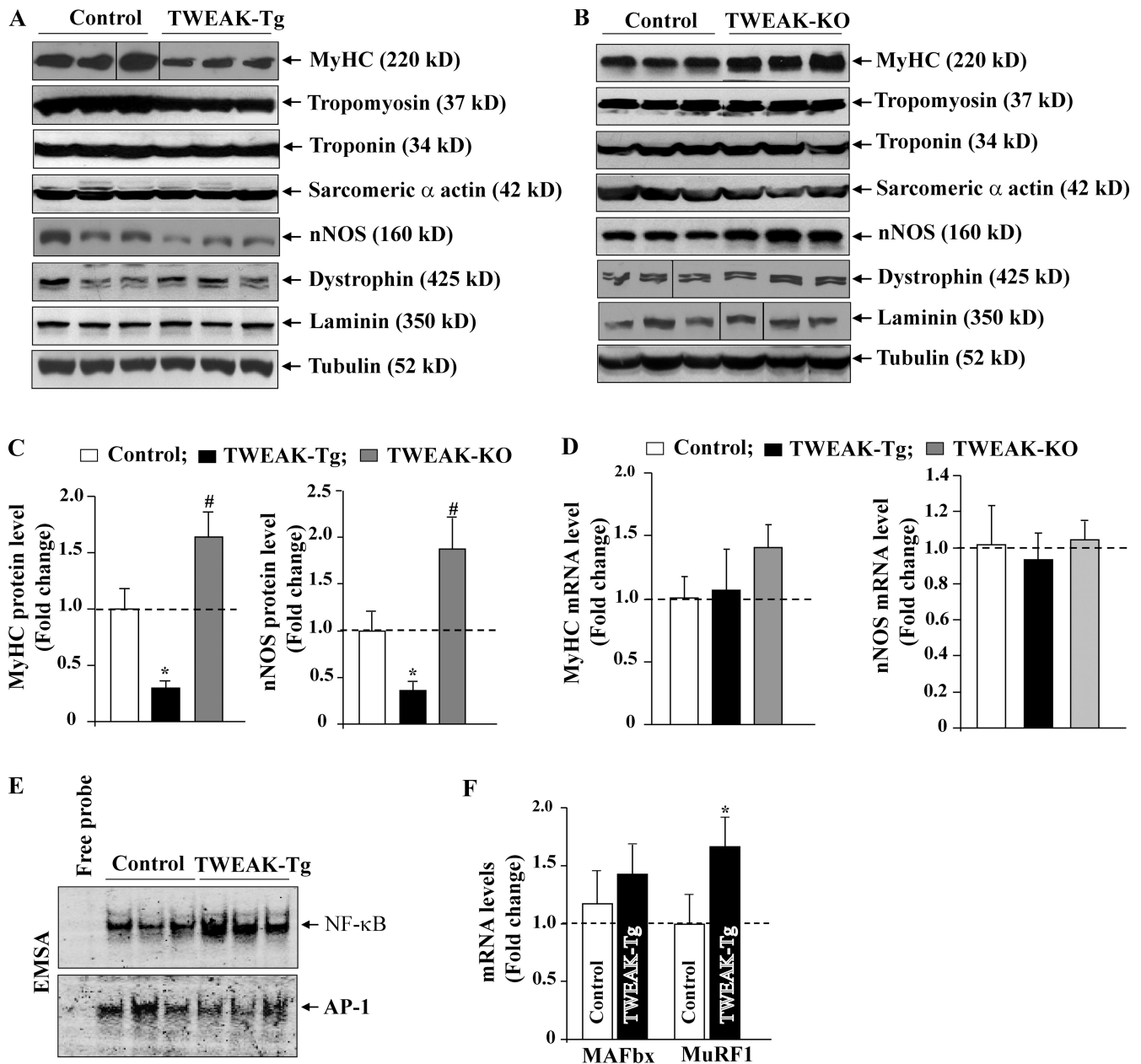


Figure 3. **Effect of TWEAK on the expression of specific muscle proteins in vivo.** (A) Representative Western blots for MyHC, nNOS, tropomyosin, troponin, sarcomeric α -actin, dystrophin, laminin, and tubulin in soleus muscle of 6-mo-old control and TWEAK-Tg mice. (B) Representative Western blots for various muscle proteins in soleus muscle of 6-mo-old control and TWEAK-KO mice. (A and B) Black lines indicate that intervening lanes have been spliced out. (C) Fold change in protein levels of MyHC and nNOS in soleus muscle of control ($n = 6$), TWEAK-Tg ($n = 5$), and TWEAK-KO ($n = 6$) mice. */#, $P < 0.05$ (values significantly different from control mice). (D) Fold change in mRNA levels of MyHC and nNOS in soleus muscle of control ($n = 6$), TWEAK-Tg ($n = 5$), and TWEAK-KO ($n = 4$) mice. (E) Activation of NF- κ B but not AP-1 transcription factor in soleus muscle of 6-mo-old TWEAK-Tg compared with littermate control mice. (F) Fold difference in mRNA levels of MAFbx and MuRF1 in control and TWEAK-Tg mice. *, $P < 0.01$ (values significantly different from level of MuRF1 in control mice). (C, D, and F) Error bars represent the SD. Dashed lines represent the basal levels in control mice.

of skeletal muscle mass. Gross analysis showed that the loss of GA muscle was increased in TWEAK-Tg mice and rescued in TWEAK-KO mice compared with control mice 10 d after denervation (Fig. 5 A). Indeed, the measurement of individual muscle weight confirmed that transgenic overexpression of TWEAK stimulates denervation-induced loss of skeletal muscle mass (Fig. 5 B). Conversely, TWEAK-KO mice showed reduced loss of skeletal muscle mass upon denervation (Fig. 5 B). We also assessed the fiber CSA after staining muscle sections

with H&E dyes (Fig. 5 C) and found that the mean fiber CSA in TA muscle was significantly reduced in TWEAK-Tg mice compared with control mice after denervation (Fig. 5 D). In contrast, the fiber CSA was significantly preserved in skeletal muscle of TWEAK-KO mice after denervation (Fig. 5, C and D). Similar results were obtained with soleus (Fig. 5, E and F) and GA and EDL muscles (not depicted).

Fibrosis is an important pathological feature in various muscular disorders and is observed in paraplegic patients, who

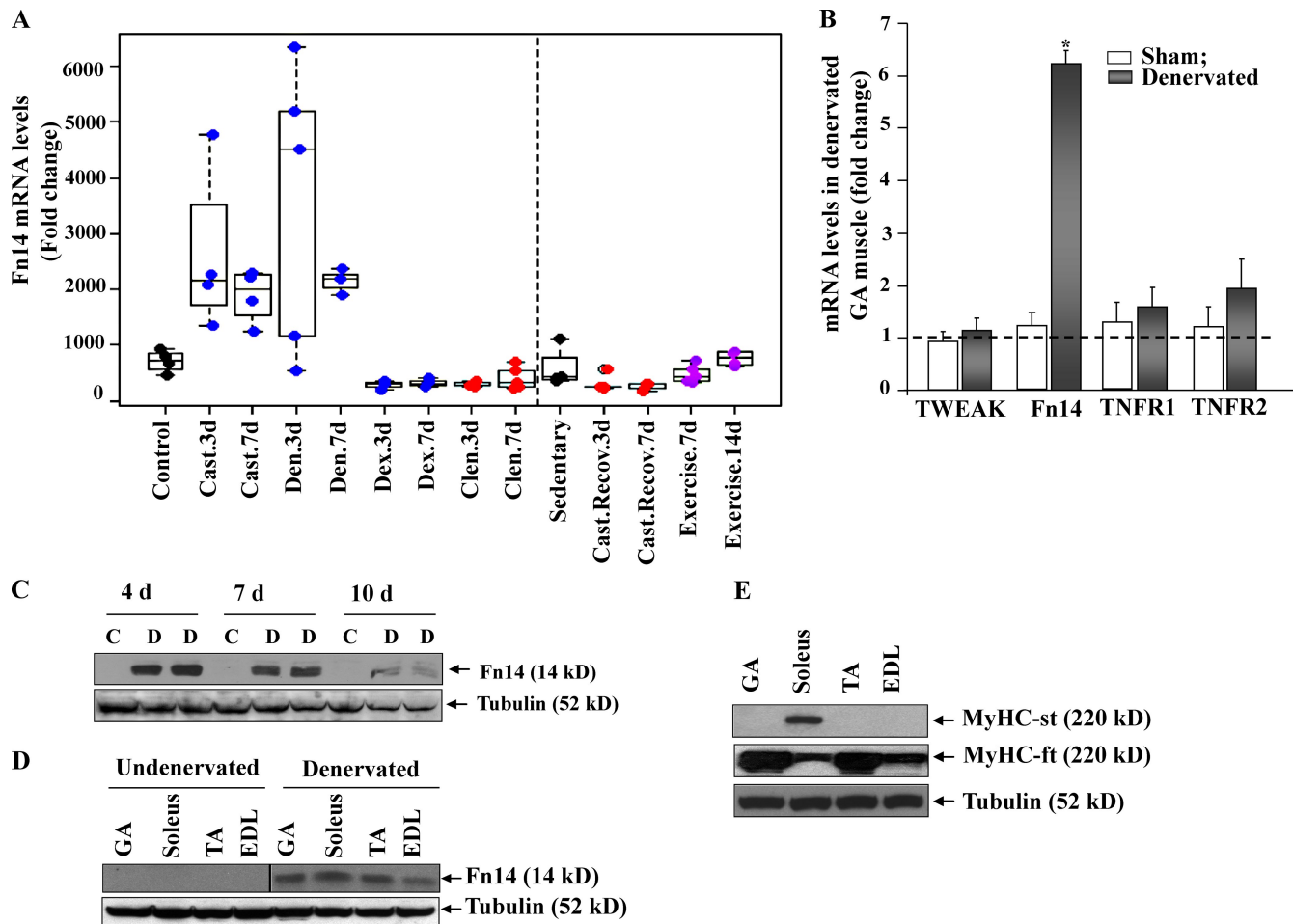


Figure 4. Expression of TWEAK and Fn14 in skeletal muscle upon denervation. (A) Expression of Fn14 in skeletal muscle in response to casting (Cast.), denervation (Den.), and dexamethasone (Dex.) treatment to induce atrophy and in response to clenbuterol (Clen.) and recovery (Recov.) from casting to induce hypertrophy. Animals were also studied under free-running exercise conditions (exercise). mRNA was taken at the time points indicated and assessed in an Affymetrix microarray study. mRNA from 10 animals ($n = 10$) were used for each condition. (B) Relative mRNA levels of TWEAK, Fn14, and TNFR1 and -2 in denervated GA muscle versus sham-operated contralateral GA muscle after 4 d from 12-wk-old C57BL/6 mice ($n = 5$). *, $P < 0.01$. (A and B) Error bars represent the SD. Dashed lines represent the basal levels in control mice. (C) Levels of Fn14 protein measured by Western blotting in control (C) and denervated (D) GA muscle of C57BL/6 mice at 4, 7, or 10 d of denervation. (D) Expression of Fn14 protein in GA, TA, soleus, and EDL muscle of mice measured 4 d after denervation. The black line indicates that intervening lanes have been spliced out. (E) Representative Western blots showing expression of slow-type (st) MyHC (using clone A4.840) and fast-type (ft) MyHC (using clone BF-F3) in GA, TA, soleus, and EDL muscle of mice.

suffer from permanent denervation. Because proinflammatory cytokines have been traditionally suggested as one of the most important mediators of fibrosis in chronic diseases (Drexler et al., 1992) and TWEAK-Tg mice showed increased fibrosis at the age of 6 mo (Fig. 2 A, middle), we next investigated whether overexpression or genetic ablation of TWEAK in mice affects the extent of fibrosis in skeletal muscle in response to denervation. Staining of muscle sections with Sirius red dye, which labels collagen fibers (Lahoute et al., 2008), showed a drastic increase in the accumulation of collagen fibers in denervated TA muscle of TWEAK-Tg mice compared with controls. Furthermore, the level of fibrosis was somewhat reduced in denervated TA muscle of TWEAK-KO mice after 21 d of denervation (Fig. 6 A). Increased fibrosis in TWEAK-Tg and its reduction in TWEAK-KO mice compared with control mice upon denervation was also confirmed by measuring the mRNA levels of collagen I (i.e., Col1a2, major collagen in skeletal muscle) in TA and soleus muscles by the QRT-PCR technique (Fig. 6 B).

Because ablation of TWEAK attenuated muscle atrophy upon denervation, we next determined whether the absence of TWEAK also affects muscle force production in isometric contractions in denervated skeletal muscle. We noted a significant decrease in soleus muscle absolute force production after denervation in control mice, which was significantly preserved in TWEAK-KO mice (Fig. 6 C). These results further support the inference that blocking TWEAK represents a promising strategy to counteracting skeletal muscle atrophy elicited by denervation.

Pharmacological inhibition of TWEAK attenuates denervation-induced skeletal muscle atrophy in mice

We also investigated whether blockade of TWEAK activity using a rat anti-mouse TWEAK-neutralizing antibody (Nakayama et al., 2003; Kamata et al., 2006) can prevent the denervation-induced skeletal muscle loss in mice. Interestingly, treatment

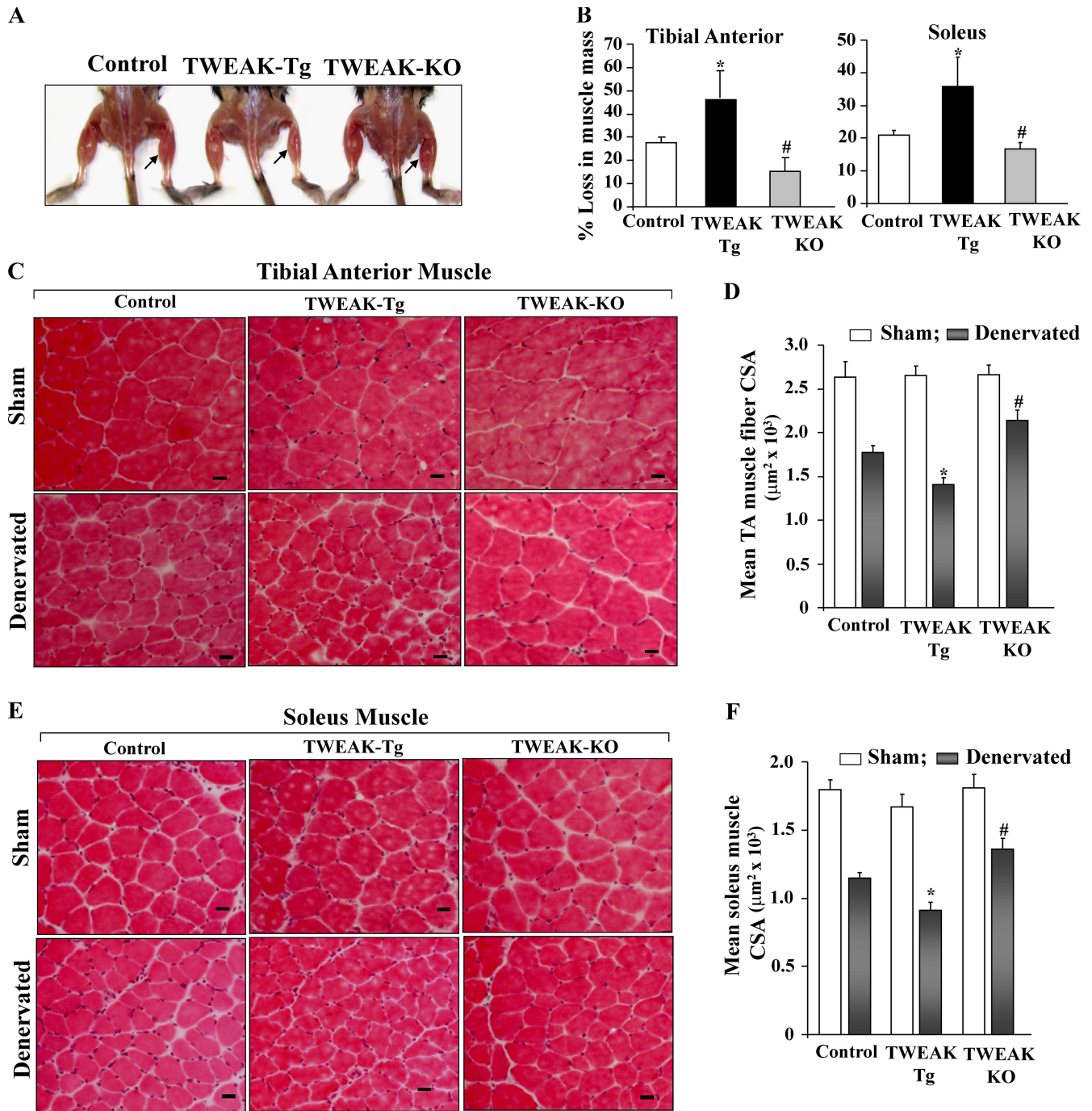


Figure 5. **Role of TWEAK in denervation-induced skeletal muscle atrophy.** (A) 3-mo-old control, TWEAK-Tg, and TWEAK-KO mice 10 d after denervation procedure. Arrows point to denervated GA muscle. (B) TA and soleus muscle were isolated from tendon to tendon from control, TWEAK-Tg, and TWEAK-KO mice 10 d after denervation ($n = 6$ per group), and their wet weight was measured. $*/\#$, $P < 0.05$ (values significantly different from corresponding muscle of control mice). (C) H&E-stained sections of TA of control, TWEAK-Tg, and TWEAK-KO mice 10 d after denervation ($n = 8$ in each group). (D) Quantification of fiber CSA of TA muscle in control, TWEAK-Tg, and TWEAK-KO mice 10 d after denervation. (E) Representative photomicrographs of H&E-stained soleus muscle sections from control, TWEAK-Tg, and TWEAK-KO mice 12 d after denervation. (F) Measurement of fiber CSA in H&E-stained soleus muscle sections in control, TWEAK-Tg, and TWEAK-KO mice 12 d after denervation ($n = 6$ in each group). (D and F) $*, P < 0.01$; and $\#, P < 0.05$ (values significantly different from that of control mice at indicated time after denervation). (B, D, and F) Error bars represent the SD. Bars, 20 μm .

of mice with MTW1 antibody significantly increased the fiber CSA in denervated TA and soleus muscle of MTW1-treated mice compared with isotype control-treated mice (Fig. 7), further suggesting that TWEAK is a mediator of denervation-induced skeletal muscle atrophy.

TWEAK is an upstream activator of NF- κ B in denervated skeletal muscle

NF- κ B is a transcription factor that causes skeletal muscle wasting and is activated by cytokines (Cai et al., 2004; Mourkioti et al., 2006). To understand the intracellular signaling mechanisms

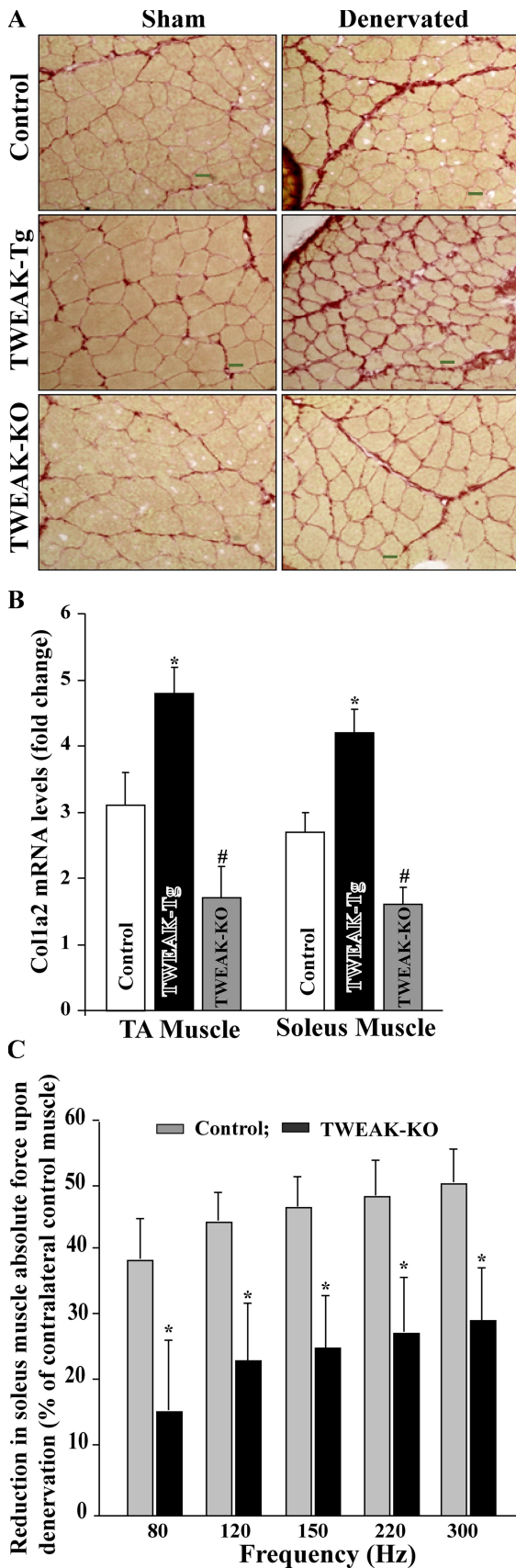


Figure 6. **Role of TWEAK in development of fibrosis and loss of muscle function.** (A) Sirius red staining performed on TA muscle sections after 21 d of denervation in control, TWEAK-Tg, and TWEAK-KO mice ($n = 4$ in each

by which TWEAK-Fn14 stimulates the loss of skeletal muscle mass in response to denervation, we measured the activation of NF- κ B in control and denervated skeletal muscle of TWEAK-Tg and TWEAK-KO mice. DNA-binding activity of NF- κ B, measured by electrophoretic mobility shift assay (EMSA), was found to be significantly increased in denervated muscle of TWEAK-Tg mice as compared with control mice (Fig. 8 A). In contrast, the denervation-induced activation of NF- κ B was significantly reduced in TWEAK-KO mice (Fig. 8 B). To confirm that the retarded bands seen in EMSA were indeed NF- κ B, we performed a supershift assay. Preincubation of nuclear extracts from denervated GA muscle of control mice with antibodies against p50 or p52 shifted the bands to higher levels of molecular weight, indicating that the NF- κ B-DNA complex analyzed by EMSA constitutes these proteins (Fig. 8 C). Furthermore, by electroporating TA muscle with NF- κ B reporter plasmid, we also confirmed that the observed changes in the DNA-binding activity of NF- κ B in denervated skeletal muscle of control, TWEAK-Tg, and TWEAK-KO mice were highly correlated with the transcriptional activation of NF- κ B (Fig. 8 D).

Besides NF- κ B, the PI3K-Akt signaling pathway is also an important regulator of skeletal muscle mass in vivo (Glass, 2003). Activation of Akt augments skeletal muscle mass in vivo by stimulating anabolic and suppressing catabolic pathways (Bodine et al., 2001b; Lai et al., 2004; Blaauw et al., 2008). It has been reported that denervation causes suppression of Akt activity in skeletal muscle, whereas constitutive activation of Akt blunts the loss of skeletal muscle mass upon denervation (Bodine et al., 2001b; Stitt et al., 2004; Mourkioti et al., 2006). Our results showed that although levels of phosphorylated Akt, mammalian target of rapamycin (mTOR), p70S6K, and GSK3- β were reduced in GA muscle 10 d after denervation, there was no noticeable difference in the levels of phosphorylated Akt, mTOR, GSK3- β , or p70S6K between denervated muscle of control, TWEAK-Tg, and TWEAK-KO mice (Fig. S3).

TWEAK acts through up-regulation of the ubiquitin-proteasome system

The amount of ubiquitin protein conjugates and the expression of the components of ubiquitin degradation pathways are significantly enhanced during atrophy (Attaix and Bechet, 2007). In genetic screens for the markers of muscle atrophy, two genes, MAFbx (also known as Atrogin-1) and MuRF1, both encoding ubiquitin ligases, were identified that are highly induced in vivo in atrophying muscle, driven by immobilization, denervation, hind limb suspension, starvation, tumor

group). (B) Fold change in the mRNA levels of collagen type 1, α 2 (Col1 α 2) in TA and soleus muscle of control ($n = 6$), TWEAK-Tg ($n = 3$), and TWEAK-KO ($n = 3$) mice 10 d after denervation measured by QRT-PCR. */#, $P < 0.01$ (values significantly different from denervated muscle of control mice). (C) Denervation-induced loss in absolute muscle force production in isometric contraction was measured in soleus muscle of control ($n = 5$) and TWEAK-KO ($n = 6$) mice at 80, 120, 150, 220, and 300 Hz. *, $P < 0.01$ (values significantly different from the denervated soleus muscle of control mice at same frequency). (B and C) Error bars represent the SD. Bars, 50 μ m.

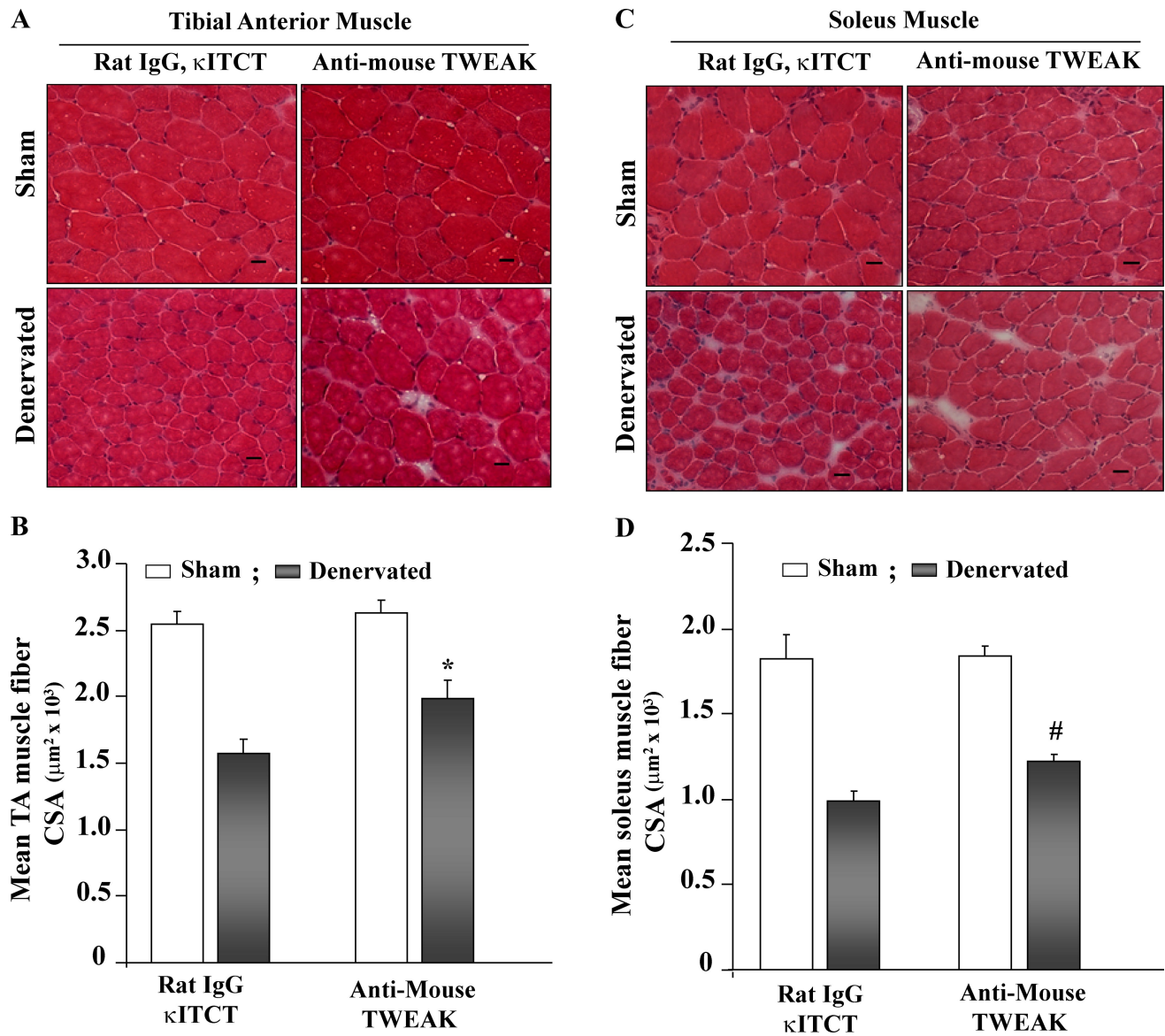


Figure 7. **TWEAK-neutralizing antibody rescues denervation-induced muscle loss in mice.** 2-mo-old C57BL/6 mice were denervated for 2 d, followed by intraperitoneal injections of 200 μg/mouse of either rat IgG1 or anti-TWEAK every third day for 12 d ($n = 4$ in each group). (A) Representative photomicrographs of H&E-stained sections of control and denervated TA muscle of isotype and anti-TWEAK-treated mice. (B) Quantification of fiber CSA in TA muscle sections of isotype and anti-TWEAK-treated mice. *, $P < 0.01$ (values significantly different from denervated TA muscle of IgG1-treated mice). (C) H&E-stained sections of control and denervated soleus muscle of mice treated with isotype or anti-TWEAK. (D) Quantification of fiber CSA in soleus muscle sections. #, $P < 0.05$ (values significantly different from denervated soleus muscle of IgG1-treated mice). (B and D) Error bars represent the SD. ITCT, isotype control. Bars, 20 μm.

load, and glucocorticoids (Bodine et al., 2001a; Gomes et al., 2001; Glass, 2005). The role of MAFbx and MuRF1 has been confirmed by the findings that the loss-of-function mutations in either of these genes reduced skeletal muscle atrophy in response to divergent catabolic stimuli, including denervation (Bodine et al., 2001a). We studied the expression patterns of MAFbx and MuRF1 by QRT-PCR in the transgenic and knockout animals versus controls. The expressions of both MAFbx and MuRF1 were significantly increased in denervated GA muscle compared with sham-operated control muscle (Fig. 9, A and B). The mRNA levels of MuRF1 were significantly increased in TWEAK-Tg and significantly decreased in TWEAK-KO mice (Fig. 9 A), which is consistent with MuRF1 being

regulated by NF-κB activation (Cai et al., 2004). In contrast, there was no significant difference in the level of MAFbx expression between denervated muscle of control, TWEAK-Tg, and TWEAK-KO mice (Fig. 9 B), which is again consistent with a prior study showing that activation of NF-κB induced MuRF1 but not MAFbx expression (Cai et al., 2004). Furthermore, by performing Western blot analysis, we found that the protein level of MuRF1 was also elevated in TWEAK-Tg and reduced in TWEAK-KO mice compared with control mice in denervated muscles (Fig. 9 C).

It should be noted that in the naive, undenervated, transgenic animals, NF-κB (Fig. 8 A) or MuRF1 (Fig. 9 B) was not up-regulated. This is because these experiments were performed

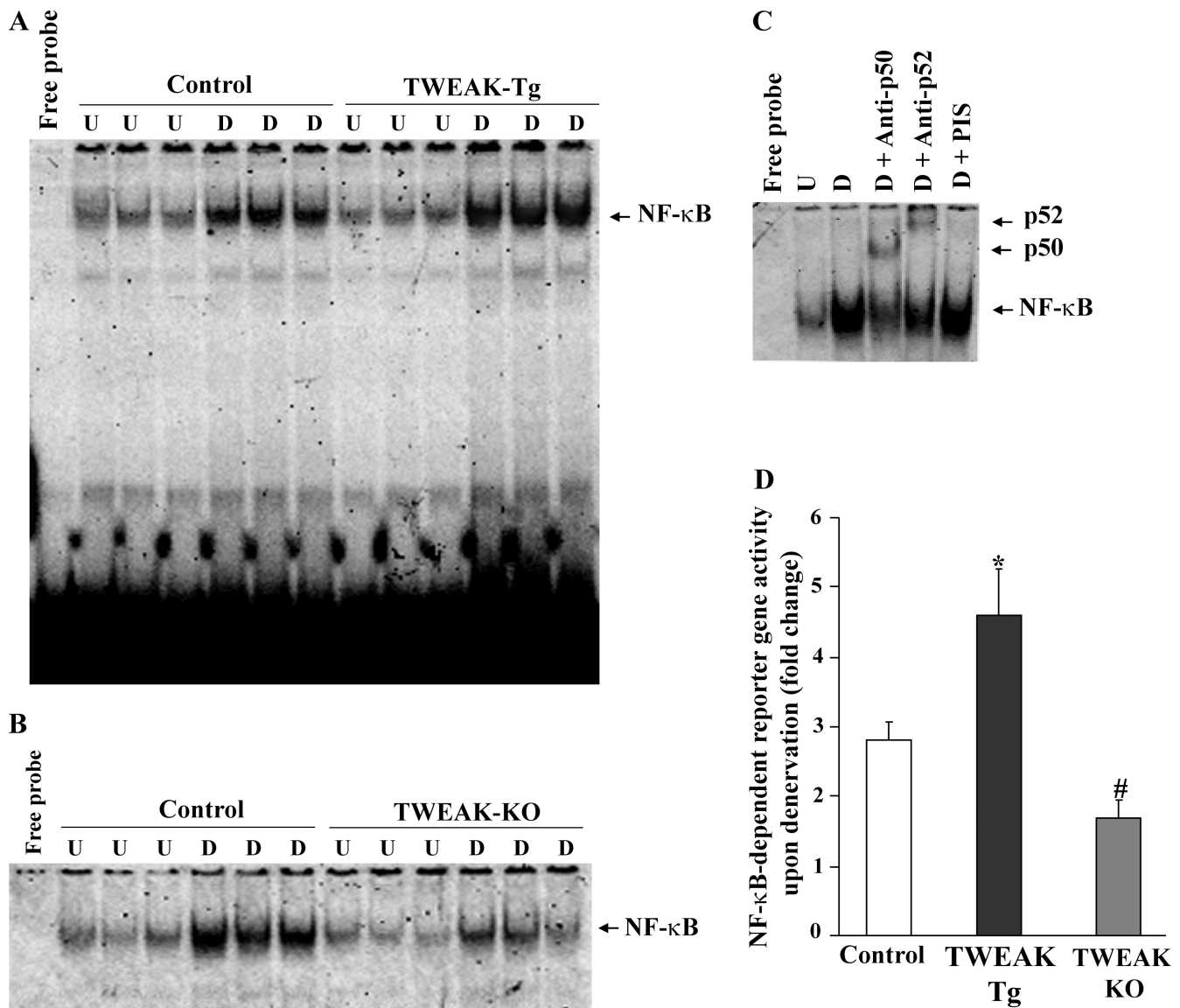


Figure 8. **Role of TWEAK in the activation of NF-κB in denervated skeletal muscles.** DNA-binding activity of NF-κB was measured by EMSA in TA muscle 10 d after denervation. (A and B) Representative EMSA gel from three independent experiments for control and TWEAK-Tg mice (A) and control and TWEAK-KO mice (B). (C) Supershift assay performed using nuclear extracts from denervated GA muscle of control mice using antibodies against p50 and p52 subunits of NF-κB. (D) Fold change in NF-κB reporter gene activity (normalized using Renilla luciferase) in TA muscle of control, TWEAK-Tg, and TWEAK-KO mice after denervation. *, $P < 0.01$; and #, $P < 0.05$ (values significantly different from corresponding control mice). Error bars represent the SD. U, undenervated; D, denervated; PIS, preimmune serum.

on 3-mo-old animals, before a phenotype is seen in the transgenics. By 6 mo of age, atrophy, fibrosis, and NF-κB and MuRF1 up-regulation were noted in unperturbed animals (Figs. 2 and 3).

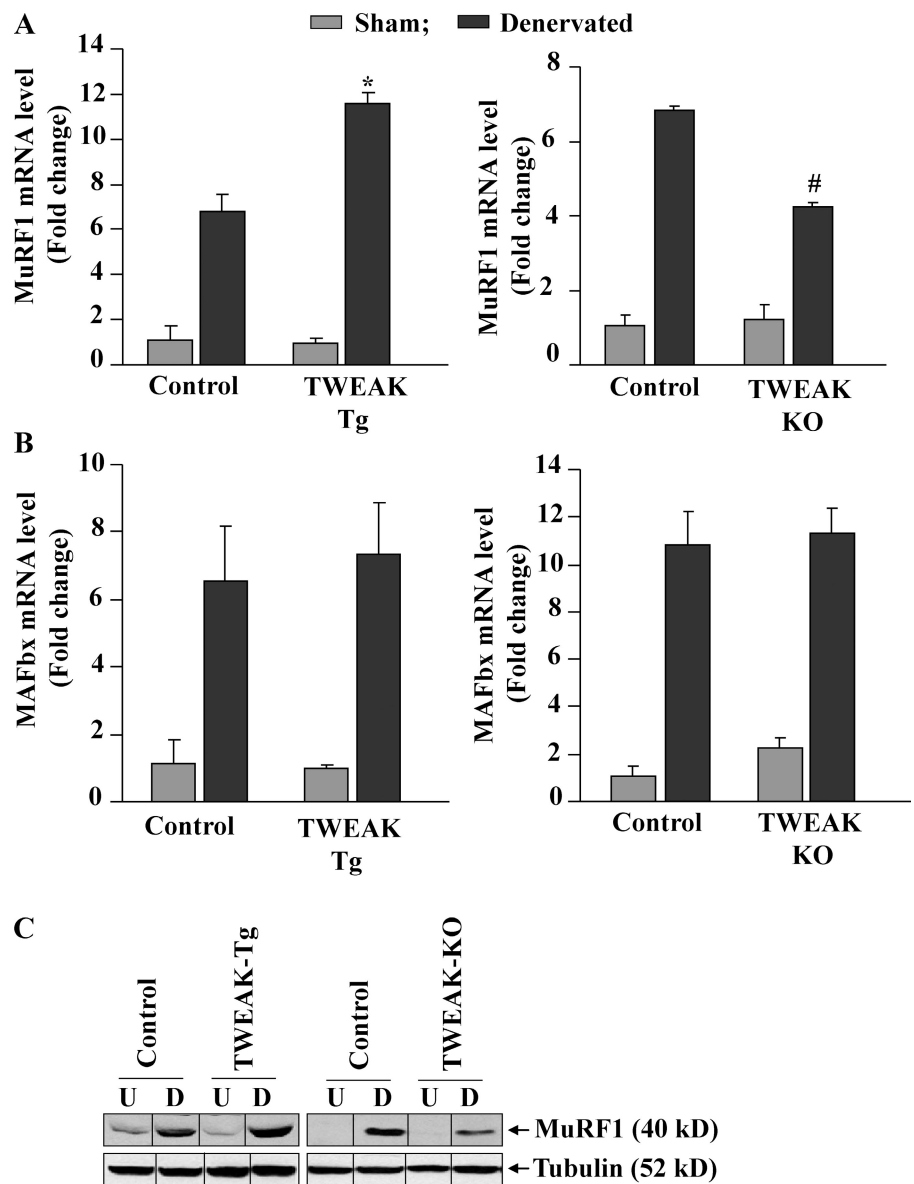
Recent evidence also suggests that lysosomal proteolysis may also contribute to the loss of muscle protein during atrophy (Mammucari et al., 2007; Zhao et al., 2007, 2008). Autophagosome formation has been demonstrated in skeletal muscle of mice in response to fasting or denervation (Mordier et al., 2000; Mammucari et al., 2007). The expression of several genes involved in the autophagy pathway has been found to be increased in denervated skeletal muscle (Zhao et al., 2007). To understand whether TWEAK affects autophagy, we compared mRNA levels of autophagy-related molecules LC3B, Beclin-1,

Atg-5, Atg-12, and Gabarapl1 in denervated muscle of control, TWEAK-Tg, and TWEAK-KO mice. Although a significant increase in mRNA levels of all of the five genes was observed in denervated skeletal muscle, there was no significant difference in the mRNA levels of any of these genes in denervated muscle of TWEAK-Tg or TWEAK-KO mice compared with control mice (Fig. S4).

Discussion

Skeletal muscle atrophy is commonly observed in disuse conditions such as denervation, unloading, and immobilization (Spätle and Schulze, 2004; Glass, 2005; Sandri, 2008). Proinflammatory cytokines have been suggested both to induce and mediate

Figure 9. **Effects of TWEAK on the expression of muscle-specific E3 ubiquitin ligases in denervated skeletal muscle.** (A) Relative mRNA levels of MAFbx in denervated GA muscle of TWEAK-Tg or TWEAK-KO versus control mice ($n = 6$ in each group). */#, $P < 0.01$ (values significantly different from corresponding control mice). (B) Relative mRNA levels of MuRF1 in denervated GA muscle of TWEAK-Tg or TWEAK-KO versus control mice ($n = 6$ per group). (A and B) Error bars represent the SD. (C) Representative immunoblots demonstrating MuRF1 protein levels in undenervated (U) and denervated (D) GA muscle of control, TWEAK-Tg, and TWEAK-KO mice. Black lines indicate that intervening lanes have been spliced out.



local catabolic mechanisms at advance stages of chronic diseases, leading to cachexia (Späte and Schulze, 2004; Argilés et al., 2005; Li et al., 2008); however, because disuse-related skeletal muscle atrophy does not involve any systematic inflammatory response, the involvement of proinflammatory cytokines in this type of atrophy has received limited attention. The results of this study provide strong evidence, for the first time, that the inflammatory cytokine TWEAK is an important mediator of denervation-induced skeletal muscle atrophy.

Overexpression of TWEAK is sufficient to cause skeletal muscle atrophy in vivo

New transgenic mice were produced, in which TWEAK was overexpressed four- to fivefold (Fig. 2, A and B). These animals live into adulthood, in contrast to prior TWEAK transgenics, in which the cytokine was expressed at 14-fold normal levels; those animals died peri- or neonatally because of excessive muscle loss (Dogra et al., 2007a).

Transgenic mice overexpressing TWEAK four- to six-fold show no gross phenotype until the animals are around 6 mo of age. At that time, a decrease in muscle fiber size can be observed, which is coincident with an increase in fibrosis, which is a similar phenotype to that seen in settings of denervation (Figs. 2 A and 6, A and B). Coincident with the onset of atrophy, there is an increase in the activity of NF- κ B transcription factor (Fig. 3 E) and the levels of the E3 ubiquitin ligase MuRF1 (Fig. 3 F) but not those of a second muscle-specific E3 ligase, MAFbx (Bodine et al., 2001a). MuRF1 and MAFbx are found to be coactivated in many settings of muscle atrophy (Bodine et al., 2001a; Gomes et al., 2001). However, it was also previously demonstrated that discrete activation of the NF- κ B pathway induces MuRF1 but not MAFbx expression, placing MuRF1 downstream of NF- κ B (Cai et al., 2004; Glass, 2005); MAFbx can be activated by p38 signaling (Li et al., 2005).

MuRF1 is a specific E3 ligase for MyHC protein (Clarke et al., 2007; Fielitz et al., 2007). It degrades MyHC and other

components of the thick filament such as myosin light chain (Cohen et al., 2009). The loss of MyHC in the 6-mo-old TWEAK-Tg animals (Fig. 3 A) indicates that the MuRF1–MyHC pathway has been activated by TWEAK–Fn14 signaling, establishing a TWEAK–Fn14–NF- κ B–MuRF1–MyHC protein degradation cascade.

It is of interest that otherwise-unperturbed TWEAK-Tg animals did not display a phenotype until they were older. This suggests the possibility that a second, age-dependent event has to occur in order for TWEAK overexpression to be sufficient to induce atrophy. The late appearance of the atrophic phenotype in TWEAK-Tg mice (i.e., around 4–5 mo) suggests that the catabolic action of TWEAK in skeletal muscle may be neutralized by active muscle formation and/or the presence of growth factors at a younger age. It is also possible that TWEAK has different roles in the acquisition and the maintenance of skeletal muscle mass *in vivo*. It has been previously reported that genetic ablation of TWEAK receptor delays the regeneration of myofibers in mice (Girgenrath et al., 2006), suggesting that TWEAK may also be essential for the proliferation of muscle progenitor cells during skeletal muscle growth. Furthermore, we found that increased concentration of TWEAK induces fibrosis in skeletal muscle (Fig. 2 A). These data are consistent with a recent study demonstrating that increased levels of TWEAK in circulation induce fibrosis in cardiac muscle (Jain et al., 2009). The increased number of type II fibers in soleus muscle of TWEAK-Tg mice also indicates that the elevated levels of TWEAK are sufficient to induce transition of slow-type fibers into fast-type fibers and its absence increases the proportion of slow-type fibers (Fig. 3, C–F).

Although it is of interest to show that ectopic, enhanced expression of TWEAK is sufficient to induce skeletal muscle atrophy, that finding by itself does not explain what happens under normal settings of atrophy. Therefore, it was interesting to see that genetic ablation of TWEAK augments fiber CSA; this finding establishes that TWEAK is a negative regulator of skeletal muscle mass in adult animals (Fig. 2, A and B).

Recent evidence suggests that muscle wasting in response to cancer cachexia involves the degradation of only selective muscle proteins (Acharyya et al., 2004, 2005; Acharyya and Guttridge, 2007). Cancer cachexia also leads to sarcolemmal abnormalities, including the reduced expressions of components of the dystrophin–glycoprotein complex in skeletal muscle (Acharyya et al., 2005). Our results demonstrate that skeletal muscle atrophy in TWEAK-Tg mice is associated with the reduced levels of contractile proteins MyHC and tropomyosin (Fig. 3, A and C). Furthermore, the level of nNOS, a protein which interacts with the dystrophin–glycoprotein complex in skeletal muscle, but not dystrophin and laminin is reduced in TWEAK-Tg mice (Fig. 3 A). nNOS dysregulation has been shown to be itself sufficient to induce muscle atrophy (Suzuki et al., 2007), and recent evidence suggests that the localization of nNOS to the sarcolemma is essential for the maintenance of normal muscle activity (Kobayashi et al., 2008). Furthermore, genetic ablation of nNOS in mice causes significant reduction in maximum tetanic force production and increased susceptibility to contraction-induced fatigue, suggesting that nNOS is

required for skeletal muscle strength *in vivo* (Percival et al., 2008). Our results indicate that the increased levels of TWEAK may destabilize the sarcolemma in part by reducing the level of nNOS, thus disturbing the equilibrium and resulting in atrophy, and by up-regulating NF- κ B–MuRF1 signaling, causing actual breakdown of the thick filament.

Genetic ablation of TWEAK spares skeletal muscle under denervation conditions

Our previous study showed that the expression of the receptor Fn14 but not TWEAK itself might be important for the catabolic action of TWEAK in skeletal muscle *in vivo* (Dogra et al., 2007a). Although the expression of TWEAK in skeletal muscle does not change, the levels of Fn14 are significantly reduced after initial stages of development (Dogra et al., 2007a). Other reports also suggest that Fn14 is expressed at relatively low level in normal tissues (Tanabe et al., 2003; Winkles, 2008). However, the expression of Fn14 is highly up-regulated in response to tissue injury, regeneration, and in chronic inflammatory diseases, supporting its role in tissue remodeling (Tanabe et al., 2003; Winkles, 2008). Although the underpinning mechanisms leading to the increased expression of Fn14 remain unknown, this study demonstrates that the activation of the TWEAK–Fn14 pathway mediates the loss of skeletal muscle mass and strength upon denervation (Figs. 5, 6 C, and 7). Additionally, increased fibrosis in the denervated muscle of the TWEAK-Tg mice further supports the contention that TWEAK is a major regulator of fibrosis in atrophying conditions (Fig. 6, A and B).

The loss of skeletal muscle proteins in atrophying muscle is mediated by the activation of the ATP-dependent ubiquitin–proteasome pathway, with an associated increase in the expression of the E3 ubiquitin ligases MAFbx and MuRF1 (Glass, 2003, 2005). Before this study, the identity of particular upstream triggers of atrophy in the various models remained unknown. The results presented in this study demonstrate that the activation of the TWEAK–Fn14 system specifically activates NF- κ B in denervated muscle (Fig. 8). Although skeletal muscle wasting might involve coordinated activation of multiple cell signaling pathways, NF- κ B activation in skeletal muscle is sufficient to cause skeletal muscle wasting (Cai et al., 2004; Li et al., 2008), in part by activating MuRF1, because muscle-wasting in IKK- β -overexpressing transgenic mice was significantly reduced by crossing them with MuRF1-KO mice (Cai et al., 2004). Furthermore, skeletal muscle-specific deletion of IKK- β in mice reduces the expression of MuRF1 and atrophy in response to denervation (Mourkioti et al., 2006). Although MAFbx was activated by denervation in these experiments (Fig. 9), which is in line with prior studies (Bodine et al., 2001a; Gomes et al., 2001), this E3 ligase was not further perturbed by the addition or deletion of TWEAK, adding further evidence to the published report that NF- κ B activation does not up-regulate MAFbx expression (Cai et al., 2004).

It is also of interest to note some of the pathways that TWEAK did not activate. Although TWEAK was found to stimulate the NF- κ B pathway, it did not affect the activation of Akt (Fig. S3) or autophagy pathway (Fig. S4) in denervated skeletal muscle. Collectively, our results suggest that the

TWEAK–Fn14 pathway specifically up-regulates the expression of the components of the ubiquitin–proteasome system (e.g., MuRF1) in denervated skeletal muscle.

The TWEAK–Fn14 pathway provides a novel signaling mechanism to regulate skeletal muscle mass under denervation conditions

These data highlight a previously undiscovered and surprising mechanism for modulating skeletal muscle atrophy. The fact that the Fn14 receptor but not TWEAK itself is up-regulated under atrophy conditions suggests a mechanism in which muscle creates a permissive setting for atrophy by modulating the TWEAK receptor. Because TWEAK itself is not perturbed, the data suggest that the receptor's expression is limiting for TWEAK to function. It is noteworthy that in younger animals, TWEAK overexpression is not sufficient to cause atrophy, but it is able to increase muscle loss under atrophy conditions. Because the TWEAK receptor Fn14 is up-regulated by denervation, this adds further data for the inference that Fn14 levels are limiting, and therefore, TWEAK requires Fn14 up-regulation to induce atrophy. Finally, one can always push back the definition of the term trigger. For example, although the current study identifies TWEAK–Fn14 signaling as being necessary to up-regulate NF- κ B–MuRF1-induced proteolysis in atrophy conditions, one can then enquire as to the mechanism by which denervation up-regulates Fn14. This is also an area of interest for future investigation.

The TWEAK–Fn14 pathway as a potential target to counter disuse-related skeletal muscle atrophy

This study marks an initial attempt to determine whether an inflammatory cytokine is involved in disuse-related skeletal muscle atrophy. The identification of the TWEAK–Fn14 dyad as a mediator of denervation-induced muscle atrophy suggests that inhibition of this pathway may be helpful under denervation and perhaps disuse conditions, especially because the deletion of TWEAK was sufficient to maintain muscle strength in addition to mass. Although Fn14 is not up-regulated in every setting of muscle atrophy, future studies will help to determine the full range of clinically relevant settings in which the TWEAK–Fn14 pathway is operative and in which inhibition of this pathway is sufficient to preserve functional muscle.

Materials and methods

Animal models

Transgenic mice expressing full-length TWEAK cDNA under the control of muscle CK promoter were generated as described previously (Dogra et al., 2007a). TWEAK-KO mice were provided by A. Ashkenazi (Genentech South San Francisco, CA; Maecker et al., 2005). All of the mice were in the C57BL/6 background, and their genotype was determined by PCR from tail DNA.

To induce atrophy, 12-wk-old mice were either casted or denervated for 3–7 d, as previously described (Bodine et al., 2001a). To study hypertrophy, the casted muscles were allowed to recover for 1 wk after the casts were removed. Also, as an additional model of hypertrophy, animals were treated with clenbuterol for 1 wk, as described previously (Kline et al., 2007). Sciatic denervation was performed by anesthetizing the mice with an

intraperitoneal injection of 100 mg/kg ketamine and 20 mg/kg xylazine, shaving the right hind quarters, making a 0.5-cm incision ~0.5 cm proximal to the knee on the lateral side of the right leg, separating the muscles and lifting out the sciatic nerve with a surgical hook or forceps, removing a 2–3-mm piece of sciatic nerve, and closing the incision with surgical staples.

For casting, mice were anesthetized, and immobilized at the knee and ankle joints, with the ankle in plantar flexion to maximize atrophy in the posterior compartment muscles. The Plaster of Paris cast encompassed one hind limb. For recovery, after 2 wk of atrophy, the cast was removed, and the animals were allowed to ambulate freely in their cages. For clenbuterol treatment, the mice received either physiological saline at 1 ml/kg per day or 3 mg/kg per day clenbuterol (Sigma-Aldrich) dissolved in water via a subcutaneous injection. 9 d after the denervation surgery, mice were anesthetized with isoflurane and euthanized by CO₂ asphyxiation. The left and right legs were shaved, the skin on each leg was resected, and the TA, soleus, and medial GA muscles were isolated, removed, and weighed. All animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to the American Physiological Society's Guiding Principles in the Care and Use of Animals. For microarrays, Affymetrix mouse 430 version 2 microarrays were used to measure gene expression values. Normalization in our analysis was performed using the GeneChip robust multiarray averaging normalization method (Irizarry et al., 2006).

To study the effects of TWEAK-neutralizing antibody (Clone MTW1; BioLegend) on denervation-induced muscle loss, 8-wk-old C57BL/6 mice were used. 2 d after denervation, the mice were treated by intraperitoneal injections of either 200 μ g/mouse MTW1 antibody or 200 μ g/ml isotype control every third day (total of three injections). After 24 h of final injection, the mice were sacrificed, and hind limb muscles were isolated for histological experiments.

Electroporation of plasmid DNA in TA muscle

The injection of plasmid DNA into TA muscle of mice and electroporation were performed according to a protocol as previously described (Schertzer et al., 2006). In brief, pNF- κ B–Luc (Takara Bio Inc.) and pRL-TK renilla luciferase (Promega) were prepared using an endotoxin-free kit (QIAGEN) and suspended in sterile saline solution in a 1:10 ratio. Mice were anesthetized, and a small portion of TA muscle of both hind limbs was surgically exposed and injected with 30 μ l of 0.5 U/ μ l hyaluronidase (EMD). 2 h later, plasmid DNA (50 μ g in 25 μ l saline) was injected in TA muscle, and 1 min after plasmid DNA injection, a pair of platinum plate electrodes was placed against the closely shaved skin on either side of the small surgical incision (such that the electrodes did not contact the muscle), and electric pulses were delivered transcutaneously. Three 20-ms square-wave pulses of 1-Hz frequency at 75V/cm were generated using a stimulator (model S88; Grass Technologies) and delivered to the muscle. The polarity was then reversed, and a further three pulses were delivered to the muscle. After electroporation, the wound was closed with surgical clips, and mice were returned to their cages and fed a standard diet. 3 d after electroporation, the left hind limb was denervated, whereas the right side was only sham operated. Finally, after 10 d of denervation, the mice were sacrificed, TA muscle was isolated, and muscle extracts made were used for measurement of luciferase and renilla activity using a Dual-luciferase reporter assay system (Promega).

RNA isolation and QRT-PCR

RNA isolation and QRT-PCR were performed using a method as previously described (Dogra et al., 2006, 2007b). In brief, RNA was extracted from homogenized tissues using TRIZOL reagent (Invitrogen) and an RNeasy Mini kit (QIAGEN) according to the manufacturers' protocol. The quantification of mRNA expression was performed using the SYBR green dye method on a sequence detection system (model 7300; Applied Biosystems). 1 μ g of purified RNA was used to synthesize first strand cDNA by reverse transcription system using oligo (dT) primer (Applied Biosystems) and the Omniscript reverse transcription kit (QIAGEN). The first strand cDNA reaction (0.5 μ l) was subjected to real-time PCR amplification using gene-specific primers. The sequence of primers used is described in Table S1.

Approximately 25 μ l of reaction volume was used for the real-time PCR assay that consisted of 2 \times of 12.5 μ l Brilliant SYBR green QPCR master mix (Applied Biosystems), 400 nM of primers (0.5 μ l each from the stock), 11 μ l water, and 0.5 μ l of template. The thermal conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min, and, for a final step, a melting curve of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. All reactions were performed in duplicate to reduce variation.

Data normalization was accomplished using the endogenous control (glyceraldehyde-3-phosphate dehydrogenase or β -actin), and the normalized values were subjected to a $2^{-\Delta\Delta Ct}$ formula to calculate the fold change between the control and experimental groups.

Western blotting

Levels of different proteins in skeletal muscle were determined by performing immunoblotting as described previously (Kumar and Boriek, 2003). In brief, tissues were washed with PBS and homogenized in Western blot lysis buffer A (50 mM Tris-Cl, pH 8.0, 200 mM NaCl, 50 mM NaF, 1 mM DTT, 1 mM Na orthovanadate, 0.3% IGEPAL, and protease inhibitors). Approximately 100 μ g of protein was resolved on each lane on 10–12% SDS-PAGE, electrotransferred onto nitrocellulose membrane, probed using anti-Fn14 (1:1,000; Cell Signaling Technology), MF-20 (1:1,000; Development Studies Hybridoma Bank), antilaminin (1:1,000; Sigma-Aldrich), antitropomyosin (1:2,000; Sigma-Aldrich), antitropinin (1:1,000; Sigma-Aldrich), anti-sarcomeric α -actin (1:1,000; Sigma-Aldrich), anti-nNOS (1:500; Santa Cruz Biotechnology, Inc.), antidystronin (1:200; Development Studies Hybridoma Bank), anti-MyHC fast type (1:1,000; clone BF-F3; Development Studies Hybridoma Bank), anti-MyHC slow type (1:1,000; clone A4.840; Development Studies Hybridoma Bank), antitubulin (1:5,000; Abcam), anti-MuRF1 (1:1,000; R&D Systems), anti-phospho-Akt (1:500; Cell Signaling Technology), anti-phospho-GSK3- β (1:1,000; Cell Signaling Technology), anti-phospho-p70S6K (1:1,000; Cell Signaling Technology), anti-phospho-mTOR (1:1,000; Cell Signaling Technology), anti-collagen I (1:1,000; Abcam), anti-collagen III (1:1,000; Abcam), and anti-collagen IV (1:500; Abcam), and detected by chemiluminescence. The bands were quantified using ImageQuant TL software (GE Healthcare). To determine the levels of collagens I and III, muscle extracts were prepared in lysis buffer lacking DTT and separated on SDS-PAGE under nonreducing conditions.

EMSA

NF- κ B activation was analyzed by EMSA as previously described (Kumar and Boriek, 2003; Li et al., 2009a). In brief, 25 μ g of nuclear extracts prepared from control or denervated muscle was incubated with 16 fmol of 32 P end-labeled NF- κ B or AP-1 consensus oligonucleotide (Promega) at 37°C for 20 min, and the DNA-protein complex was resolved on a 7.5% native polyacrylamide gel. A 5% gel was used for supershift analysis. For supershift assays, nuclear extracts were first incubated with 1 μ g of anti-p50 (Santa Cruz Biotechnology, Inc.), anti-p52 (Cell Signaling Technology), or preimmune serum for 30 min before performing probe-binding reaction. The radioactive bands from the dried gel were visualized and quantified by PhosphorImager (GE Healthcare) using ImageQuant TL software.

Muscle histology, immunohistochemistry, and morphometric measurements

All experiments involving the evaluation of TWEAK-Tg or TWEAK-KO and control were conducted on at least six mice per group. Hind limb muscles (soleus, GA, and TA) from 1- to 6-mo-old mice were removed, frozen in isopentane cooled in liquid nitrogen, and sectioned in a microtome cryostat. For the assessment of tissue morphology or visualization of fibrosis, 10- μ m-thick transverse sections of muscles were stained, respectively, with H&E and Sirius red (American Master Tech) and examined under a light microscope (Eclipse TE 2000-U; Nikon). The amount of fibrosis in paraffin-embedded soleus muscle sections was also determined using Masson's trichrome staining kit (American Master Tech) according to a protocol suggested by the manufacturer.

For immunohistochemistry study, the sections were blocked in 1% bovine serum albumin in PBS for 1 h and incubated with primary antibodies in blocking solution at 4°C overnight under humidified conditions. The sections were washed briefly with PBS before incubation with secondary antibodies for 1 h at room temperature and then washed three times for 30 min with PBS. The slides were mounted using fluorescence medium (Vector Laboratories), and staining was visualized at room temperature on an Eclipse TE 2000-U microscope using a Plan 10 \times NA 0.25 PH1 DL or Plan-Fluor ELWD 20 \times NA 0.45 Ph1 DM objective lens (Nikon), a digital camera (Digital Sight DS-Fi1; Nikon), and NIS Elements BR 3.00 software (Nikon). Image levels were equally adjusted using Photoshop CS2 software (Adobe).

The primary antibodies were antilaminin (1:100), anti-MyHC fast type (1:200; NCL-MHCf; Novocastra Laboratories Ltd), anti-MyHC slow type I (1:200; clone A4.840), anti-Mac-1 (1:250; Development Studies Hybridoma Bank), anti-collagen I (1:300), anti-collagen III (1:300), and anti-collagen IV (1:500). Alexa Fluor 488- or Alexa Fluor

596-conjugated secondary antibodies (1:3,000; Invitrogen) were used for detection. Fiber CSA was analyzed in H&E- or laminin-stained soleus or TA muscle sections. For each muscle, the distribution of fiber CSA was calculated by analyzing 200–500 myofibers as described previously (Dogra et al., 2007a; Li et al., 2009b).

Skeletal muscle functional analysis

The skeletal muscle force production in isometric contraction was performed as previously described (Li et al., 2009b). In brief, soleus muscle from control or denervated hind limb of mice was rapidly excised and placed in Krebs-Ringer solution. The muscle was mounted between a Fort25 force transducer (World Precision Instruments) and a micromanipulator device in a temperature controlled myobath (World Precision Instruments). The muscle was positioned between platinum wire stimulating electrodes and stimulated to contract isometrically using electrical field stimulation (supramaximal voltage, 1.2-ms pulse duration) from the S88 stimulator. In each experiment, muscle length was adjusted to optimize twitch force (optimal length, L_0). The muscle was rested for 15 min before the tetanic protocol was started. The output of the force transducer was recorded in a computer using Lab-Trax-4 software (World Precision Instruments). To evaluate a potentially different frequency response between groups, tetani were assessed by sequential stimulation at 80, 120, 150, 220, and 300 Hz with a 2-min rest in between.

Statistical analysis

Results are expressed as mean \pm SD. The Student's *t* test or analysis of variance was used to compare quantitative data populations with normal distributions and equal variance. A value of *P* < 0.05 was considered statistically significant unless otherwise specified.

Online supplemental material

Fig. S1 shows the expression of collagen I, II, and III in soleus muscle of control and TWEAK-Tg mice analyzed by fluorescence microscopy and Western blotting. Fig. S2 examines the concentration of macrophages in soleus muscle of control, TWEAK-Tg, and TWEAK-KO mice. Fig. S3 examines the activation of the Akt signaling pathway in denervated skeletal muscle of control, transgenic, and knockout mice. Fig. S4 demonstrates the expression of autophagy-related genes in denervated skeletal muscle of control, transgenic, and knockout mice. Table S1 describes the sequence of the primers used in QRT-PCR assays. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200909117/DC1>.

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