Myogenin and Class II HDACs Control Neurogenic Muscle Atrophy by Inducing E3 Ubiquitin Ligases

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SUMMARY

Maintenance of skeletal muscle structure and function requires innervation by motor neurons, such that denervation causes muscle atrophy. We show that myogenin, an essential regulator of muscle development, controls neurogenic atrophy. Myogenin is upregulated in skeletal muscle following denervation and regulates expression of the E3 ubiquitin ligases MuRF1 and atrogin-1, which promote muscle proteolysis and atrophy. Deletion of myogenin from adult mice diminishes expression of MuRF1 and atrogin-1 in denervated muscle and confers resistance to atrophy. Mice lacking histone deacetylases (HDACs) 4 and 5 in skeletal muscle fail to upregulate myogenin and also preserve muscle mass following denervation. Conversely, forced expression of myogenin in skeletal muscle of HDAC mutant mice restores muscle atrophy following denervation. Thus, myogenin plays a dual role as both a regulator of muscle development and an inducer of neurogenic atrophy. These findings reveal a specific pathway for muscle wasting and potential therapeutic targets for this disorder.

INTRODUCTION

Maintenance of muscle mass depends on a balance between protein synthesis and degradation. Innervation of skeletal muscle fibers by motor neurons is essential for maintenance of muscle size, structure, and function. Numerous disorders, including amyotrophic lateral sclerosis (ALS), Guillain-Barré syndrome, polio, and polyneuropathy, disrupt the nerve supply to muscle, causing debilitating loss of muscle mass (referred to as neurogenic atrophy) and eventual paralysis.

Loss of the nerve supply to muscle fibers results in muscle atrophy mainly through excessive ubiquitin-mediated proteolysis via the proteasome pathway (Beehler et al., 2006). Other pathologic states and systemic disorders, including cancer, diabetes, fasting, sepsis, and disuse, also cause muscle atrophy through ubiquitin-dependent proteolysis (Attaix et al., 2008; Attaix et al., 2005; Medina et al., 1995; Tawa et al., 1997). The muscle-specific E3 ubiquitin ligases MuRF1 (also called Trim63) and atrogin-1 (also called MAFbx or Fbxo32) are upregulated during muscle atrophy and appear to represent final common mediators of this process (Bodine et al., 2001; Clarke et al., 2007; Gomes et al., 2001; Kedar et al., 2004; Lecker et al., 2004; Li et al., 2004; Li et al., 2007; Willis et al., 2009). However, the precise molecular mechanisms and signaling pathways that control the expression of these key regulators of muscle protein turnover have not been fully defined and it remains unclear whether all types of atrophic signals control these E3 ubiquitin ligase genes through the same or different mechanisms. Further understanding of the molecular pathways that regulate muscle mass is a prerequisite for the development of novel therapeutics to ameliorate muscle-wasting disorders.

Myogenin is a bHLH transcription factor essential for skeletal muscle development (Hasty et al., 1993; Nabeshima et al., 1993). After birth, myogenin expression is downregulated in skeletal muscle but is reinduced in response to denervation (Merlie et al., 1994; Tang et al., 2008; Williams et al., 2009). Upregulation of myogenin in denervated skeletal muscle promotes the expression of acetylcholine receptors and other components of the neuromuscular synapse (Merlie et al., 1994; Tang and Goldman, 2006; Williams et al., 2009). However, it has not been possible to address the potential involvement of myogenin in neurogenic atrophy because myogenin null mice die at birth due to failure in skeletal muscle differentiation (Hasty et al., 1993; Nabeshima et al., 1993). Histone acetylation has been implicated in denervation-dependent changes in skeletal muscle gene expression, and histone deacetylase (HDAC) inhibitors block the expression of
myogenin in response to denervation (Tang and Goldman, 2006). In this regard, the class Ila HDACs, HDAC4 and HDAC5, which act as transcriptional repressors (Haberland et al., 2009; McKinsey et al., 2000; Potthoff et al., 2007), are upregulated in skeletal muscle upon denervation and repress the expression of Dach2, a negative regulator of myogenin (Cohen et al., 2007; Tang et al., 2008).

To investigate the potential involvement of myogenin, HDAC4, and HDAC5 in neurogenic atrophy, we performed denervation experiments in mutant mice in which these transcriptional regulators were deleted in adult skeletal muscle. We show that adult mice lacking myogenin fail to upregulate the E3 ubiquitin ligases MuRF1 and atrogin-1 following denervation and are resistant to muscle atrophy. We demonstrate that myogenin binds and activates the promoter regions of the MuRF1 and atrogin-1 genes, in vitro and in vivo. Similar to adult mice lacking myogenin, mice lacking Hdac4 and Hdac5 in skeletal muscle do not upregulate myogenin following denervation and are resistant to muscle atrophy. Conversely, overexpression of myogenin in skeletal muscle is sufficient to upregulate the expression of MuRF1 and atrogin-1 and promote neurogenic atrophy in mice lacking Hdac4 and Hdac5. These findings reveal a key role of myogenin and class Ila HDACs as mediators of neurogenic atrophy and potential therapeutic targets to treat this disorder.

RESULTS

Adult Mice Lacking Myogenin Are Resistant to Muscle Atrophy upon Denervation

To bypass the requirement of myogenin for skeletal muscle development and investigate its functions in muscle of adult mice, we used a conditional myogenin null allele (Knapp et al., 2006), which could be deleted in adult muscle with a tamoxifen-regulated Cre recombinase transgene (Hayashi and McMahon, 2002; Knapp et al., 2006). Tamoxifen was administered to mice 2 months of age, and 89% deletion of the conditional myogenin allele occurred as measured by PCR genotyping from genomic DNA 1 week after tamoxifen injection (see Figure S1 available online). Hereafter, we refer to these mice with deletion of myogenin during adulthood as Myog−/− mice.

To examine the role of myogenin in denervated skeletal muscle, the sciatic nerve was severed one month following tamoxifen administration, and muscle atrophy was assessed 14 days later by weighing denervated and contralateral tibialis anterior (TA) muscles. Wild-type (WT) denervated TA showed approximately a 40% decrease in weight following denervation in comparison to the contralateral TA (Figure 1A). In contrast, denervated TA from Myog−/− mice showed a minimal decrease in muscle weight (~20%) compared to the contralateral TA (Figure 1A), suggesting that Myog−/− mice were partially resistant to muscle atrophy. Because we deleted myogenin in adult mice, muscle development and growth occurred normally prior to tamoxifen administration. As expected, the muscle weights of the nondenervated contralateral TA in Myog−/− and WT mice were similar (WT TA = 37.82 ± 0.87 mg; Myog−/− TA = 36.27 ± 0.54 mg; t test = 0.19). Comparable resistance to atrophy was observed in the gastrocnemius and plantaris (GP) weight of Myog−/− mice (Figure 1A).

Immunostaining for laminin of TA cross-sections clearly delineated a decrease of muscle fiber size in the WT denervated TA in comparison to the contralateral muscle, indicative of muscle atrophy (Figure 1B). In contrast, the decrease in fiber size was less evident in the Myog−/− denervated TA (Figure 1B). Morphometric analysis of TA cross-sections highlighted a significant difference in myofiber size between WT and Myog−/− muscles following denervation, confirming the latter were resistant to muscle atrophy (Figure 1C).

As expected, seven days after denervation, MuRF1 and atrogin-1 expression was dramatically upregulated in the GP of denervated WT mice (Figure 1D). Remarkably, this upregulation was significantly reduced in Myog−/− denervated GP (Figure 1D), suggesting that the lack of upregulation of MuRF1 and atrogin-1 in denervated Myog−/− muscles was responsible for resistance to atrophy. Deletion of myogenin mRNA from adult Myog−/− muscle was confirmed by real-time PCR (Figure 1D). Of note, expression of MyoD (Myod1), another bHLH myogenin regulatory factor (Davis et al., 1987), was highly upregulated in both the contralateral and denervated GP of the Myog−/− mice, seven days after denervation (Figure 1D). These data show that myogenin does not regulate Myod1 expression following denervation. The dramatic upregulation of Myod1 following denervation of Myog−/− mice, which are resistant to atrophy, also argues against a major role of Myod1 in promoting neurogenic atrophy. Accordingly, Myod1 null mice are not resistant to muscle atrophy following denervation (Jason O’Rourke and E. Olson, unpublished data).

Denervation is known to affect skeletal myofiber composition (Herbison et al., 1979; Midrio et al., 1992; Nwoye et al., 1982; Patterson et al., 2006; Sandri et al., 2006; Sato et al., 2009). To determine whether the resistance to muscle atrophy observed in mice lacking myogenin was due to differences in fiber type composition, we performed fiber type analysis of soleus muscles 2 weeks after denervation. Our findings revealed no difference in fiber type composition between WT and Myog−/− mice (Figure S2). These findings suggest that myogenin, which is upregulated following denervation, is required for maximal induction of E3 ubiquitin ligase genes and neurogenic atrophy.

We next tested whether myogenin was necessary for mediating other forms of atrophy, such as occurs in response to fasting. As shown in Figure 1E, the GP muscles of WT and Myog−/− mice displayed comparable loss in mass following a 48 hr fast. We observed the upregulation of MuRF1 and atrogin-1 upon fasting in both WT and Myog−/− mice and validated the deletion of myogenin in Myog−/− mice (Figure 1F). These data clearly demonstrate that myogenin is not required for starvation atrophy, but rather is a specific mediator of neurogenic atrophy.

Myogenin Activates MuRF1 and Atrogin-1 Transcription

Because upregulation of MuRF1 and atrogin-1 was impaired in Myog−/− mice, we analyzed the promoter regions of the MuRF1 and atrogin-1 genes for E boxes (CANNTG) that might confer sensitivity to myogenin. Indeed, three E boxes are located...
in the promoter of the MuRF1 gene, E1 (−143 bp), E2 (−66 bp), and E3 (−44 bp), and one conserved E box is located 79 bp upstream of the atrogin-1 gene (Figure S3A). The E boxes upstream of MuRF1 are contained in a genomic region near the binding site for FoxO transcription factors (Waddell et al., 2008), but several kilobases away from a region shown to be regulated by NFκB (Cai et al., 2004). The E box upstream of atrogin-1 is embedded in a region containing multiple FoxO-binding sites (Sandri et al., 2004).

To confirm the binding of myogenin to the MuRF1 and atrogin-1 promoters, we performed chromatin immunoprecipitation (ChIP) assays using differentiated C2C12 myotubes, as Myogenin expression correlates with MuRF1 and atrogin-1 expression during muscle cell differentiation (Figure S3B) (Spencer et al., 2000). After six days of differentiation, chromatin from C2C12 myotubes was immunoprecipitated with antibodies against myogenin or immunoglobulin G (IgG) as a control. Using primers flanking the E boxes in the MuRF1 and atrogin-1 promoters, DNA was amplified by PCR (Figure 2A and Figure S3C). Clear enrichment of the corresponding promoter sequences in the DNA immunoprecipitated with antibodies against myogenin compared to IgG was indicative of myogenin binding to the endogenous MuRF1 and atrogin-1 promoters.

We validated in vivo binding of myogenin to the endogenous MuRF1 and atrogin-1 promoters by performing ChIP assays using sonicated chromatin extracts from TA muscles harvested from mice at 3 days and 7 days after denervation (Figure 2B and Figure S3D). Direct binding of myogenin as a heterodimer with E12 proteins to the E boxes E2 and E3 in the MuRF1 promoter and to the E box in the atrogin-1 promoter was shown by gel mobility shift assays (Figure S3E).
We further tested the ability of myogenin to activate the MuRF1 and atrogin-1 promoter regions in vitro by constructing luciferase reporter plasmids containing the 600 bp genomic DNA fragment upstream of the MuRF1 gene (MuRF1-Luc) or 712 bp upstream of the atrogin-1 gene (atrogin-1-Luc) upstream of a luciferase reporter. Mutant versions of these promoter regions were generated by mutating the myogenin-binding sites in the promoters. By transfecting C2C12 cells, activation of luciferase was detected in response to myogenin using the wild-type promoters (Figure 2C). This activation was blunted by mutation of the E boxes in the promoters (Figure 2C), indicating that the MuRF1 and atrogin-1 promoter regions contain responsive myogenin-binding sites. Similar results were obtained in transfected COS1 cells (Figure S3F).

To test the responsiveness of the E3 ligase gene promoters to atrophic signals in vivo, transgenic mice were generated harboring the same upstream regions of the genes ligated to a lacZ reporter (Kothary et al., 1989; Williams et al., 2009). Transgenic mice with the mutated versions of these promoter regions were also generated (MuRF1-Emut-lacZ and atrogin-1-Emut-lacZ). Seven days following denervation, β-galactosidase expression controlled by the wild-type promoters was upregulated in denervated GP muscle fibers compared to the innervated contralateral leg muscles (Figure 2D). The expression of lacZ in only a subset of myofibers likely reflects the mosaicism of F0 transgenic mice and, perhaps, variable upregulation of the E3 ubiquitin ligase genes in different myofibers in response to denervation (Moriscot et al., 2010). In contrast to the obvious upregulation of the wild-type transgenes following denervation, mutation of the E boxes in these promoters abrogated β-galactosidase expression, revealing an essential role for myogenin in denervation-dependent activation of MuRF1 and atrogin-1 in vivo (Figure 2D). These results show that the MuRF1 and atrogin-1 genes are targets of myogenin transcriptional activation in response to denervation.
Mice Null for Class II HDACs Are Resistant to Muscle Atrophy upon Denervation

Previous studies showed that the class II HDACs, HDAC4 and HDAC5, are upregulated in skeletal muscle in response to denervation (Bodine et al., 2001; Cohen et al., 2007; Tang et al., 2008) and are responsible for the repression of Dach2, a negative regulator of Myogenin (Cohen et al., 2007; Tang et al., 2008). In light of the role of myogenin in promoting muscle atrophy, we hypothesized that mice lacking HDAC4 or HDAC5 in skeletal muscle would be resistant to atrophy following denervation owing to a block of Myogenin expression via Dach2. Mice with global deletion of Hdac4 display lethal bone abnormalities (Vega et al., 2004), so we deleted Hdac4 specifically in skeletal muscle using a conditional allele and a myogenin-Cre transgene (Cohen et al., 2007; Tang et al., 2008). Previous studies showed that the class II HDACs, HDAC4 and HDAC5, are upregulated in skeletal muscle in response to denervation (Bodine et al., 2001; Cohen et al., 2007; Tang et al., 2008), so we generated double knockout (dKO) mice by crossing Hdac4 sk KO or Hdac5 KO mice (hereafter referred to as Hdac4 sk KO or Hdac5 KO) (Potthoff et al., 2007). The absence of HDAC4 protein upon Hdac4 gene deletion was confirmed by western blot analysis (Figure S4). Since mice null for Hdac5 do not display a phenotype (Chang et al., 2004), we used Hdac5−/− mice (hereafter referred to as Hdac5 KO) for these experiments. Fourteen days following denervation, WT denervated TA showed approximately a 50% decrease in weight in comparison to the contralateral TA (Figure 3A). In contrast, denervated TA muscles from Hdac4 sk KO or Hdac5 KO mice showed a decrease of about 30% in muscle weight in comparison to the contralateral muscles (Figure 3A), suggesting that these mice were partially resistant to muscle atrophy. The weight of the contralateral TA was similar among the mice (data not shown).

HDAC4 and HDAC5 display functional redundancy in different tissues and in a variety of developmental and pathological settings (Backs et al., 2008; Haberland et al., 2009; Potthoff et al., 2007), so we generated double knockout (dKO) mice by crossing Hdac4 sk KO with Hdac5 KO mice to further investigate the role of HDAC4 and HDAC5 in skeletal muscle atrophy. The dKO mice were viable and fertile and showed no obvious phenotype under normal conditions (data not shown). Strikingly, fourteen days after denervation, the TA of denervated dKO mice showed a decrease in weight of only ~10% compared to the contralateral TA (Figure 3A), revealing that the dKO mice were more resistant to muscle atrophy compared to Hdac4 sk KO or Hdac5 KO mice. The weight of the contralateral TA was comparable among the mice (data not shown). Similar differences were also observed among GP muscles between WT and dKO mice (Figure S5).

Immunostaining for laminin 14 days after denervation clearly demonstrated that the denervated TA fibers from Hdac4 sk KO and Hdac5 KO mice were larger than the denervated WT fibers and that the denervated TA from dKO mice had a minimal decrease in muscle fiber size compared to the contralateral dKO TA (Figure 3B). Morphometric analysis on TA sections revealed that, although WT mice showed a reduction of ~70% in the myofiber cross-sectional area between denervated and contralateral TA, Hdac4 sk KO denervated TA displayed ~30% reduction in myofiber cross-sectional area. Hdac5 KO denervated TA also showed a substantial reduction in myofiber area (~50%) when compared to the contralateral TA, whereas in dKO mice this reduction was only ~25% (Figure 3C). From these results, we conclude that HDAC4 and HDAC5 redundantly regulate skeletal muscle atrophy and mice lacking these HDACs in skeletal muscle are resistant to muscle atrophy upon denervation.

Aberrant Transcriptional Responses to Denervation in HDAC Mutant Mice

We compared the transcriptional responses to denervation in WT and dKO mice by real-time PCR analysis of denervation-responsive transcripts. As reported previously (Cohen et al., 2007; Tang et al., 2008), Dach2 expression was dramatically downregulated upon denervation in WT mice. However, Dach2 was only modestly downregulated in the dKO mice (Figure 4). Consistent with the repressive influence of Dach2 on Myogenin expression, in WT mice, Myogenin and Myod1 were strongly upregulated three days after denervation, as were MuRF1 and atrogin-1 (Figure 4). In contrast, neither Myogenin nor Myod1 transcripts were upregulated following denervation of dKO mice.
mice (Figure 4). The upregulation of *MuRF1* and *atrogin-1* was also completely abolished in dKO denervated GP (Figure 4), suggesting that the lack of upregulation of *MuRF1* and *atrogin-1* in denervated dKO muscles was in part responsible for resistance to atrophy.

**Myogenin Overexpression in dKO Muscle Restores Neurogenic Atrophy**

To examine whether forced expression of myogenin was sufficient to overcome the resistance of the dKO TA muscle to denervation-induced atrophy, we electroporated the TA of dKO mice with either a myogenin expression plasmid or an empty expression plasmid. Gene delivery efficiency was monitored by coelectroporation with a GFP vector (Dona et al., 2003; Rana et al., 2004). Three days after electroporation, which is sufficient time for the electroporated plasmids to be expressed in skeletal muscle (Dona et al., 2003), we denervated one leg of the dKO mice by cutting the sciatic nerve; the TA muscles were harvested 10 days after denervation. As seen in Figure 5A, laminin immunostaining of dKO TA muscles clearly revealed a decrease in

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**Figure 4. dKO Mice Show Altered Gene Expression upon Denervation**

Expression of the indicated mRNAs was detected by real-time PCR in WT and dKO denervated GP and normalized to the expression in the contralateral muscle. Data are represented as mean ± SEM. *p < 0.005 versus dKO. n = 6 for each time point.

**Figure 5. Ectopic Expression of Myogenin Induces Muscle Atrophy in dKO Mice Following Denervation**

(A) Immunostaining for laminin (red) of cross-section of contralateral and denervated dKO TA electroporated with GFP expression plasmid and control plasmid (HDAC4/5 dKO Control) or GFP plasmid and myogenin (HDAC4/5 dKO + Myogenin), 10 days after denervation. Histology shows that the dKO denervated GFP-positive fibers coelectroporated with myogenin are smaller than denervated GFP-positive fibers coelectroporated with control plasmid. Scale bar = 20 microns.

(B) Morphometric analysis performed on GFP-positive fibers of contralateral (−) and denervated (+) dKO TA muscles electroporated with GFP expression plasmid and control plasmid (Control) or GFP plasmid and myogenin (Myogenin), 10 days after denervation. Values indicate the mean of cross-sectional area of GFP-positive muscle fibers as a percentage of the contralateral control fibers ± SEM. *p < 0.05 versus control. n = 7 for each condition.

(C) Expression of Myogenin, *MuRF1*, and *atrogin-1* in contralateral (−) and denervated (+) dKO TA muscles electroporated with GFP expression plasmid and a control plasmid (Control) or GFP plasmid and myogenin (Myogenin), 10 days after denervation. Values are normalized to the expression in the contralateral control muscles. Data are represented as mean ± SEM. *p < 0.05 versus control. n = 3 for each sample. See also Figure S6.
myofiber size in the denervated TA of dKO mice overexpressing myogenin compared to the denervated dKO TA electroporated with the control vector. Morphometric analysis performed on GFP-positive myofibers showed a significant decrease in the size of myofibers of the denervated dKO TA electroporated with myogenin versus control vector (Figure 5B). Real-time PCR analysis validated the overexpression of myogenin in electroporated TA muscle of dKO mice and showed an upregulation of the expression of MuRF1 and atrogin-1 (Figure 5C), confirming the myogenin-dependent regulation of the E3 ubiquitin ligases.

The potential role of myogenin in driving muscle atrophy was further investigated by overexpressing myogenin in the TA muscle of WT mice. Morphometric analysis performed on GFP-positive myofibers showed no significant size difference between myofibers electroporated with control or myogenin expression plasmid (Figures S6A and S6B). Real-time PCR analysis validated the overexpression of myogenin in electroporated TA muscle of WT mice and showed an upregulation of the expression of MuRF1 and atrogin-1 (Figure S6C). Taken together, these findings demonstrate that overexpression of myogenin is necessary but not sufficient to induce muscle atrophy.

**DISCUSSION**

The results of this study demonstrate a key role of myogenin, well known for its function as an essential regulator of myogenesis, in controlling neurogenic atrophy. Myogenin promotes muscle atrophy upon denervation by directly activating the expression of MuRF1 and atrogin-1, which encode E3 ubiquitin ligases responsible for muscle proteolysis. Upregulation of Myogenin in response to denervation is controlled by a retrograde signaling pathway that promotes reinnervation of denervated myofibers (Figure 6). Thus, skeletal muscle responds to denervation by activating an elaborate network of transcriptional and epigenetic pathways, involving positive and negative feedback loops that modulate nerve-muscle interactions and muscle growth and function (Figure 6).

**Dual Roles of Myogenin in Muscle Development and Atrophy**

Our findings reveal the gene regulatory circuitry for muscle development is reemployed in adulthood to control aspects of muscle disease and stress responsiveness. Thus, myogenin can exert opposing effects on skeletal muscle—either promoting differentiation or degradation—depending on the developmental or pathological setting. These contrasting activities of myogenin likely reflect differential modulation by signaling pathways and cofactors that enable myogenin to regulate distinct sets of target genes.

Similar to myogenin, Dach2 is a transcription factor involved in both muscle development and muscle atrophy. Dach2 is expressed in the developing somites prior to the onset of myogenesis and has been shown to regulate myogenic specification by interacting with the Eya2 and Six1 transcription factors (Heanue et al., 1999; Kardon et al., 2002). Indeed, Dach proteins are required for activation of Six1 targets (Li et al., 2003), suggesting a possible role of Dach proteins in the Six1-mediated regulation of muscle development (Laclef et al., 2003) or fiber type specification (Grifone et al., 2004). Following denervation, Dach2 plays a role in connecting neuronal activity with myogenin expression (Cohen et al., 2007; Tang and Goldman, 2006; Tang et al., 2008).

The finding that forced expression of myogenin in HDAC4/5 mutant mice is sufficient to restore muscle atrophy following denervation indicates that myogenin is a key downstream mediator of the proatrophic functions of these HDACs. It is...
regulating proteolysis, endocytosis, signal transduction (Hicke, et al., 2009), their potential roles in myogenesis have not been explored. Considering the important role of ubiquitination in cancer cachexia, and sarcopenia, cause muscle atrophy and upregulated upon denervation and spinal cord isolation (Hyatt et al., 2003), but is not induced in response to other forms of atrophy, such as fasting, cancer cachexia, or diabetes (Lecker et al., 2004; Sacheck et al., 2007). In this regard, we have found that Myog−/− mice display a normal loss of skeletal muscle mass in response to fasting, further demonstrating that myogenin is dedicated to neurogenic atrophy and sensing the state of motor innervation. The fact that MuRF1 and atrogin-1 are upregulated in other atrophy conditions in the absence of myogenin upregulation (Lecker et al., 2004; Sacheck et al., 2007) strongly suggests that other transcription factors known to regulate the expression of these ubiquitin ligases, such as the FoxO family or NFκB (Bodine et al., 2001; Sandri et al., 2004; Waddell et al., 2008), play a role in driving muscle atrophy in a myogenin-independent manner.

Our finding that myogenin, in addition to HDAC4 and HDAC5, acts as a regulator of neurogenic muscle atrophy through the activation of E3 ubiquitin ligases provides a new perspective on potential therapies for muscle wasting disorders. Class II HDACs are regulated by a variety of calcium-dependent signaling pathways that control their nuclear export through signal-dependent phosphorylation (Backs et al., 2008; McKinsey et al., 2000). In a pathological condition such as muscle denervation, HDAC4 and HDAC5 are upregulated, shuttle into the myonuclei adjacent to neuromuscular junctions (Cohen et al., 2007), and are critical regulators of muscle atrophy. Modulation of the activity of class II HDACs, through pharmacologic inhibition compatible with the maintenance of steady-state transcription of genes regulated by class II HDACs, may represent a new strategy for ameliorating muscle atrophy following denervation.

**EXPERIMENTAL PROCEDURES**

**Mouse Lines**
Mice used in this study are described in the Extended Experimental Procedures.

**Denervation**
In anaesthetized adult mice, the sciatic nerve of the left leg was cut and a 3 mm piece was excised. The right leg remained innervated and was used as control. Mice were sacrificed after 3, 7, 10, or 14 days.

**DNA Delivery by Electroporation**
For gene delivery by electroporation, adult dKO mice were anesthetized; TA muscles exposed, injected with 30 μg of DNA in a solution of 5% mannitol, and immediately subjected to electroporation. Electroporation was performed by delivering 10 electric pulses of 20 V each (five with one polarity followed by five with inverted polarity). A pair of 3 × 5 mm GenePaddle electrodes (BTX, San Diego, CA) placed on opposite sides of the muscle was used to deliver the electric pulses. pCMV-Snap25-GFP (provided by Tulio Pozzan, University of Padua, Padua, Italy) was used in a 1:1 ratio with pcDNA3.1 (Invitrogen) or EMSV-myogenin plasmid (Rana et al., 2004).

**Immunohistochemistry**
Cryosections of TA or soleus were fixed in 4% paraformaldehyde in PBS for 10 min at 4°C and washed in PBS. After incubating 30 min with 0.1% Triton X-100 in PBS, the samples were fixed for 1 hr in 15% goat serum in PBS supplemented with M.O.M. Mouse IgG blocking reagent (Vector Laboratories) (BB) at room temperature. Primary antibodies were incubated overnight at 4°C (1:100 dilution of rabbit polyclonal anti-laminin antibody; 1:16000 anti-type I myosin heavy chain (MHC) (Sigma). Primary antibodies were detected by Alexa Fluor-488 or -555 goat anti-rabbit antibody (Invitrogen) diluted 1:800 in BB. DAB staining (Vector Laboratories) was used on soleus muscle for detecting type I MHC. Soleus muscles were used for metachromatic ATPase staining as described elsewhere (Ogilvie and Feeback, 1990).
Staining of transgenic lines positive for β-galactosidase was performed on GP muscles, as previously described (Williams et al., 2009).

**Morphometric Analysis**
Myofiber area was assessed on TA cryosections using ImageJ software (http://rsb.info.nih.gov/ij/) (NIH). Three H&E-stained cross-sections from three different mice for each genotype were analyzed. Between 100 and 350 GFP-positive fibers were analyzed for each electroporated TA muscle. The values are calculated as the percentage of the average of the cross-sectional area of each TA over the average cross-sectional area of the contralateral TA fibers.

**RNA Isolation and RT-PCR**
Total RNA was isolated from GP muscles using Trizol reagent (Invitrogen) following the manufacturer’s instructions. Three micrograms of RNA was converted to cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). Gene expression was assessed using real-time PCR with the ABI PRISM 7000 sequence detection system and TaqMan or with SYBR green Master Mix reagents (Applied Biosystems). Real-time PCR values were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A list of Taqman probes and Sybr Green primers are available in the Extended Experimental Procedures.

**Plasmid Constructs**
A list of the plasmids used in this study is available in the Extended Experimental Procedures.

**Cell Culture**
COS cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). C2C12 myoblasts were grown in DMEM supplemented with 20% FBS and antibiotics and differentiated in DMEM supplemented with 2% horse serum and antibiotics.

**Chromatin Immunoprecipitation Assay**
ChIP assays were performed using C2C12 myotubes at day six of differentiation or using TA muscles three and seven days after denervation with the ChIP assay kit (Upstate) following the manufacturer’s instructions. Chromatin was immunoprecipitated with antibodies against immunoglobulin G (Sigma) or myc or using TA muscles three and seven days after denervation with the ChIP assay kit (Upstate) following the manufacturer’s instructions. The sequences of the ChIP primers are available in the Extended Experimental Procedures.

**Luciferase assay**
C2C12 transfections were performed using Lipofectamine 2000 (Invitrogen) as previously described (Mercer et al., 2005). COS cells were plated and transfected 12 hr later using FuGENE (Roche Applied Science) following the manufacturer’s instructions. The MuRF1 and atrogin-1 reporter plasmid cloning strategy is described in the Extended Experimental Procedures. Luciferase assays were performed with the Luciferase Assay kit (Promega) according to the manufacturer’s instructions.

**Site-Directed Mutagenesis**
Mutations were introduced into E boxes E2 and E3 of the MuRF1 promoter region and in the E box of the atrogin-1 promoter by using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The same E box mutations as those used in electrophoretic mobility shift assays were introduced within each E box site in the promoters.

**Statistical Analysis**
Data are presented as mean ± standard error of the mean (SEM). Statistical significance was determined using two-tailed t test with a significance level minor of 0.05.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2010.09.004.

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EXTENDED EXPERIMENTAL PROCEDURES

Mice lines
Hdac5^{+/−} mice were described previously (Chang et al., 2004). Hdac4 conditional mutant mice were generated by flanking exon 6 with loxP sites, which results in a frame shift mutation in the Hdac4 allele (Potthoff et al., 2007). Transgenic mice harboring genomic DNA fragments ligated to the hsp68 basal promoter upstream of a LacZ reporter gene were generated as previously described (Kothary et al., 1989; Williams et al., 2009). Among various lines of transgenic mice, three out of five for MuRF1-WT-lacZ and three out of seven for atrogin-1-WT-lacZ expressed the lacZ transgene in denervated myofibers. Among various lines of transgenic mice harboring mutant constructs, three out of nine for MuRF1-Emut-lacZ and two out of six for atrogin-1-Emut-lacZ expressed the lacZ transgene, as seen in denervated myofibers.

Mice harboring the Myog^{flex} allele mated to CAGGCre-ERTM transgenic mice, which ubiquitously express a conditional Cre-recombinase that is activated by intraperitoneal injection of tamoxifen (10 mg/40 g body weight), have been previously described (Knapp et al., 2006). Tamoxifen injections were performed in two month-old mice. The deleted Myog allele was detected by quantitative PCR genotyping from genomic DNA 1 week after tamoxifen injection, using primer sequences described previously (Knapp et al., 2006). Denervation experiments were performed one month after tamoxifen injection and myogenin deletion was confirmed by quantitative real time PCR. Littermates injected with tamoxifen, but not expressing the Cre-recombinase, were used as controls. All mouse studies were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Plasmid Constructs
The following plasmids were used: pCMV-Snap25-GFP (provided by Tullio Pozzan, University of Padua, Padua, Italy); EMSV-myogenin; pcDNA-E12; pcDNA 3.1 and CMV-lacZ.

Immunoblot
Immunoblots were performed from GP muscles as previously described (Vega et al., 2004). Antibodies against HDAC4 (1:500 in TBST; Sigma) and α-tubulin (1:3,000 in 5% milk TBST; Sigma) were used. ECL Advance Western Blotting Detection Kit (Amersham Biosciences) was used for signal detection.

Electrophoretic Mobility Shift Assays
Oligonucleotides were synthesized (Integrated DNA Technology) corresponding to the E box sites and mutated binding sites for generating probes used in the gel mobility shift assays. Unlabeled competitor was 200X of labeled probe. Myogenin and E12 were transcribed and translated together using a coupled in vitro transcription/translation system (Promega) and DNA binding assays were performed as described (Lu et al., 1999). Oligonucleotides were synthesized as follows (plus strand sequences are shown with the binding sites in bold and the mutations underlined):

- **MuRF1** E2: GGAATGCTCGAGCTGCTCCCTC, TCTTGC
- **MuRF1** E3: CTGGGGCTCATGTGACAGAGGT, TCGTGCC
- **atrogin-1** E box: CCCGAGGCACGTGGCTTTGTT, TCTTGC

Taqman Primers

- **Hdac4**: Mm01299542_g1
- **Hdac5**: Mm00515917_m1
- **Myog**: Mm00446194_m1
- **Myod1**: Mm00440387_m1
- **MuRF1**: Mm01185221_m1
- **Atrogin-1**: Mm01207878_m1
- **GAPDH**: Mm99999915_g1

Sybr Green Primers

- **Dach2** for: 5’-ACTGAAAGTGCTTTTGATAA-3’;
- **Dach2** rev: 5’-TTTAGAGCTTTGGCATGTA-3’

ChIP Primers
Oligonucleotide primers for amplification of E boxes on the MuRF1 promoter are:
Oligonucleotide primers for amplification of the E box on the *atrogin-1* promoter are:

```
For: 5′-CGGCAGGCAACAGCGATTT-3′
Rev: 5′-GTCTTGCTGAGGCCCTC-3′.
```

Oligonucleotide primers for amplification of GAPDH are:

```
For: 5′-ATC CAC GAC GGA CAC ATT GG-3′
Rev: 5′-TGGTGCTGCAAA GGCTGT GG-3′
```

**Luciferase assay**

The *MuRF1* reporter plasmid was constructed by ligating 600 bp of murine genomic region upstream of the 5′ UTR of the *MuRF1* gene into the pGL3-Basic reporter (Promega). The *MuRF1* genomic fragment was generated by PCR using the following primers:

```
For: 5′-GGTACCGCAGCAAGCCCCTTTACCAC-3′
Rev: 5′-CTCGAGGCTGCACCTCTGTCACATGA-3′
```

The *atrogin-1* reporter plasmid was constructed by linking 712 bp of murine genomic region upstream of the 5′ UTR of the *atrogin-1* gene into the pGL3-Basic reporter. The *atrogin-1* genomic fragment was generated by PCR using the following primers:

```
For: 5′-GGTACCAGAGCGCGACCGACCAGCGACCAGA-3′
Rev: 5′-CTCGAGTGACCCCTACCCGTCCACCA-3′
```
Figure S1. Deletion of Myog Allele in Myog\textsuperscript{−/−} Mice, Related to Figure 1
Quantitative PCR genotyping was performed using genomic DNA from Myog\textsuperscript{−/−} and WT mice 1 week after tamoxifen injection. n = 8 for each sample. Data are represented as mean ± SEM.
Figure S2. Myog−/− Mice Do Not Show Changes in Fiber Type Composition, Related to Figure 1
(A) Fiber type composition was determined by metachromatic ATPase staining of contralateral and denervated soleus from WT and Myog−/− mice, 14 days after denervation. Type I fibers stain dark blue. Type II fibers stain light blue. Scale bar = 20 microns.
(B) Type I myosin heavy chain (MHC) immunostaining of contralateral and denervated soleus muscle of WT and Myog−/− mice, 14 days after denervation. Scale bar = 20 microns.
Figure S3. Myogenin Binds and Activates MuRF1 and atrogin-1 Transcription In Vitro and In Vivo, Related to Figure 2

(A) Sequence alignment of a fragment of the promoter region of the mouse MuRF1 and atrogin-1 genes from different species shows the conserved upstream region containing E boxes. Position (0) denotes the transcriptional start site.

(B) Real-time PCR expression of Myogenin, MuRF1, and atrogin-1 in C2C12 cells, 2 and 6 days after switching from growth to differentiation medium. Values are

(C) Western blot analysis of MuRF1 and atrogin-1 expression in C2C12 cells 3 and 7 days after differentiation.

(D) ChIP analysis of Myogenin binding to the MuRF1 and atrogin-1 promoters.

(E) EMSA analysis of Myogenin binding to E boxes in the MuRF1 and atrogin-1 promoters.

(F) Luciferase assays with Myogenin binding to MuRF1-Luc and atrogin-1-Luc reporter constructs.
normalized to transcript expression in growth medium. n = 3 for each time point. Data are represented as mean ± SEM.

(C) Representative ChIP assay performed using C2C12 myotubes shows myogenin binding on the MuRF1 and atrogin-1 genes. Chromatin was immunoprecipitated with antibodies against immunoglobulin G (IgG), or myogenin (Myog). A negative control was performed with no antibody (NoAb). Primers flanking the E boxes on MuRF1 and atrogin-1 promoters were used for amplifying DNA by PCR. GAPDH primers were used as negative control; Input shows similar amount of chromatin in each sample.

(D) Representative ChIP assay performed using denervated TA muscle at 3 and 7 days following denervation shows myogenin binding on the MuRF1 and atrogin-1 genes. GAPDH primers were used as negative control; Input shows similar amount of chromatin in each sample.

(E) Electrophoretic mobility shift assay shows direct binding of Myogenin/E12 heterodimers to the E box consensus sequences in the MuRF1 (E2 and E3) and atrogin-1 (E box) promoters. Competitor consists of the E box consensus sequence (w) or a mutated E box (m).

(F) Luciferase assays using extracts of COS cells transfected with luciferase reporter plasmids ligated to WT (MuRF1-Luc) (atrogin-1-Luc), or the mutant (E-mut) constructs of MuRF1 and atrogin-1 genes and increasing amounts of a myogenin expression plasmid. Empty vector is used as control. Data are represented as mean ± SEM.
Figure S4. Absence of HDAC4 Expression in Skeletal Muscle of dKO Mice, Related to Figure 3
Representative immunoblot for HDAC4 in contralateral (Con) and denervated (Den) GP of WT and dKO mice, 7 days after denervation. α-Tubulin antibody is used as loading control.
Figure S5. dKO Mice Are Resistant to Muscle Atrophy upon Denervation, Related to Figure 3
Percentage of GP muscle weight of WT and dKO mice 14 days after denervation, expressed relative to contralateral muscle. **p < 0.005 versus WT. n = 6 for each sample. Data are represented as mean ± SEM.
Figure S6. Ectopic Expression of Myogenin Does Not Induce Muscle Atrophy in WT Mice, Related to Figure 5

(A) Immunostaining for laminin (red) of cross-section of TA WT muscles electroporated with GFP expression plasmid and control plasmid (WT Control) or GFP plasmid and myogenin (WT + Myogenin). Scale bar = 20 microns.

(B) Morphometric analysis shows that GFP-positive myofibers have no significant (NS) size difference between WT myofibers electroporated with control or myogenin expression plasmid. n = 7 for each sample. Data are represented as mean ± SEM.

(C) Expression of Myogenin, MuRF1 and atrogin-1 in WT TA muscles electroporated with GFP plasmid and a control plasmid (Control) or GFP plasmid and myogenin (Myogenin). Values are normalized to the expression of the control muscles. n = 3 for each sample. Data are represented as mean ± SEM.