A Histone Deacetylase 4/Myogenin Positive Feedback Loop Coordinates Denervation-dependent Gene Induction and Suppression

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Muscle activity contributes to formation of the neuromuscular junction and affects muscle metabolism and contractile properties through regulated gene expression. However, the mechanisms coordinating these diverse activity-regulated processes remain poorly characterized. Recently, it was reported that histone deacetylase 4 (HDAC4) can mediate denervation-induced myogenin and nicotinic acetylcholine receptor gene expression. Here, we report that HDAC4 is not only necessary for denervation-dependent induction of genes involved in synaptogenesis (nicotinic acetylcholine receptor and muscle-specific receptor tyrosine kinase) but also for denervation-dependent suppression of genes involved in glycolysis (muscle-specific enolase and phosphofructokinase). In addition, HDAC4 differentially regulates genes involved in muscle fiber type specification by inducing myosin heavy chain IIA and suppressing myosin heavy chain IIB. Consistent with these regulated gene profiles, HDAC4 is enriched in fast oxidative fibers of innervated tibialis anterior muscle and HDAC4 knockdown enhances glycolysis in cultured myotubes. HDAC4 mediates gene induction indirectly by suppressing the expression of Dach2 and MITR that function as myogenin corepressors. In contrast, HDAC4 is directly recruited to myocyte enhancer factor 2 sites within target promoters to mediate gene suppression. Finally, we discovered an HDAC4/myogenin positive feedback loop that coordinates gene induction and repression underlying muscle phenotypic changes after muscle denervation.

INTRODUCTION

Skeletal muscle is composed of a spectrum of fiber types with different structural and functional properties. The composition of myosin heavy chain (MHC) isoforms specifies muscle contractile properties. In rodents, MHC IIA, IIB, or both are enriched in fast twitch fibers, whereas MHC I is enriched in slow twitch fibers. These fibers also express different enzymes that result in fiber type-specific metabolic properties. Type IIB fibers are rich in glycolytic enzymes, whereas type I and IIA fibers are rich in enzymes necessary for oxidative metabolism. The extent to which a fiber is glycolytic or oxidative is largely determined by the activity pattern of its innervating motor neuron (Gundersen et al., 1988). Nerve activity also plays a crucial role in maintaining muscle mass (Jackman and Kandarian, 2004) and in shaping the formation of the neuromuscular junction (NMJ) (Sunesen and Changeux, 2003; Kummer et al., 2006).

Although many of the genes contributing to muscle fiber phenotype are regulated by muscle activity, not all are regulated in the same direction. For example, in the fast tibialis anterior (TA) muscle some genes, such as those encoding the MyoD family of basic helix-loop-helix transcription factors, and synaptic components such as nicotinic acetylcholine receptors (nAChRs) and muscle-specific kinase (MuSK) are induced (Goldman et al., 1985, 1986; Eftimie et al., 1991; Dutton et al., 1993; Tang et al., 2006), whereas other genes, such as those encoding muscle-specific enolase (MSE), phosphofructokinase (PFK), and MHC IIB, are suppressed after muscle denervation (Huey and Bodine, 1998; Nozais et al., 1999; Tang et al., 2000; Raffaello et al., 2006). Interestingly, in contrast to denervation-dependent MHC IIB mRNA suppression in fast TA muscle (Huey and Bodine, 1998), muscle inactivity induces its expression in the slow soleus muscle (Pandolfi et al., 2006), suggesting muscle fiber-type–specific regulation.

A key molecule mediating denervation-dependent nAChR and MuSK gene regulation is myogenin (Mgn), a member of the basic helix-loop-helix family of transcription factors, whose expression is regulated by muscle depolarization (Tang et al., 2006). Muscle denervation induces Mgn gene expression, which may be mediated by HDAC4. Although HDAC4 is enriched in fast oxidative fibers of innervated tibialis anterior muscle, HDAC4 knockdown enhances glycolysis in cultured myotubes. This suggests that HDAC4 contributes to muscle fiber type specification by inducing MHC IIA and suppressing MHC IIB.
expression, and this induction requires histone deacetylase (HDAC) activity (Tang and Goldman, 2006). HDAC activity serves to relieve Mgn gene inhibition by suppressing expression of Dach2, a Mgn gene corepressor whose levels are high in innervated muscle and low in denervated muscle (Tang and Goldman, 2006). Dach2 repression after muscle denervation seems to be mediated, in part, via HDAC4 expression (Cohen et al., 2007). In addition, the HDAC9 splice variant MTR is also induced in innervated muscle and contributes to activity-dependent Mgn suppression by functioning as a corepressor in a complex with the myocyte enhancer factor (MEF) 2 transcription factor (Mejat et al., 2005). These two deacetylases (HDAC4 and -9) belong to a subgroup of class II HDACs that include HDAC5 and -7 (Khochbin et al., 2003; Yang and Seto, 2008). Although the mechanisms underlying activity-dependent gene regulation in muscle are emerging (Chin et al., 1998; Liu et al., 2005; Mejat et al., 2005; Tang and Goldman, 2006), relatively little is known about how the muscle is able to coordinate activity-dependent gene induction and gene suppression.

Here, we report that HDAC4 is induced in denervated TA muscle and is not only necessary for the denervation-dependent induction of Mgn and synaptic protein gene expression but also for the denervation-dependent suppression of genes involved in glycylcotic metabolism and fiber-type specification. Investigation of the mechanisms underlying HDAC4-dependent gene regulation showed that HDAC4 mediates gene repression by recruitment to MEF2 sites in the promoters of repressed genes, whereas HDAC4 indirectly contributes to gene induction by coordinately inhibiting the expression of the Mgn gene corepressors Dach2 and MTR. Consistent with its role in regulating the expression of genes involved in muscle metabolism, we found that HDAC4 is preferentially enriched in myonuclei of fast oxidative fibers in innervated muscle and its knockdown in myotubes enhances glycolysis. Finally, we found that HDAC4 and Mgn positively regulate each other’s expression to allow maintenance and coordination of gene activation and repression after muscle denervation.

MATERIALS AND METHODS

Plasmids, Cells, and Reporter Gene Assay

Plasmids pcSC2-green fluorescent protein (GFP) and pcSC2-Bgalactosidase (β-gal) were gifts from D. Turner (University of Michigan, Ann Arbor, MI) and pmGluMgnhsRNA (mogunin-targeting short hairpin RNA [shRNA] under control of the mouse U6 promoter) were described previously (Tang et al., 2004). pcDNA3-HDAC4-GFP was a gift from T. Kousourides (Curdon Institute, Cambridge, United Kingdom); pcDNA3.1-HDAC4-3SA was described previously (Wang et al., 2000). pECEPN1-HDAC4-GFP was a gift from F. Sanchez-Madrid (Universidad Autonoma de Madrid, Madrid, Spain). The human 1.6-kb HDAC4 promoter (NT 022173) was cloned into the HindIII/BamHI sites of pXP2 by using genomic DNA from human embryonic kidney (HEK) 293 cell line with primers 5'-CTGCACAGTCCAGTCTGTCATCCTCTCCAGATGACGTAAGTCTG-3' and 5'-ATCCGCCTGAATTGGTGGATCGCTATCCTGCGAGAGATGTGTGCGAGATGTG. The MEF2 mutation in the MSE promoter was generated by electroporation as described previously (Tang et al., 2005). As a control for electroporation efficiency, we observed using an Axiohot microscope (Carl Zeiss, Thornwood, NY) equipped with fluorescence optics and a 10 × 0.3 NA objective (Figure 5A), a 40 × 0.85 NA objective (Figures 5, B–D, and 6A), or a 100 × 1.3 NA oil immersion objective. A Sony digital camera was used to capture images with Digital Acquire software (Optronics, Goleta, CA). Neuromuscular junctions were sometimes observed using a Fluoview FV1000 confocal imaging system (Olympus, Tokyo, Japan) by using 60 × 1.2 NA water immersion objective (Figure 5D). Confocal images were acquired with FV10-ASW 1.7 software (Olympus). Standard procedures were used for Western blotting.

Muscle Electroporation and RNA Analysis

Stealth siRNA (Invitrogen) or plasmid DNA was delivered into TA muscle by electroporation as described previously (Tang et al., 2006). Briefly, TA or soleus muscle was injected with 2 μl of plasmid DNA containing 2–5 μg of pcSC2EGFP plasmid for identifying electroporated fibers and stealth siRNA (Invitrogen) (160 pM) targeting either HDAC4, MTR, Mgn, or a control small interfering RNA (siRNA). At least two different siRNAs targeting different sequences were used for each gene-specific knockdown to ensure results were not due to off-target effects of siRNAs. Control siRNAs have a similar GC content as the experimental siRNA but do not target any known gene. The molar ratio of stealth siRNA to enhanced green fluorescent protein (EGFP) plasmid DNA was 300-fold so that the amount of siRNA was much less than the amount of plasmid DNA. Muscle cell uptake was facilitated by placing electrodes, coated with ultrasound transmission gel, on either side of the leg and using a BTX square wave electroporator to deliver six pulses of 140 V/cm of 60-ms duration with an interpulse interval of 1 ms. Animals were injected to receive delivery of electroporation or isolation of electroporated/GFP+ fibers by using a stereo microscope equipped with fluorescence optics. A 12-d postelectroporation recovery ensures muscle fibers had recovered from any electroporation-induced damage.
**Table 1. PCR primers**

<table>
<thead>
<tr>
<th>Primer name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer sequence (5’ to 3’) forward</th>
<th>Primer sequence (5’ to 3’) reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChR</td>
<td>CGT-CTG-GTG-GCA-AAG-CT</td>
<td>CCG-CTC-TCC-ATG-AAG-CT</td>
</tr>
<tr>
<td>CPT-1</td>
<td>ACC-TGG-CAT-ACA-CCG-AGA-CT</td>
<td>CTT-TGG-CTA-CTG-GGT-AGT-CA</td>
</tr>
<tr>
<td>CS</td>
<td>TGC-AGA-ATT-TTC-CAA-ACA-ATC-T</td>
<td>ATT-GTG-TGA-CCA-CTC-TAG-CC</td>
</tr>
<tr>
<td>Cyto B</td>
<td>CTT-CTA-GGA-GTC-TGG-CTA-AT</td>
<td>GGC-ACC-TCA-GAA-TAT-TTG</td>
</tr>
<tr>
<td>Dach2</td>
<td>ACT-GAA-AGT-GCG-ATT-TGG-GTA</td>
<td>TIC-AGA-CCG-CTTGG-ATT-GTA</td>
</tr>
<tr>
<td>FBPI</td>
<td>CGT-ATA-TCC-ACC-GCA-CCT-TG</td>
<td>TCG-ATC-TGA-CCT-GAG-GA</td>
</tr>
<tr>
<td>GLUT4</td>
<td>GCA-GGC-GCC-AGT-GAC-CAA-GGA-A</td>
<td>CCA-GCC-AGT-CAT-TGT-AGG</td>
</tr>
<tr>
<td>GS1</td>
<td>TCT-CCA-GAA-CCC-CTC-TCC-T</td>
<td>CTC-AGG-GAT-CTG-GTA-TGT-GT</td>
</tr>
<tr>
<td>GS2</td>
<td>AGA-GTT-TGT-CCG-AGG-TTG-TCA</td>
<td>TAT-GTA-GGA-GGG-TCA-GCA-T</td>
</tr>
<tr>
<td>HDAC1</td>
<td>TCA-ATG-TTG-GTG-AGT-ACG-CT</td>
<td>CTG-GAA-GGT-GGG-TCA-ACA-T</td>
</tr>
<tr>
<td>HDAC2</td>
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<td>GTA-CTA-GGA-GGG-TCA-GCA-GC</td>
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<tr>
<td>HDAC3</td>
<td>ATG-TGT-TGA-ATA-GAG-GAT-GT</td>
<td>GCT-ATC-GAT-CTG-CCC-TCC-T</td>
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<tr>
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<td>CAG-GAG-ATG-GTG-GCC-AGT-AA</td>
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<tr>
<td>HDAC5</td>
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<td>CAT-CTC-GAT-GGG-GAT-TGC</td>
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<td>AGG-CTC-AGT-ACA-TG-CTG-CT</td>
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<tr>
<td>HDAC7</td>
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<td>AGC-TGG-GTG-AAG-TAA-TCC</td>
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<td>CCA-GCT-GCC-ACT-TTG-TGC</td>
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<td>HDAC9 (MIFR)</td>
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<td>TGG-AGA-GCT-TCC-CTG-GAG-GG</td>
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<tr>
<td>Madh2</td>
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<td>TGG-AGA-GGT-TGG-GCT-GCA-GC</td>
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<tr>
<td>MHC-1β</td>
<td>ACA-AGA-TGC-GCC-AGT-GCA-GAG</td>
<td>GCT-CTG-GAT-GGG-GTA-GGA-GG</td>
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<tr>
<td>MHCIIα</td>
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<td>TTT-GCA-GTA-GGG-TCA-GCA-GC</td>
</tr>
<tr>
<td>MHCIIβ</td>
<td>AAA-AGT-TGA-CAA-ATT-TAT-GAT-GA</td>
<td>AAT-CAT-GAA-CCT-CTG-CAT-GG</td>
</tr>
<tr>
<td>MIFR</td>
<td>ACT-CTC-GTC-CCA-ATG-TAA-AAA</td>
<td>TCC-AGG-TAC-AGT-TTG-GG-TAA</td>
</tr>
<tr>
<td>Mgn</td>
<td>CGT-ATG-TAA-GCA-AAG-TCC-TCC</td>
<td>ATT-GCT-CCA-GGG-CCC-GGA-GGA-GG</td>
</tr>
<tr>
<td>PFKm</td>
<td>TGC-CAC-TAA-GAT-GGG-TG-TG</td>
<td>GTC-GTG-CTC-AGC-TGC-GCA</td>
</tr>
<tr>
<td>PGC-1a</td>
<td>CGG-ACA-ACT-CAG-CAG-CAA-GTC-CCT</td>
<td>CTT-GCT-GGC-CGC-AAG-GGC-GCA</td>
</tr>
<tr>
<td>SDHαa</td>
<td>CGA-GAA-ATT-TGC-GAT-CAG-CAC-G</td>
<td>CTA-GAT-TCC-GTC-GAG-GG</td>
</tr>
<tr>
<td>SDHb</td>
<td>AGC-CTT-ATC-TGA-AGA-AGG</td>
<td>TAC-TGT-TCC-GTT-CAG-GAG</td>
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<tr>
<td>SDHc</td>
<td>GAA-GAA-CAC-GAC-TGG-AAA-CCG</td>
<td>AAA-GTT-CCC-AGG-AAG-CAG-GA</td>
</tr>
</tbody>
</table>

<sup>a</sup> CPT-1, carnitine palmitoyltransferase-1; CytoB, cytochrome b; CS, citrate synthase; FBP, fructose 2,6-bisphosphatase; GS, glycogen synthase; GLUT4, glucose transporter 4; PGC-1α, peroxisome proliferators-activated receptor γ coactivator 1α.

as determined by anatomy and gene expression (Bertrand et al., 2003; Golzio et al., 2005; Mejia et al., 2005; Sadasivam et al., 2005; Tang et al., 2006). In some experiments, we first denervated the lower hindlimb muscles and then 9 d later we electroporated the TA muscle with siRNAs before harvesting electroporated muscle 14–15 d after denervation. Both denervation/electroporation regimens gave essentially identical results. GFP<sup>+</sup> fibers were dissected in ice-cold phosphate-buffered saline (PBS) and then immediately lysed in TritonX (Invitrogen) for RNA isolation. This process takes ~10–20 min. RNA was reverse transcribed with oligo(dT) primer and SuperScript II (Invitrogen), and 1/20 of the cDNA mixture served as template for PCR reactions. Real-time PCR was performed on a iCycler (Bio-Rad, Hercules, CA) by using SYBR green dye on cDNA samples. Pyruvate assay kit (Biovision, Mountain View, CA) was used to measure the concentration of intracellular pyruvate following the manufacturer’s protocol. Briefly, cultured C2C12 cells were transfected with either control or HDAC4 siRNAs (60 nM) by using Lipofectamine 2000. Transfected cells were then switched to pyruvate-free differentiation medium (5% horse serum, high glucose DMEM), and 2 d later cells were collected and lysed with 0.5 ml of pyruvate assay buffer. Pyruvate is oxidized by pyruvate oxidase, to generate a fluorogenic product that can be measured at excitation/emission 335/590 nm. Results were measured on a FluoroStar OPTIMA 96-well plate reader (BMG Labtech, Chicago, IL). Values were normalized to total protein.

**RESULTS**

**HDAC Activity Contributes to Gene Induction and Repression in Developing Myotubes and Denervated Muscle**

We demonstrated previously that HDAC inhibitors sodium butyrate (NaB) or TSA, block muscle differentiation- and denervation-dependent myogenin (Mgn) gene induction (Tang and Goldman, 2006). Here, we investigated whether HDAC activity was also necessary for differentiation- and denervation-dependent gene suppression. We chose the...
tion. Values are normalized to

HDAC1-9

Radioactive PCR showing

MSE

obtained with the HDAC inhibitor TSA.

We next investigated whether HDAC inhibition also pre-

We chose TSA for these experiments because it is specific for class II

HDACs, and it was more potent than NaB in blocking HDAC activity in vivo in preliminary studies. These experiments revealed that HDAC inhibition blocks denervation-dependent Mgn induction and MSE suppression (Figure 1B).

**HDAC4 and HDAC6 Are Induced in Denervated Muscle**

The above-mentioned data suggest specific HDACs may be necessary for both denervation-dependent gene induction and suppression. We suspected that the relevant HDACs may themselves be regulated by muscle activity. To identify these candidate deacetylases, we assayed HDAC1–9 mRNA levels in innervated and denervated TA muscle. Interestingly, like Mgn, HDAC4 and HDAC6 mRNA levels increased in 5- and 15-d denervated muscle (Figure 1C), whereas HDAC7 and HDAC8 mRNA levels increased transiently at 5 d after denervation, but returned to basal levels at 15 d after denervation (Figure 1C). Real-time PCR analysis of denervation-dependent gene expression at early times after muscle denervation showed temporal changes in HDAC4 mRNA levels but not HDAC6 most closely reflects the temporal changes in Mgn and MSE mRNA levels (Figure 1D). This time course of denervation-dependent Mgn and HDAC4 gene expression was also reflected in protein levels with HDAC4 induction preceding Mgn induction (Figure 1E).

**HDAC4 Regulates Genes Involved in Synapse Formation, Glycolytic Metabolism, and Fiber-Type Specification**

Although the temporal pattern of HDAC4 gene expression suggests it is a better candidate than HDAC6 in mediating denervation-dependent Mgn gene regulation, they are both induced in denervated muscle and they are both likely to contribute to activity-dependent gene regulation. To determine whether these HDACs are necessary for denervation-dependent Mgn gene regulation, we knocked down their expression by using siRNAs. Consistent with the temporal pattern of their expression, HDAC4 but not HDAC6 knockdown relieved suppression of Mgn mRNA induction (Figure 2, A and B). As expected, the denervation-dependent increase in nAChR α-subunit and MusSK mRNA expression, shown previously to be induced by Mgn (Tang et al., 2006), was also blocked after HDAC4 knockdown (Figure 2C). We also investigated the effect that HDAC4 knockdown had on the expression of genes involved in muscle contraction and metabolism. Interestingly, HDAC4 knockdown reciprocally regulated the expression of MHC isoforms by blocking denervation-dependent induction of MHC IIa and relieving denervation-dependent suppression of MHC Iib (Figure 2, C and E). In addition, HDAC4 knockdown relieved suppression of MSE and PFK that normally occurs in denervated TA muscle (Figure 2D, top; and E). Similar results were observed in the denervated slow soleus muscle where denervation-dependent induction of Mgn and MHC IIa mRNAs was suppressed and denervation-dependent suppression of MSE and MHC Iib mRNAs was relieved by HDAC4 knockdown (Figure 2D, bottom). The extremely low expression of MHC Iib mRNA in soleus is consistent with the preponderance of slow-type MHC I and IIa fibers in this muscle. Quantification of the effects of HDAC4 knockdown on a variety of glycolytic, oxidative, and contractile protein encoding mRNAs in TA muscle showed that those involved in glycolysis (PFK and MSE)
and muscle contraction (MHC IIa and IIb) were the most influenced, whereas genes related to oxidative metabolism were less influenced (Figure 2E).

The above-mentioned data suggest HDAC4 is necessary for both denervation-dependent gene induction and suppression. To determine whether HDAC4 is sufficient for mediating these changes in gene expression in response to muscle denervation, we expressed the mutant HDAC4-3SA in innervated TA muscle. This mutant harbors three mutations, S246A, S467A, and S632A, conferring its resistance to nuclear export (Grozinger and Schreiber, 2000; Wang et al., 2000). Quantitative real-time PCR revealed a 3.5-fold increase in the HDAC4 mRNA level after electroporation of TA muscle with this expression vector (Figure 2F). This increased expression of nuclear localized HDAC4 in innervated muscle mimicked the effect of muscle denervation by suppressing MSE and MHC IIb mRNA levels and increasing Mgn mRNA levels (Figure 2F).

Because HDAC4 suppresses both MSE and PFK gene expression and because PFK is a rate-limiting enzyme in glycolysis, we investigated whether HDAC4 influences glycolysis. For these experiments, we transfected HDAC4 siRNA into C2C12 muscle cells and measured pyruvate production. After HDAC4 knockdown, intracellular pyruvate increased by ∼50%, indicating HDAC4 does inhibit glycolysis in muscle cells (Figure 2G).

**HDAC4 Coordinates the Suppression of Multiple Repressors to Allow for Denervation-dependent Mgn Gene Induction**

The Mgn corepressors Dach2 and MITR are induced in innervated muscle and suppressed in denervated muscle...
HDAC4 Directly Suppresses MSE Gene Expression

HDAC4 represses genes involved in glycolytic metabolism (Figure 2, D and E), and we suspected this may result by recruitment of HDAC4 to ME2 sites residing in glycolytic enzyme gene promoters (McKinsey et al., 2002). To investigate whether HDAC4 contributes to this regulation, we expressed HDAC4-3SA in innervated muscle (Figure 3A) and knocked down HDAC4 in denervated TA muscle (Figure 3B). Consistent with the idea that HDAC4 contributes to denervation-dependent Dach2 and MITR gene repression, we found HDAC4-3SA suppressed the expression of both of these corepressors in innervated muscle and HDAC4 knockdown partially relieved their suppression in denervated muscle.

Because HDAC4 expression is necessary for denervation-dependent Mgn gene induction and knockdown of HDAC4 in denervated muscle returned it to a more innervated pattern of gene expression (Figure 2), we investigated whether the effect of HDAC4 knockdown on Mgn gene expression is due to increased Dach2 and MITR expression. For this, we evaluated Mgn mRNA levels in HDAC4-siRNA electroporated TA muscle from wild-type mice, Dach2−/− mice (Davis et al., 2006), and Dach2−/− mice that also had MITR levels reduced by RNA interference (Figure 3C). Consistent with the idea that HDAC4 mediates its effects on Mgn gene expression by suppressing both Dach2 and MITR expression, we found that HDAC4-knockdown in Dach2 null animals partially relieved Mgn suppression, whereas the addition of MITR knockdown (~65% knockdown) in Dach2 null animals abrogated Mgn suppression more dramatically (Figure 3C). Therefore, HDAC4 seems to coordinate only suppress Dach2 and MITR expression after muscle denervation, and suppression of both these genes is required for robust denervation-dependent Mgn gene induction.

HDAC4 is Enriched in Oxidative Skeletal Muscle Fibers and Nuclear Import Is Stimulated by Muscle Denervation

We next analyzed HDAC4 expression in innervated TA muscle that contains predominantly type II fibers (99%) that can be further subdivided into a mixture of small oxidative fibers and larger glycolytic fibers (Gorza, 1990). Interestingly, immunohistochemistry revealed a mosaic pattern of HDAC4 expression, with small oxidative fibers tending to have more intense staining than the relatively larger glycolytic fibers (Figure 5A). We confirmed that HDAC4 expres-
Figure 4. HDAC4 suppresses MSE promoter activity via recruitment to the MSE promoter’s MEF2 element. (A) Nuclear-localized HDAC4-3SA suppresses expression from a cotransfected MSE:Luciferase expression vector in C2C12 myotubes. Confluent C2C12 cells were transfected with a CMV:HDAC4-3SA expression vector, MSE: luciferase expression vector, and a CMV:β-gal expression vector that was used for normalization. Cells were differentiated in low serum, and 48 h later they were harvested for luciferase and β-gal assays. Reported MSE promoter activity is luciferase activity normalized to β-gal activity. Error bars are standard deviations, n = 5. (B) The MSE promoter’s MEF2 site is required for HDAC4-dependent regulation. C2C12 cells were transfected with wild-type or a MEF2 mutant MSE:luciferase expression vector along with either a control or HDAC4-targeting siRNA and a normalizing CMV:β-gal vector as described above. Samples were analyzed as described in A. (C) Autoradiogram showing that overexpression of MEF2 in HEK293 cells increases binding to the MSE promoter’s MEF2 element. HEK293 cells were transfected with increasing amounts of CMV:MEF2 or an empty vector (−) and 48 h later they were harvested and nuclear extracts were prepared. Nuclear extracts with (+) and without (−) overexpressed MEF2 were incubated with a radiolabeled oligonucleotide containing the MSE promoter’s MEF2 site in the absence (−) or presence (+) of a 50-fold excess of unlabeled MEF2 oligonucleotide (competitor). Samples were resolved on a native polyacrylamide gel that was then dried and exposed to x-ray film. Shown is a representative autoradiogram. (D) ChIP assay shows that after muscle denervation HDAC4 or overexpressed myc-HDAC4 binds to the MSE promoter’s MEF2 site but not the Mgn promoter’s MEF2 site. Samples were either 5-d denervated TA muscle or 5-d denervated TA muscle that was electroporated with a myc-HDAC4 expression vector 12 d before denervation. Real-time PCR with primers flanking either the Mgn or MSE promoter’s MEF2 site was used to quantify the amount of immunoprecipitated DNA.

Figure 5. HDAC4 is enriched in TA muscle small oxidative fibers and localizes to the subsarcolemma region and NMJ. (A) Innervated TA muscle was immunostained with an anti-HDAC4 antibody (HDAC4) or assayed for SDH activity. Arrows in the low-magnification (100×) panel (left) identify representative HDAC4-positive small oxidative fibers. Asterisks in higher magnification (400×) panels (middle and right) identify small oxidative fibers expressing both HDAC4 and SDH. (B) Immunofluorescence showing HDAC4 immunoreactivity colocalizes with COX1 (400× magnification). (C and D) HDAC4 immunoreactivity colocalizes with BTX-stained NMJs (in merge [C], blue color is 4,6-diamidino-2-phenylindole-stained nuclei). Bars, 40 μm.

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pression (Figures 1 and 2). The molecular mechanisms mediating HDAC4 induction in denervated muscle have not been investigated. A previous study suggested that the HDAC4 promoter harbors GC-rich regulatory elements that bind Sp proteins and that mithramycin treatment of cultured cells, which impedes Sp protein binding to GC-rich sequences (Nehls et al., 1993), down-regulates HDAC4 gene expression (Liu et al., 2006). Therefore, we investigated whether mithramycin affected denervation-dependent induction of HDAC4 in vivo. For these experiments, we bathed innervated and denervated sternomastoid muscle in 100 μM mithramycin for 12 h, and we found mithramycin suppressed HDAC4 and Mgn gene expression in innervated but not denervated muscle (Figure 7A). Therefore, GC-binding proteins seem to play a role in maintaining the basal expression of HDAC4 and Mgn genes in innervated muscle, but they are not required for maintaining high levels of expression in denervated muscle.

Because E-box sequences (CANNTG) are known to mediate denervation-dependent induction of nAChR and MuSK promoters via their interaction with Mgn (Tang et al., 2004, 2006), we looked for similar elements residing in the HDAC4 promoter. Indeed, three E-boxes (−572, −924, and −1383 relative to the translation initiation codon) were found upstream of the HDAC4 coding sequence. Interestingly, these E-boxes conform to the CACCTG or CACGTG sequences that seem to be required for denervation-dependent activation of human, rat, mouse, and chicken nAChR and MuSK promoters (Tang et al., 2006), suggesting that Mgn may also mediate denervation-dependent HDAC4 gene induction. Indeed, we found Mgn overexpression in C2C12 cells increased HDAC4 promoter activity (Figure 7B) and that shRNA-mediated Mgn knockdown reduced HDAC4 promoter activity (Figure 7C).

The above-mentioned data suggest Mgn activates the HDAC4 promoter; therefore, we investigated whether Mgn induction after muscle denervation influences HDAC4 gene expression. For this analysis, we measured endogenous HDAC4 mRNA before and after siRNA-mediated Mgn knockdown in denervated TA muscle. Interestingly, Mgn knockdown blocked denervation-dependent HDAC4 gene induction (Figure 7D). As shown in Figure 7E, similar results were obtained when we assayed denervation-dependent HDAC4 induction in conditional Mgn null mice in which the Mgn gene was deleted in adult muscle (Knapp et al., 2006). These data suggest that Mgn, via its effect on HDAC4 expression, may also be necessary for denervation-dependent suppression of Mse, Pfk, and MhcIIb gene expression. To test this idea, we used Mgn null mice and compared denervation-dependent regulation of Mse, Pfk, and MhcIIb mRNA expression with that found in normal mice (Figure 7F). Consistent with the idea that Mgn and HDAC4 regulate each others expression, denervation-dependent suppression of Mse, Pfk, and MhcIIb mRNAs was impaired in the

Figure 6. Muscle inactivity stimulates nuclear accumulation of HDAC4. (A) Triple fluorescence immunostaining for laminin, HDAC4, and nuclei on innervated and denervated TA muscle sections. Arrowheads point to HDAC4+/+ myonuclei located within the muscle fibers basal lamina; 600× magnification. (B) HDAC4 immunoreactivity shows low-level nuclear accumulation of HDAC4 in innervated small oxidative fibers and high level nuclear accumulation in denervated small oxidative and larger glycolytic fibers. Arrowheads point to nuclei with weak HDAC4 immunoreactivity in oxidative fibers of innervated muscle and high HDAC4 immunoreactivity in both oxidative and glycolytic denervated muscle fibers; 1000× magnification. Qualitative assessment of HDAC4 expression in cytoplasm and nuclei is presented in the table. (C) Western blot analysis of HDAC4 nuclear levels (NE) and cytoplasmic levels (CE) in active (−TTX) and inactive (+TTX) primary mouse myotube cultures. (D) Inactivity induced nuclear accumulation of HDAC4 in cultured primary mouse myotubes. Primary myotubes were transfected with indicated expression vectors and either kept inactive with TTX or allowed to spontaneously contract (−TTX); 400× magnification. Bars, 40 μm. Inn, innervated; Den, denervated.
Herein identify an HDAC4/Mgn positive feedback loop that couples muscle activity to muscle phenotype. The data described here provide insights into the mechanisms coupling changing demands of their environment. A major goal of research on muscle biology is to understand the molecular mechanisms underlying this regulation.

Muscle Denervation
HDAC4 Coordinates Gene Induction and Suppression after Muscle Denervation

Although muscle denervation is known to cause activation and suppression of gene expression (Buonanno et al., 1998), the molecular mechanisms underlying this regulation have remained elusive. We found that HDAC4 is necessary for both denervation-dependent gene induction and suppression (Figure 2). HDAC4 nuclear localization is regulated by calcium and calcium-regulated protein kinases (McKinsey et al., 2002; Liu et al., 2005) and denervation-dependent accumulation of HDAC4 in the nucleus seems to initiate changes in gene expression (McKinsey et al., 2002). We demonstrated that HDAC4 contributes indirectly to Mgn induction by coordinately inhibiting the expression of the Mgn gene coregulated with HDAC4.

Figure 7. HDAC4 and Mgn positively regulate each others expression. (A) Mithramycin inhibits HDAC4 and Mgn mRNA expression in innervated (Inn) but not denervated (Den) sternomastoid muscle. Inn and Den sternomastoid muscle was bathed in mithramycin (100 mM) or vehicle (−), in vivo, for 12 h before harvesting tissue for assaying HDAC4, Mgn, and γ-actin mRNA levels by using real-time PCR. HDAC4 and Mgn mRNA levels are normalized to γ-actin RNA levels. Experiments were performed as described in the legend of Figure 1B and in Materials and Methods. (B) Mgn overexpression induces HDAC4 promoter activity in C2C12 myotubes. C2C12 myotubes were transfected with an HDAC4 promoter luciferase and CMV-γ-actin expression vector, +/− a CMV:Mgn expression vector. Forty-eight hours later, cells were harvested for luciferase and β-gal activities. Reported HDAC4 promoter activity is luciferase activity normalized to β-gal activity. (C) Mgn knockdown reduces HDAC4 promoter activity in C2C12 myotubes. C2C12 myotubes were transfected with an HDAC4 promoter luciferase and CMV-γ-actin expression vector, +/− a U6:Mgn-shRNA expression vector for knocking down Mgn expression. Forty-eight hours later, cells were harvested for luciferase and β-gal activities. Reported HDAC4 promoter activity is luciferase activity normalized to β-gal activity. (D) Mgn knockdown in denervated TA muscle blocks denervation-dependent induction of HDAC4. Innervated TA muscle was electroporated with control or Mgn-targeting siRNAs; and 12 d later, muscles either remained innervated (Inn) or were denervated for 5 d (Den) before harvesting electroporated muscle fibers for analysis of mRNA expression. Two different Mgn-targeting siRNAs were used in this experiment, and radioactive PCR was used to visualize Mgn, HDAC4 and γ-actin mRNA levels. (E) Conditional deletion of the Mgn gene in adult mice blocks denervation-dependent induction of HDAC4 mRNA. Adult mice harboring a conditionally expressed Mgn gene were either treated with tamoxifen (Mgn+/−) or remained untreated (WT). The left hindlimb muscles were denervated for 5 d, whereas the right hindlimb remained innervated. Five days after denervation, innervated and denervated TA muscles were isolated and Mgn, HDAC4, and γ-actin mRNA levels were assayed by real-time PCR. Mgn and HDAC4 mRNA levels were normalized to γ-actin mRNA. –Fold difference from innervated RNA level is reported. (F) Denervation-dependent suppression of PFK, MSE, and MHC IIb mRNA levels are abrogated in mice where the Mgn gene is deleted in adult animals. Experimental protocol was as described in E. Error bars are SDs, n = 3.
pressors Dach2 and MITR (Figure 3). HDAC4 contributes to gene suppression via its interaction with MEF2 transcription factors (Lu et al., 2000; Miska et al., 1999), and we identified putative MEF2 elements ~980 base pairs upstream of the Dach2 coding sequence and 742 base pairs, 1568 base pairs, 2558 base pairs, and 2771 base pairs upstream of the MITR coding sequence. We also found HDAC4 inhibits MEF2 activity.

Consistent with the idea that HDAC4 mediates inhibition by interaction with MEF2, we found HDAC4 is recruited to the MEF2 promoter’s MEF2 site. The MEF2 promoter also harbors MEF2 sites at positions -172 and -1780 relative to the translation initiation codon, respectively, which likely serve as HDAC4 docking sites.

Our observation that HDAC4 is recruited to the MEF2 promoter, and not the Mgn promoter’s MEF2 site is intriguing and may suggest that HDAC4 recruitment to MEF2 sites is context dependent. Whether the MEF2 site and flanking sequence or the constellation of transcription factors and cofactors that bind these promoters affect HDAC4 recruitment is not currently known.

Subcellular Localization of HDAC4 in Innervated and Denervated Muscle

We observed enrichment of HDAC4 in small oxidative fibers of innervated TA muscle that is composed predominantly of fast oxidative and glycolytic fiber types (Figure 5). Because HDAC4 can suppress glycolytic enzyme gene expression and induce Mgn expression (Figure 2) and because Mgn expression is highest in oxidative fibers (Hughes et al., 1993), we found that the expression of oxidative enzymes (Hughes et al., 1999; Ekmark et al., 2003), it seems reasonable to propose that HDAC4-regulated gene expression contributes to the oxidative phenotype of innervated fast muscle fibers.

After muscle denervation, HDAC4 is enriched in nuclei of both fast oxidative and glycolytic fibers (Figure 6). Muscle denervation is also accompanied by an inhibition of glycolytic enzyme expression (Gundersen et al., 1988), and we showed that this is mediated by an HDAC4-dependent mechanism (Figure 2). Although Mgn is reported to increase expression of oxidative enzymes in innervated muscle, we did not find HDAC4/Mgn-dependent induction of oxidative enzymes in denervated muscle. This is consistent with previous reports showing muscle denervation is not correlated with increased oxidative metabolism (Gundersen et al., 1988) and may suggest Mgn differentially regulates oxidative enzyme gene expression in innervated and denervated muscle.

In addition to its nuclear localization, we found HDAC4 concentrated beneath the sarcolemma and also at the NMJ (Figure 5). Interestingly, mitochondria are found in both these regions (Ogata and Yamasaki, 1985; Perkins et al., 2001) and colocalize with HDAC4 (Figure 5). We suspect that mitochondria, or other cellular components distributed in a similar manner as mitochondria, serve as docking sites for HDAC4 and function either to sequester HDAC4 from nuclear entry and/or provide novel substrates for HDAC4-dependent modification. Of relevance, HDAC7 has also been found to localize to mitochondria (Bakin and Jung, 2004). More interestingly, HDAC4 interacts with the sarcolemma Z-disk-associated muscle LIM protein (MLP) in cardiac sarcomeres and impacts muscle contractile activity (Gupta et al., 2008). Whether MLP also associates with HDAC4 in skeletal muscle is not known. These unexpected findings suggest that HDAC4 localizes to the cytoplasm not just simply as a passive regulatory mechanism but may indicate that novel regulatory mechanisms remain to be elucidated.

An HDAC4/Mgn Positive Feedback Loop Coordinates and Maintains Denervation-dependent Transcriptional Programs

Our results reveal a novel positive feedback loop coordinating the induction and repression of genes after muscle denervation (Figure 8). We found that in denervated muscle HDAC4 expression is necessary for Mgn gene induction and that Mgn induction is necessary for increased HDAC4 gene expression (Figures 2 and 7). This signaling loop seems to be initiated upon muscle denervation by reduced intracellular calcium levels that stimulate nuclear retention of HDAC4. Increased nuclear levels of HDAC4 repress expression of the Mgn transcriptional repressors Dach2 and MITR. Relief of Mgn transcriptional repression, along with activating factors, results in increased Mgn levels that can then stimulate transcription of the HDAC4 gene.

We have shown that the HDAC4/Mgn regulatory loop is capable of coordinating gene induction and suppression in denervated muscle. Muscle fibers are defined by the type of MHC protein expressed and their unique program of gene expression. In rodents, slow muscle fibers express MHC I and are rich in oxidative metabolism, whereas fast muscle fibers express a variety of MHC II isoforms (IIa, IIx, and IIb) to various extents. At one extreme, MHC IIa fibers are more like slow muscle in that they are rich in oxidative enzymes and exhibit a high resistance to fatigue; at the other extreme are MHC IIb fibers that have high glycolytic metabolism and fatigue easily. Endurance training will often result in a shift of fiber type to the more fatigue-resistant MHC I and MHC IIa type fibers. Similarly denervation of fast muscle is often accompanied by a shift to the more fatigue resistant type IIa
phenotype (Gundersen et al., 1988). It seems that the HDAC4/Mgn signaling pathway contributes to this shift in muscle phenotype by suppressing expression of the fast MHC IIb contractile protein along with a variety of glycolytic enzymes and inducing expression of the more fatigue-resistant MHC IIa contractile protein (Figure 2).

Because HDAC4 nuclear localization is regulated by muscle activity and calcium levels (Liu et al., 2005), it is uniquely poised to respond to the changing demands put on muscle. In this respect, it is similar to the calcineurin/nuclear factor of activated T cells (NFAT) signaling complex that seems to drive expression of genes that are involved in determining a slow fiber phenotype (Chin et al., 1998). In this case, calcineurin responds to increased muscle calcium by dephosphorylating cytoplasmic NFAT, inducing its nuclear translocation where it can act on genes involved in determining the slow fiber phenotype. Thus, these data suggest that the HDAC4/Mgn signaling pathway, along with the calcineurin/NFAT pathway, represent master controls of muscle phenotype in response to the changing demands put on muscle by neural input.

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