Histone deacetylase degradation and MEF2 activation promote the formation of slow-twitch myofibers

Matthew J. Potthoff, Hai Wu, Michael A. Arnold, John M. Shelton, Johannes Backs, John McAnally, James A. Richardson, Rhonda Bassel-Duby, and Eric N. Olson

Department of Molecular Biology, Department of Internal Medicine, and Department of Pathology, University of Texas Southwestern Medical Center, Dallas, Texas, USA.

Skeletal muscle is composed of heterogeneous myofibers with distinctive rates of contraction, metabolic properties, and susceptibility to fatigue. We show that class II histone deacetylase (HDAC) proteins, which function as transcriptional repressors of the myocyte enhancer factor 2 (MEF2) transcription factor, fail to accumulate in the soleus, a slow muscle, compared with fast muscles (e.g., white vastus lateralis). Accordingly, pharmacological blockade of proteasome function specifically increases expression of class II HDAC proteins in the soleus in vivo. Using gain- and loss-of-function approaches in mice, we discovered that class II HDACs suppress the formation of slow twitch, oxidative myofibers through the repression of MEF2 activity. Conversely, expression of a hyperactive form of MEF2 in skeletal muscle of transgenic mice promotes the formation of slow fibers and enhances running endurance, enabling mice to run almost twice the distance of WT littermates. Thus, the selective degradation of class II HDACs in slow skeletal muscle provides a mechanism for enhancing physical performance and resistance to fatigue by augmenting the transcriptional activity of MEF2. These findings provide what we believe are new insights into the molecular basis of skeletal muscle function and have important implications for possible therapeutic interventions into muscular diseases.

Introduction
Skeletal muscle fibers of adult vertebrates differ markedly with respect to their contractile and metabolic properties, which reflect different patterns of gene expression (1). Slow-twitch or type I myofibers exhibit an oxidative metabolism, are rich in mitochondria, heavily vascularized, and resistant to fatigue. In contrast, fast-twitch or type II fibers exhibit glycolytic metabolism, are involved in rapid bursts of contraction, and fatigue rapidly. Myofibers can be further classified as either type I, IIA, IIX/d, or IIB, depending on the type of myosin heavy chain (MHC) isoform expressed (2). The heterogeneity of skeletal myofibers is reflected at the molecular level in that almost every protein involved in contraction (MHC, myosin light chain, troponin I, troponin T, troponin C, actin, etc.) has at least 2 isoforms expressed discretely in slow (type I) and fast (type II) fibers (3). In adult animals, specialized myofiber phenotypes remain plastic and vary in response to contractile load, hormonal milieu, and systemic diseases (4). Functional overload or exercise training results in transformation of preexisting fast fibers to a slow-twitch, oxidative phenotype (5). Conversely, decreased neuromuscular activity induced by spinal cord injury, limb immobilization, space flight, or blockade of action potential conduction causes a slow-to-fast myofiber conversion (6).

Functional demands modulate skeletal muscle phenotypes by activating signaling pathways that modify the gene expression profile of the myofiber. The signaling pathways involved in myofiber remodeling are of particular interest because of their relevance to several human disorders, including muscle dystrophy, metabolic disorders, and muscle atrophy (7). Increasing the abundance of slow, oxidative fibers in the mdx mouse model of Duchenne muscular dystrophy, for example, reduces the severity of the dystrophic phenotype (8, 9). Skeletal muscles also play an important role in whole-body metabolism, such that increasing the number of type I fibers enhances insulin-mediated glucose uptake and protects against glucose intolerance (10), which could have important therapeutic implications for diabetes and other metabolic diseases.

The myocyte enhancer factor 2 (MEF2) transcription factor, a key regulator of muscle development, is preferentially activated in slow, oxidative myofibers (11) and responds to calcium-dependent signaling pathways that promote the transformation of fast, glycolytic fibers into slow, oxidative fibers (12). The transcriptional activity of MEF2 is repressed by class II histone deacetylases (HDACs) (13–15). However, the potential involvement of MEF2 and class II HDACs in regulating myofiber identity in vivo has not been explored.

In this study, we show class II HDACs are selectively degraded by the proteasome in slow, oxidative myofibers, enabling MEF2 to activate the slow myofiber gene program. Consistent with these conclusions, forced expression of class II HDACs in skeletal muscle or genetic deletion of Mef2c or Mef2d blocks activity-dependent fast-to-slow fiber transformation whereas expression of a hyperactive MEF2 protein promotes the slow-fiber phenotype, enhancing endurance and enabling mice to run almost twice the distance of WT littermates. These findings provide new insights into the
molecular basis of skeletal muscle performance and have important implications for possible therapeutic manipulation of muscle function for amelioration of muscular diseases.

Results

Reduction of class II HDAC proteins in soleus muscle. We speculated that variations in MEF2 activity patterns seen among different types of muscles, as revealed by both RT-PCR and Northern blot analyses (Figure 1; supplemental material available online with this article; doi:10.1172/JCI31960DS1). The relatively low level of class II HDAC protein expression in soleus appeared to reflect a posttranscriptional mechanism since mRNA transcripts encoding class II HDACs were more abundant in soleus than in VW muscles, as revealed by both RT-PCR and Northern blot analyses (Figure 1B and Supplemental Figure 2).

Downregulation of class II HDACs in transgenic skeletal muscles transformed toward a slow, oxidative phenotype. Forced expression of constitutively active calcineurin or calcium/calmodulin-dependent protein kinase IV (CaMKIV) in adult fast, glycolytic fibers of transgenic mice results in an increase in the number of slow fibers (17, 18). We used these transgenic mouse models to determine whether fast- to slow-fiber transformation correlated with a downregulation of class II HDACs, as might be expected if class II HDACs are involved in the fiber-type switch. The levels of class I HDACs (HDAC1, -2, and -3) were similar in VW muscles from WT and transgenic mice whereas the transformation of VW muscles toward a slow myofiber identity was associated with diminished expression of class II HDAC (HDAC4, -5, and -7) proteins (Figure 1C), consistent with the possibility that class II HDACs repress the expression of slow-fiber genes in fast myofibers.

Class II HDACs redundantly regulate slow, oxidative fiber expression. To directly examine the potential role of class II HDACs in regulating fiber-type identity, we analyzed adult skeletal muscles from mutant mice lacking 1 or more class II HDACs. Hdac5−/− and Hdac9−/− mice are viable (15, 19), so we were able to analyze the fiber-type composition of these homozygous mutants with global Hdac gene deletion in all tissues. However, because Hdac4−/− mice die at birth from skeletal defects (20) and Hdac7−/− mice die during embryogenesis from vascular defects (21), we used floxed alleles to delete these genes specifically in skeletal muscle using a skeletal muscle–specific Cre recombinase transgene (Myo-Cre) (SkM-KO mice), thereby avoiding lethality (22). Mice lacking individual class II HDACs did not display abnormalities in fiber-type switching or skeletal muscle development (Figure 2A). In contrast, soleus muscles from Hdac5−/−Hdac9−/− and Hdac4−/−Myo-creHdac5−/− double-knockout mice showed an increase in the percentage of slow myofibers from 48% ± 2.6% to 70% ± 3% (P < 0.003) and 63% ± 6.6%...
The fiber-type composition of the soleus of 
Hdac5+/-Hdac9–/– mice and 
Hdac4+/-Hdac5–/–mice was identical to that of WT mice (data not shown) whereas 
Hdac4+/-Hdac5–/–Hdac9+/-mutant mice showed an increase in slow 
fibers comparable to that of double-mutant mice (Figure 2A), sug-
gesting that deletion of any combination of 4 alleles of 
Hdac4, -5, or -9 results in enhanced slow-fiber gene expression.

Analysis of the expression of transcripts encoding the individual 
MHC isoforms revealed an increase in expression of oxidative genes 
(MHC type I [MHC-I] and-IIa) in soleus and PLA muscles of 
Hdac5–/–Hdac9–/–mutant mice compared with 
Hdac5+/–Hdac9+/–or 
Hdac5+/–Hdac9+/– littermates. These results suggested that a reduction 
in expression of class II HDAC proteins below a specific threshold 
results in an increase in slow and oxidative fibers.

Forced expression of HDAC5 blocks fiber-type switching. Exercise train-
ing transforms preexisting fast fibers to an oxidative phenotype (5). 
To determine whether class II HDACs modulated fiber-type switch-
ing in response to exercise, we constructed an inducible skeletal 
muscle–specific transgenic system using the myogenin promoter/
MEF2 enhancer (Myo) to drive expression of the tetracycline trans-
activator (tTA) (Myo-tTA), which acts in trans to activate a tetracycline-responsive transgene. In this system, the transgene is not 
expressed in the presence of doxycycline (DOX) but is induced when 
the drug is removed. To verify the spatial expression of tTA in the 
Myo-tTA transgenic mice, we generated transgenic mice harboring 
a lacZ reporter gene cloned behind the tetracycline-responsive (tet-
responsive) expression cassette (tet-lacZ). When the Myo-tTA trans-
genome mice were crossed to the tet-lacZ reporter mice in the absence 
of DOX, lacZ expression was observed specifically in skeletal muscle 
without preference for adult slow or fast fibers (data not shown).

The Myo-tTA transgenic mice were bred to responder mice bearing 
a tet-responsive transgene encoding a signal-resistant FLAG-tagged 
human HDAC5 mutant protein (HDAC5S/5) (23). Expression of the 
FLAG-tagged HDAC5 protein was detected by Western blot analysis
with an anti-FLAG antibody using total protein extracts from gastrocnemius and PLA (GP) muscles of 4-week-old tet-HDAC5/Myo-tTA transgenic mice that had DOX removed 10 days earlier (Figure 2D).

Overexpression of HDAC5 in transgenic mice was confirmed by probing with an HDAC5 antibody (Figure 2D). FLAG-HDAC5 was not detected in the presence of DOX (Figure 2D).

To analyze the influence of HDAC5 on fiber-type switching, DOX was removed from 6-week-old tet-HDAC5/Myo-tTA double-transgenic mice, and these mice were provided free access to a running wheel for 4 weeks, a time period shown previously to allow the transformation of fast, glycolytic fibers to oxidative fibers (12). Tet-HDAC5/Myo-tTA transgenic mice and control mice ran voluntarily at comparable intensities (Supplemental Figure 3, A and B).

Metachromatic ATPase staining of skeletal muscles showed a pronounced increase in type I and IIa fibers within GP muscles of exercised tet-HDAC5 mice (without tTA) compared with unexercised mice (Figure 2E). In contrast, GP muscles from exercised tet-HDAC5/Myo-tTA double-transgenic mice without DOX did not show an increase in type I and type IIa fibers compared with sedentary mice (Figure 2E). Quantification of slow fibers revealed an approximate 10-fold reduction in the number of slow fibers from exercised HDAC5 transgenic mice compared with exercised WT mice (data not shown). Sedentary tet-HDAC5/Myo-tTA double-transgenic mice with and without DOX displayed normal fiber-type distributions (data not shown). We conclude that continuous repression of class II HDAC target genes in adult skeletal muscle is sufficient to inhibit exercise-induced fiber-type switching.

Requirement of MEF2 for establishing slow, oxidative myofiber identity.

To examine whether class II HDAC regulation of fiber-type switching occurs through repression of MEF2 activity, we analyzed the skeletal muscles from individual MEF2 knockout mice. Conditional alleles of Mef2c and Mef2d (24, 25) were deleted specifically in skeletal muscle using transgenic mice that express Cre recombinase under the control of the myogenin promoter/MEF2 enhancer (Myo-Cre) (22), which is active in both fast and slow fibers (data not shown). As shown in Figure 3A, skeletal muscle–specific deletion (SkM-KO) of Mef2c or Mef2d using Myo-Cre resulted in a reduction in slow fibers within the soleus whereas the abundance of slow fibers was unaltered in Mef2a–/– mice (Figure 3A) or Mef2c+/– and Mef2d+/– mice (data not shown).

To further validate the reduction of slow fibers following skeletal muscle–specific deletion of Mef2c, we performed immunohistochemistry for type I fibers using a MHC-I specific antibody. As shown in Figure 3B, skeletal muscle lacking Mef2c displayed a loss of type I fibers in the GP and a reduction in number (and intensity) of type I fibers in the soleus. Moreover, using glycerol gradient silver staining of MHC isoforms from the Mef2c SkM-KO soleus muscle, we discovered that these muscles display a reduction in MHC-I (Figure 3C). Specifically, a decrease in the percentage of slow myofibers from 48% ± 2.6% to 25% ± 3.6% (P < 0.002) and 33% ± 11.4% (P < 0.001) was observed in the soleus of the Mef2c SkM-KO and Mef2d SkM-KO mice, respectively (Figure 3D). These findings demonstrate that MEF2C and MEF2D activate slow-fiber genes and that repression of fiber-type switch-
Activated MEF2 is sufficient to increase slow-fiber expression. (A) Western blot analysis of Myo-MEF2C-VP16 transgene expression using an anti-VP16 antibody. Expression of the slow-fiber–specific troponin I and oxidative markers myoglobin and cytochrome c in protein extract of GP muscles of Myo-MEF2C-VP16 transgenic mice. (B) Metachromatic ATPase staining of gastrocnemius and PLA muscles of WT and Myo-MEF2C-VP16 transgenic mice. Original magnification, ×4. Scale bar: 300 μm. Dashed red lines delineate gastrocnemius muscle from PLA. (C and D) Exercise endurance and muscle performance showing total time running (min; C) and total distance run (m; D) of Myo-MEF2-VP16 transgenic muscles were analyzed by forced treadmill exercise. Eight-week-old Myo-MEF2C-VP16 transgenic and WT male mice with similar body weights were subjected to forced treadmill exercise (n = 5 for each group) on a 10% incline.

increase in running time and a 94% increase in distance, respectively, compared with WT mice. Thus, activation of MEF2 is sufficient to enhance skeletal muscle oxidative capacity and mitochondrial content, thereby diminishing muscle fatigability and augmenting endurance.

Class II HDACs are ubiquitinated and degraded by the proteasome.

To begin to understand the mechanistic basis for the lack of accumulation of class II HDAC proteins in slow skeletal muscle fibers, we examined the half-life of HDAC5 in vitro using a stable C2C12 muscle cell line that constitutively expressed FLAG-tagged HDAC5. Inhibition of protein synthesis with cycloheximide for 4 hours resulted in a precipitous decrease in the level of HDAC5 protein in skeletal myocytes (Figure 5A). In contrast, α-tubulin was stable over this time period. The proteasome inhibitor MG132 blocked the degradation of HDAC5 (Figure 5B), suggesting that HDAC5 is degraded by the proteasome pathway.

Since ubiquitination is a prerequisite for degradation by the proteasome (26), we examined whether HDAC5 was ubiquitinated. Indeed, ubiquitinated HDAC5 was readily detectable when HA-tagged ubiquitin was expressed in the HDAC5-expressing cell line in the presence of MG132 (Figure 5C). The signal-resistant HDAC5/S3 mutant (13), lacking the regulatory phosphorylation sites (serines 259 and 498), was ubiquitinated to the same extent as the WT HDAC5 protein (Figure 5C), indicating that phosphorylation of HDAC5 on these residues is not a prerequisite for ubiquitination. FLAG-HDAC4, -7, and MITR (a splice variant of HDAC9) were also ubiquitinated in C2C12 cells (data not shown).

To determine whether ubiquitination of HDAC5 occurs in the nucleus or cytoplasm, we compared the ubiquitination of WT HDAC5, which is expressed primarily in the nucleus (Figure 5D), and an HDAC5 mutant with a mutation in the nuclear localization signal, HDAC5(K270R) (Figure 5D). As shown in Figure 5E, the K270R mutant was not ubiquitinated, whereas fusion of an SV40–nuclear localization signal (SV40-ALS) to the HDAC5(K270R) protein restored nuclear localization and ubiquitination. Together these results demonstrate that HDAC5 ubiquitination occurs in the nucleus.

Blockade to HDAC degradation in vivo by MG132. To determine whether class II HDACs are degraded via the proteasome pathway in slow, oxidative myofibers in vivo, 8-week-old, WT C57BL/6 male mice were injected with either DMSO or MG132 and expression of HDAC4 and -5 was examined in the soleus, GP, tibialis anterior, and EDL skeletal muscles after 6 hours, a time period shown previously to provide proteasome inhibition in vivo (27). Treatment with MG132 in vivo increased the level of HDAC4 and HDAC5, confirming specificity for HDAC5 degradation by the proteasome.

To begin to understand the mechanistic basis for the lack of accumulation of class II HDAC proteins in slow skeletal muscle fibers, we examined the half-life of HDAC5 in vitro using a stable C2C12 muscle cell line that constitutively expressed FLAG-tagged HDAC5. Inhibition of protein synthesis with cycloheximide for 4 hours resulted in a precipitous decrease in the level of HDAC5 protein in skeletal myocytes (Figure 5A). In contrast, α-tubulin was stable over this time period. The proteasome inhibitor MG132 blocked the degradation of HDAC5 (Figure 5B), suggesting that HDAC5 is degraded by the proteasome pathway.

Since ubiquitination is a prerequisite for degradation by the proteasome (26), we examined whether HDAC5 was ubiquitinated. Indeed, ubiquitinated HDAC5 was readily detectable when HA-tagged ubiquitin was expressed in the HDAC5-expressing cell line in the presence of MG132 (Figure 5C). The signal-resistant HDAC5/S3 mutant (13), lacking the regulatory phosphorylation sites (serines 259 and 498), was ubiquitinated to the same extent as the WT HDAC5 protein (Figure 5C), indicating that phosphorylation of HDAC5 on these residues is not a prerequisite for ubiquitination. FLAG-HDAC4, -7, and MITR (a splice variant of HDAC9) were also ubiquitinated in C2C12 cells (data not shown).

To determine whether ubiquitination of HDAC5 occurs in the nucleus or cytoplasm, we compared the ubiquitination of WT HDAC5, which is expressed primarily in the nucleus (Figure 5D), and an HDAC5 mutant with a mutation in the nuclear localization signal, HDAC5(K270R) (Figure 5D). As shown in Figure 5E, the K270R mutant was not ubiquitinated, whereas fusion of an SV40–nuclear localization signal (SV40-ALS) to the HDAC5(K270R) protein restored nuclear localization and ubiquitination. Together these results demonstrate that HDAC5 ubiquitination occurs in the nucleus.

Blockade to HDAC degradation in vivo by MG132. To determine whether class II HDACs are degraded via the proteasome pathway in slow, oxidative myofibers in vivo, 8-week-old, WT C57BL/6 male mice were injected with either DMSO or MG132 and expression of HDAC4 and -5 was examined in the soleus, GP, tibialis anterior, and EDL skeletal muscles after 6 hours, a time period shown previously to provide proteasome inhibition in vivo (27). Treatment with MG132 in vivo increased the level of HDAC4 and HDAC5, confirming specificity for HDAC5 degradation by the proteasome pathway.

To begin to understand the mechanistic basis for the lack of accumulation of class II HDAC proteins in slow skeletal muscle fibers, we examined the half-life of HDAC5 in vitro using a stable C2C12 muscle cell line that constitutively expressed FLAG-tagged HDAC5. Inhibition of protein synthesis with cycloheximide for 4 hours resulted in a precipitous decrease in the level of HDAC5 protein in skeletal myocytes (Figure 5A). In contrast, α-tubulin was stable over this time period. The proteasome inhibitor MG132 blocked the degradation of HDAC5 (Figure 5B), suggesting that HDAC5 is degraded by the proteasome pathway.

Since ubiquitination is a prerequisite for degradation by the proteasome (26), we examined whether HDAC5 was ubiquitinated. Indeed, ubiquitinated HDAC5 was readily detectable when HA-tagged ubiquitin was expressed in the HDAC5-expressing cell line in the presence of MG132 (Figure 5C). The signal-resistant HDAC5/S3 mutant (13), lacking the regulatory phosphorylation sites (serines 259 and 498), was ubiquitinated to the same extent as the WT HDAC5 protein (Figure 5C), indicating that phosphorylation of HDAC5 on these residues is not a prerequisite for ubiquitination. FLAG-HDAC4, -7, and MITR (a splice variant of HDAC9) were also ubiquitinated in C2C12 cells (data not shown).

To determine whether ubiquitination of HDAC5 occurs in the nucleus or cytoplasm, we compared the ubiquitination of WT HDAC5, which is expressed primarily in the nucleus (Figure 5D), and an HDAC5 mutant with a mutation in the nuclear localization signal, HDAC5(K270R) (Figure 5D). As shown in Figure 5E, the K270R mutant was not ubiquitinated, whereas fusion of an SV40–nuclear localization signal (SV40-ALS) to the HDAC5(K270R) protein restored nuclear localization and ubiquitination. Together these results demonstrate that HDAC5 ubiquitination occurs in the nucleus.

Blockade to HDAC degradation in vivo by MG132. To determine whether class II HDACs are degraded via the proteasome pathway in slow, oxidative myofibers in vivo, 8-week-old, WT C57BL/6 male mice were injected with either DMSO or MG132 and expression of HDAC4 and -5 was examined in the soleus, GP, tibialis anterior, and EDL skeletal muscles after 6 hours, a time period shown previously to provide proteasome inhibition in vivo (27). Treatment with MG132 in vivo increased the level of HDAC4 and HDAC5, confirming specificity for HDAC5 degradation by the proteasome pathway.
Figure 5
Ubiquitination and degradation of class II HDACs in vitro and in vivo. (A) C2C12 cells stably expressing FLAG-tagged HDAC5 (C2C12-HDAC5) were treated with cycloheximide (CHX, 25 μM) for 0, 1, 2, or 4 hours before cells were lysed and FLAG-HDAC5 expression was measured by Western blot analysis using anti-FLAG M2 antibody. Tubulin immunoblot showed equivalent loading of each lane. (B) C2C12-HDAC5 cells were treated with cycloheximide and MG132, singly or in combination for 4 hours, and FLAG-HDAC5 expression was analyzed. (C) C2C12-HDAC5 and C2C12 cells stably expressing CaMK-resistant HDAC5 (S259/498A) were transfected with or without HA-tagged ubiquitin (HA-Ub) and treated with or without MG-132 (25 μM) for 4 hours; the ubiquitination status of WT and mutant HDAC5 was analyzed. FLAG expression in inputs shows equal loading. (D) Subcellular localization of Myc-HDAC5 (WT), Myc-HDAC5(K270R), or Myc-SV40 NLS-HDAC5(K270R) in C2C12 cells. (E) The ubiquitination status of cytoplasmic HDAC5 [Myc-HDAC5(K270R)] or nuclear HDAC5 [Myc-SV40 NLS-HDAC5(K270R)] was analyzed. (F and G) WT C57BL/6 males (8 weeks old) were IP injected with DMSO or MG132 for 6 hours. Protein was isolated from SOL, GP, tibialis anterior (TA), and EDL muscles and analyzed for expression of (F) HDAC4 or (G) HDAC5. Tubulin shows equal loading. (H) Treatment with MG132 decreases MEF2 activation. Six hours after DesMEF-lacZ mice were injected with DMSO or MG132, mice were run for approximately 3 hours using forced treadmill exercise. Skeletal muscles were then isolated from DMSO- and MG132-treated DesMEF mice and analyzed for lacZ expression. LacZ expression was reduced in MG132-treated muscles (where class II HDAC expression was increased).
The contractile properties of skeletal myofibers reflect a combination of developmental and extrinsic inputs. During embryogenesis, fast- and slow-twitch fibers are patterned by specific developmental cues (3). After birth, the pattern of motor innervation plays a key role in influencing muscle fiber type. Phasic motor neuron firing at high frequency (100–150 Hz) promotes the formation of fast fibers, which display brief, high-amplitude calcium transients and low ambient calcium levels (<50 nM) whereas tonic motor neuron stimulation (10–20 Hz) favors the formation of slow fibers, which maintain higher intracellular calcium levels (100–300 nM) (29). Calcineurin and CaMK have been implicated in the transduction of calcium-dependent signals that upregulate the expression of oxidative, slow-fiber–specific genes in skeletal muscle (17, 18, 30). However, the precise mechanisms whereby these signaling pathways modulate the slow-fiber phenotype have not been defined (30).

Our results show that skeletal muscle of transgenic mice that have undergone a fast-to-slow myofiber transformation in response to activated calcineurin and CaMK displays a reduction in abundance of class II HDAC proteins, suggesting that these calcium-dependent signaling pathways act, at least in part, by enhancing degradation of class II HDAC proteins. The calcineurin and CaMK signaling pathways have also been shown to promote the phosphorylation of class II HDACs on a series of conserved serine residues, which mediate signal-dependent nuclear export and derepression of MEF2 target genes (13, 14). Thus, it seems likely that some combination of the 2 mechanisms — proteolysis and regulated nuclear export — regulates class II HDACs and thereby MEF2 activity and myofiber identity.

Nuclear factor of activated T cells (NFAT) transcription factors, which serve as transcriptional mediators of calcineurin signaling, have also been implicated in the control of slow-fiber gene expression (30, 31), as has the nuclear receptor PPARβ (32) and PGC-1α (33). Recently, PGC-1β was implicated in regulating type IIx/d fiber formation (34). MEF2 interacts with NFAT (35) and PGC-1α (36) and also regulates PGC-1α expression (23). Indeed, the MEF2-FP16 superactivator upregulated PGC-1α but not PGC-1β expression in skeletal muscle (Supplemental Figure 4). Thus, MEF2 serves as a nodal point for the control of multiple downstream transcriptional regulators of the slow-fiber phenotype and can potentially confer calcium sensitivity to other factors via its signal-dependent interaction with class II HDACs.

Our results show that ubiquitination of class II HDACs occurs in the nucleus. Nuclear ubiquitination of transcriptional activators has also been described as a mechanism to regulate the extent and duration of activation of transcriptional activators (37). The ubiquitination of class II HDACs provides another mechanism by which transcriptional activators may become activated. Class II HDACs and MEF2 are also sumoylated, which enhances the repressive activity of class II HDACs (38, 39). Whether sumoylation might be regulated during myofiber specification in vivo has not been addressed.

The mechanism that directs ubiquitination and degradation of class II HDACs in response to calcium signaling remains to be determined. It is tempting to speculate that phosphorylation of HDACs serves as a signal for ubiquitination by recruiting specific E3 ligases. Identification of the E3 ligase(s) for class II HDACs and the signals regulating their degradation are currently under investigation.

Our results show that adult skeletal muscle phenotypes are dictated by the extent of repression of MEF2 by class II HDACs and that the fast-fiber phenotype results from the absence of MEF2 activity. The fact that 4 Hdac alleles needed to be deleted...
to observe an increase in slow-fiber gene expression suggests that there is substantial functional redundancy among different HDACs with respect to repression of the slow-fiber gene program. Forced expression of a signal-resistant mutant of HDAC5 is sufficient to suppress the slow-fiber phenotype, whereas expression of a hyperactive MEF2-VP16 chimera is sufficient to override the repressive influence of endogenous class II HDACs and drive the slow-fiber phenotype. These findings suggest that the fast-fiber phenotype represents a “default” gene program resulting from the absence of MEF2 activity.

**Therapeutic potential.** The ability of activated MEF2 to enhance oxidative capacity and endurance of skeletal muscle suggests opportunities for therapeutically enhancing muscle performance by stimulating MEF2 activity. Increasing the number of slow fibers in skeletal muscle via MEF2 also represents a potential method for treating metabolic and muscular diseases (8, 9). One could imagine augmenting MEF2 activity by interfering with the repressive activity of class II HDACs by modulating the signaling pathways that control HDAC phosphorylation, subcellular localization, or activity of class II HDACs by modulating the signaling pathways that control HDAC phosphorylation, subcellular localization, or degradation. In this regard, HDAC inhibitors have recently been shown to suppress muscle pathology associated with muscular dystrophy (40, 41) in mice.

In addition to regulating skeletal muscle gene expression and function, MEF2 signaling has been shown to drive pathological cardiac growth and remodeling, which result from signal-dependent phosphorylation and nuclear export of class II HDACs in cardiac myocytes (19). These adverse consequences of MEF2 activation pose interesting challenges to the goal of enhancing MEF2 activity in skeletal muscle while avoiding possible cardiotoxicity of such strategies and, conversely, to pharmacologically preventing cardiac dysfunction without diminishing skeletal muscle function.

**Methods**

**Plasmid constructs, tissue culture, and cell transfection.** The expression vector encoding HA-ubiquitin was described previously (42). Myo-NLS-HDAC5 was a kind gift from Tim McKinsey. The K270R point mutation was generated by site-directed mutagenesis (Stratagene). Myo-tTA and Myo-MEF2C-VP16 transgenic constructs were generated by cloning the cTA cassette from the pUDH 15-1 vector (43), fusing a VP16 activation domain in frame to the C terminus of full-length MEF2C, respectively, and cloning them from the pUDH 15-1 vector (43), fusing a VP16 activation domain in frame to the C terminus of full-length MEF2C, respectively, and cloning them into the HindIII site of a pBSISK(+) vector containing a hGHPolyA tail. The myogenin promoter/MEF2C enhancer (22) was then cloned upstream of the cassettes into KpnI/XhoI sites.

C2C12 myoblasts were grown in DMEM supplemented with fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). For transient transfection assays, cells were plated and transfected 12 hours later using FuGENE (Roche Applied Science) following the manufacturer’s instructions. Cells were harvested 24–48 hours after transfection. C2C12 stable lines expressing FLAG-tagged HDAC5 or FLAG-tagged HDAC5/- were established by G418 selection of C2C12 clones transfected with plasmids encoding WT HDAC5 or a CAK-resistant HDAC5 mutant, which in Ser259 and Ser498 were mutated into alamines (13). Cells were treated with cycloheximide (Sigma-Aldrich) and/or MG132 (Calbiochem; EMD Biosciences) at indicated concentrations.

**Generation of transgenic mice and knockout mice.** Transgenic mice that express constitutively active forms of calcineurin or CaMKIV under the control of a muscle-specific enhancer from the muscle creatine kinase (MCK) gene are described elsewhere (17, 18). Tet-HDAC5/- transgenic mice are described elsewhere (23). Myo-tTA, tet-lacZ, and Myo-MEF2C-VP16 transgenic mice were generated by injecting linearized constructs into the pronuclei of fertilized oocytes as previously described (44).

Hdac5/−/− (15), Hdac9/−/− (19), and Hdac7 conditional knockout mice had been generated previously (21). Hdac4 conditional mice were generated by flanking exon 6 with loxP sites, which results in an out-of-frame mutation in the Hdac4 allele. Mef2c/−/− and Mef2c and Mef2d conditional knockout mice have been described previously (24, 45). Correct gene targeting was confirmed by Southern blot analysis, genomic sequencing, and RT-PCR.

**Immunoprecipitation and immunoblotting.** Immunoprecipitations were performed as previously described (20). Antibodies against HA (1:1,000; Sigma-Aldrich), FLAG M2 (1:4,000; Sigma-Aldrich), Myc (1:1,000; Santa Cruz Biotechnology Inc.), α-tubulin (1:5,000; Sigma-Aldrich), HDAC1, -2, and -3 (1:1,000 for all; Sigma-Aldrich), HDAC4 (1:500; Santa Cruz Biotechnology Inc.), HDAC5 (1:1,000; Millipore), HDAC7 (1:1,000; Cell Signaling Technology), MEF2A (1:1,000; Millipore), MEF2C (1:1,000; Santa Cruz Biotechnology Inc.), MEF2D (1:2,500; BD Biosciences), troponin I slow (1:2,500; Santa Cruz Biotechnology Inc.), cytochrome c (1:2,500; BD Biosciences—Pharmingen), and myoglobin (1:3,000; Dako) were used for immunoblot analyses. For analyzing endogenous class II HDAC proteins, the ECL Advance Western Blotting Detection Kit (Amersham Biosciences) was used.

**In vivo pharmacological studies.** Myo-tTA and tet-HDAC5/−/− transgenic mice were bred while receiving DOX (200 μg/ml) in water as previously described (23). Myo-tTA/tet-HDAC5/−/− transgenic mice were maintained on DOX as needed. Voluntary wheel-running experiments were performed and measured as previously described (12). Animals were allowed to acclimatize to running cages for 4 days prior to running recordings. DOX was removed from the mice for the days of acclimation so that the animals could begin expressing the transgene. After 4 days, wheel-running activity was measured continuously for 4 weeks.

Forced treadmill exercise experiments for analyzing Myo-MEF2C-VP16 transgenic mice were performed as follows. Prior to exercise, mice were accustomed to the treadmill (Columbus Instruments) with a 5-minute run at 7 m/min once per day for 2 days. The exercise test was performed on a 10% incline for 10 m/min for the first 60 minutes, followed by 1 m/min increment increases for 3- to 15-minute intervals, then 45 minutes at 13 m/min, followed by 1 m/min increment increases at 15-minute intervals until exhaustion. Forced exercise ended when mice were unable to avoid repeated electric shocks. MEF2 reporter mice (DesMEF lacZ) were run for approximately 3 hours at 9 m/min.

In vivo proteasome inhibition experiments were performed by intraperitoneally delivering DMSO or MG132 as previously described for 6 hours (27), except 30 μM/kg body weight MG132 was used for injections. All experiments involving animals were reviewed and approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center.

**RNA isolation and analysis.** Total RNA was prepared from mouse tissues using TRIzol (Invitrogen) following the manufacturer’s instructions. Of total RNA, 1.5 μg was converted to cDNA using oligo dT primer and SuperScript II Reverse Transcriptase (Invitrogen). For PCR reactions, 2% of the cDNA pool was amplified. PCR cycles were optimized for each set of primers. Sequences for HDAC PCR primers have been described previously (46). Quantitative real-time PCR was performed for indicated MHC isoforms using SYBR Green (Applied Biosystems). Northern blots were performed with 20 μg of total RNA in each lane and probed in ULTRAhyb (Ambion) with labeled HDAC4, -5, or β-actin cDNA.

**Fiber-type and immunohistological analysis.** Soleus and GP muscles were harvested from mice and flash frozen in embedding medium or fixed in 4% paraformaldehyde as previously described (47). Fiber-type analysis using metachromatic ATPase staining (48) and glycerol gradient silver...
staining were performed as previously described (47). Paraffin sections were stained with a MHC-I antibody, followed by treatment with DAB (Vector Laboratories).

**Statistics.** Data are presented as mean ± SEM. Differences between groups were tested for statistical significance using the unpaired 2-tailed Student’s t test. Values of P < 0.05 were considered significant.

**Acknowledgments.** We thank Andrew Williams, Yuri Kim, Dillon Phan, Bryan Young, and Cheryl Nolen for technical assistance and Alisha Tizzen for assistance with graphics. This work was supported by grants from the NIH, the D.W. Reynolds Clinical Cardiovascular Research Center, the Texas Advanced Technology Program, the Muscular Dystrophy Association (to E.N. Olson), and the Robert A. Welch Foundation.

Received for publication February 27, 2007, and accepted in revised form May 29, 2007.

**Address correspondence to:** Eric N. Olson, Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9148, USA. Phone: (214) 648-1187; Fax: (214) 648-1196; E-mail: ericolson@utsouthwestern.edu.

---

**References:**


