

HDAC4 Regulates Muscle Fiber Type-Specific Gene Expression Programs

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Fiber type-specific programs controlled by the transcription factor MEF2 dictate muscle functionality. Here, we show that HDAC4, a potent MEF2 inhibitor, is predominantly localized to the nuclei in fast/glycolytic fibers in contrast to the sarcoplasm in slow/oxidative fibers. The cytoplasmic localization is associated with HDAC4 hyper-phosphorylation in slow/oxidative-fibers. Genetic reprogramming of fast/glycolytic fibers to oxidative fibers by active CaMKII or calcineurin leads to increased HDAC4 phosphorylation, HDAC4 nuclear export, and an increase in markers associated with oxidative fibers. Indeed, HDAC4 represses the MEF2-dependent, PGC-1 α -mediated oxidative metabolic gene program. Thus differential phosphorylation and localization of HDAC4 contributes to establishing fiber type-specific transcriptional programs.

INTRODUCTION

Skeletal muscle is composed of heterogeneous myofibers with different contractile and metabolic properties. This arrangement enables skeletal muscle to effectively carry out different tasks, from maintaining body posture to performing rapid movements. Myofibers are grossly classified into slow/oxidative fibers and fast/glycolytic fibers. The slow/oxidative fibers are more resistant to fatigue, rich in mitochondria, and preferentially use oxidative phosphorylation to provide ATP for contraction (thus termed oxidative fibers). The fast/glycolytic fibers, in contrast, are fast in contractility, contain fewer mitochondria and use glycolysis as the main source of energy (Spangenburg and Booth, 2003). The functionality of a myofiber is determined by the expression of unique sets of contractile proteins and metabolic enzymes at the transcriptional level. Thus, in slow fibers, type I myosin heavy chain (MHC) is the dominant form of myo-

sin heavy chain, while in fast fibers type IIb or IIx MHC are the main isoforms (Spangenburg and Booth, 2003). In terms of the metabolic program, the transcriptional co-factor peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) is expressed at higher levels in slow/oxidative fibers and is a major regulator of the mitochondrial oxidative phosphorylation program (Lin et al., 2002).

Adult skeletal muscle is highly adaptive and can undergo changes in contraction, metabolism, and size to meet a variety of functional demands [reviewed in (Bassel-Duby and Olson, 2006)]. Established and subsequent re-programming of fiber-specific gene transcription has important physiological and pathological implications. In metabolic diseases (i.e. diabetes and metabolic syndrome) a slow/oxidative-to-fast/glycolytic fiber transition correlates with disease severity (Marin et al., 1994; Nyholm et al., 1997; Schrauwen and Hesselink, 2004). Conversely, endurance training promotes a more oxidative muscle fiber phenotype reflecting a transformation from type IIx/IIb to IIa (Fitzsimons et al., 1990; Pette, 2002). The transition to oxidative fibers involves PGC-1 α , which promotes oxidative energy utilization and mitochondria biogenesis through the co-activation of MEF2-dependent gene expression (Lin et al., 2002). Supporting its instructive role in specifying the fiber type program, paradigms that promote slow/oxidative fiber formation (e.g. endurance training) frequently induce PGC-1 α expression (Terada et al., 2002; Russell et al., 2003; Sandri et al., 2006). In fact, PGC-1 α over-expression can convert fast/glycolytic fibers to slow/oxidative fibers in a mouse model (Lin et al., 2002). Promoter analysis and cell-based studies have identified MEF2 as a key transcriptional activator for PGC-1 α expression in skeletal muscle (Akimoto et al., 2004b; Czubryt et al., 2003). Consistent with this proposal, MEF2 is selectively more active in slow/oxidative than fast/glycolytic fibers (Wu et al., 2000; 2001). The molecular mechanism that differentially regulates the MEF2-PGC-1 α transcriptional program in slow vs fast myofibers has not been fully elucidated.

HDAC4 is the founding member of the class IIa HDACs, best known for their ability to bind and repress MEF2 (Black and Olson, 1998; McKinsey et al., 2000; Zhao et al., 2001). Despite its potent inhibitory activity on MEF2, HDAC4 is expressed in terminally differentiated skeletal muscles where MEF2 activity is essential (Choi et al., 2012; Cohen et al., 2007; Potthoff et al., 2007a). This finding indicates that HDAC4 is likely subjected to negative regulation, allowing MEF2-dependent muscle gene transcription. Analysis of HDAC members in skeletal muscle has indicated that protein levels of class IIa HDACs, including HDAC4, are much lower in slow/oxidative fibers compared to

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fast/glycolytic fibers (Potthoff et al., 2007b). Reduced abundance of class IIa HDAC members could certainly contribute to elevated MEF2 activity observed in slow/oxidative fibers. On the other hand, cell-based analyses have also convincingly demonstrated that phosphorylation of HDAC4 by calcium/calmodulin-dependent kinases (CaMKs) or other kinases activate HDAC4 nuclear export, leading to de-repression/activation of MEF2 target genes (Backs et al., 2006; Cohen et al., 2009; McKinsey et al., 2000; 2002; Vega et al., 2004; Zhao et al., 2001). The intracellular movement of HDAC4, in principle, could also modulate MEF2 activity. Whether phosphorylation-dependent intracellular trafficking of HDAC4 is involved in the MEF2-dependent and fiber type-specific transcriptional program is not known.

In this report, we present evidence that HDAC4 is differentially phosphorylated and localized in distinct subcellular compartments of different muscle types. In fast/glycolytic fibers, HDAC4 is less phosphorylated and more nuclear localized whereas in slow/oxidative fibers, the opposite phenotype is observed. Supporting its functional relevance to MEF2-dependent fiber type-specific transcription, we show that HDAC4 inhibits slow fiber-specific gene transcription and its phosphorylation leads to nuclear exclusion and activation of markers for slow/oxidative fibers. Our results implicate a regulatory role for HDAC4 in the maintenance and/or reprogramming of fiber type-specific gene transcription in adult skeletal muscle.

MATERIALS AND METHODS

Cell culture/plasmids/antibodies

C2C12 myoblasts were cultured in 20% FBS/DMEM and differentiated in 2% horse serum/DMEM. Stable myoblast lines were used as described previously (Cohen et al., 2009). GFP-CaMKII plasmid was from Dr. Anthony Means (Duke University) and the T287D mutant was generated by the standard protocol for PCR-mediated mutagenesis, with the mutant confirmed via DNA sequencing. MHC I and IIb reporters were provided by Dr. Steven Swoap (Williams College). PGC-1 α reporters were from Dr. Bruce Spiegelman (Addgene plasmid database). The following antibodies were used: MY-32 antibody from Sigma; cytochrome c and COX IV antibodies from BD Bioscience; GFP antibody from Santa Cruz; myogenin and tubulin antibodies from Sigma; BAF-8 antibody (Akimoto et al., 2004a); and GAPDH antibody from Cell Signaling. HDAC4 (clone 186) and P467-HDAC4 (clone 19111) antibodies were previously described (Cohen et al., 2007).

Mouse procedures

C57BL/6 mice were purchased from Jackson Laboratory. Calcineurin mice were used as described previously (Naya et al., 2000). *In vivo* electroporation of siRNAs or plasmids to TA muscle were performed as described previously (Cohen et al., 2007; 2009). HDAC4 siRNA sequences were as follows (Invitrogen): 5'-CACCGGAACCGAACCACUGCAUUU-3'. Briefly, mice were anesthetized with a ketamine/xylazine mixture. After removing hair, 200 pmol of siRNAs or 25 μ g of plasmids, including 5 μ g of GFP plasmid to identify electroporated fibers, were injected into TA muscles using cemented MicroSyringe (VWR). An ECM830 electroporator (BTX) was used for electroporations. Mice were allowed to recover in their cages. All mice were housed in at the Duke University mouse facilities, and all animal procedures were approved by the Institutional Animal Care and Use Committee at the Duke University.

RNA analysis

RNA extraction and qRT-PCR were performed as described

previously (Cohen et al., 2009). For RNA analysis, GFP-positive fibers were isolated using a dissecting microscope. Muscles were lysed in Tri reagent and RNA was extracted by standard protocols. cDNA was synthesized using iScript RT kit (Bio-Rad). Real-time PCR was performed using iQ Syber Green Supermix on the iCycler iQ detection system (Bio-Rad). PCR primer sequences are as follows: actin: 5'-ACCCAGGCATTGCTGACAGGATGC-3' and 5'-CCATCTAGAAGCATTTCGCGTGGACG-3'; PGC-1 α : 5'-CGATGTGTGCGCTTCTTGCT-3' and 5'-CGAGAGCGCATCCTTTGG-3', and myoglobin: 5'-CATGGTTGCCA-CCGTGCTCACAG-3' and 5'-GAGCCCATGGCTCAGCCC-TG-3'.

Western analysis

Muscles and C2C12 myotubes were lysed as described previously (Cohen et al., 2007; 2009). Muscles were dounce-homogenized in lysis buffer (0.05 M NaCl, 0.02 M Tris, pH 7.6, 1 mM EDTA, supplemented with leupeptin, aprotinin, phenylmethylsulfonyl fluoride, NaF, sodium orthovanadate, and 1 mM dithiothreitol). Lysates were then incubated with 1X detergent (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) by adding 10X stock solution. C2C12 myotubes were harvested at indicated time points and lysed in 150 mM NETN buffer (0.5% NP-40, 20 mM Tris, pH 8.0, 1 mM EDTA, and 150 mM NaCl) for 30 min on ice and centrifuged. Lysates were subjected to Western blotting using SDS-PAGE, transferred to membranes, and incubated with indicated antibodies.

Immunofluorescence

Muscle staining was performed as described previously (Cohen et al., 2007; 2009). Mouse muscles were dissected, frozen by OCT solution (Tissue-Tek) in methylbutane chilled in liquid nitrogen, and cryosectioned. Cross-sections were fixed for 10 min in 4% paraformaldehyde. After washing with PBS, sections were blocked in 5% normal goat serum (NGS) for 2 h at room temperature and incubated with primary antibodies overnight. Sections were washed in PBS, blocked with NGS for 1.5 h, and incubated with secondary antibody. Nuclei were stained using Hoechst dye. Sections were imaged using a Zeiss AxioScope compound microscope (Carl Zeiss).

Luciferase assay

Luciferase assay was performed as described previously (Cohen et al., 2009). Briefly, C2C12 myoblasts were plated at 20,000 cells per well in a 24-well plate. Each reporter was co-transfected with β -gal in combination with the plasmids using Fugene (Promega). Myoblasts were then differentiated in 2% horse serum/DMEM. Myotubes were lysed in luciferase lysis buffer (50% glycerol, 2.5% TX-100, 3 g Tris base, and 0.695 g CDTA per 200 ml, pH 7.8) for 20 min at room temperature. Ten μ l of cell lysate was used with 200 μ l of luciferase assay buffer (15 mM MgSO₄, 15 mM K₂HPO₄, 4 mM EGTA, 1 mM DTT, and 1 mM ATP). For normalization, 20 μ l of lysate was used in β -gal assay (10 mg ONPG, 17 μ l of β -mercaptoethanol per 5 ml Z-buffer). Each experiment was performed in triplicate.

RESULTS

More cytoplasmic localization of HDAC4 is present in slow/oxidative muscle fiber

Given that MEF2 is more active in slow/oxidative fibers, we hypothesized that its repressor, HDAC4, would be inactivated in slow/oxidative fibers. Since nuclear export of HDAC4 leads to MEF2 activation, we investigated if HDAC4 shows differential

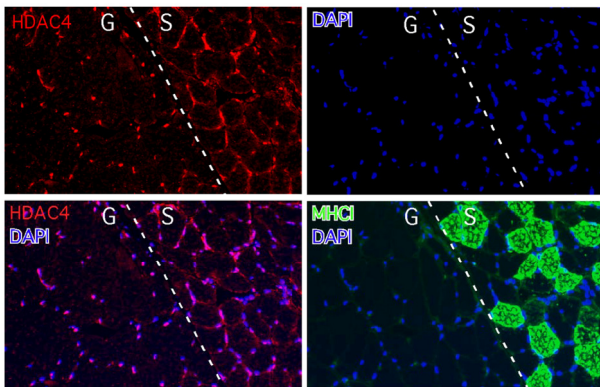


Fig. 1. Distinct subcellular localization of HDAC4 in slow and fast muscle fibers. HDAC4 localization was determined by immunostaining slow soleus (Sol) and neighboring fast-rich gastrocnemius (GA) muscles. Frozen muscle cross-sections were co-stained with HDAC4 and fast myosin-specific antibodies. GA and Sol muscles are marked as G and S, respectively. DNA is visualized by Hoechst staining.

localization in myofibers (Fig. 1). Immunostaining with an HDAC4-specific antibody revealed a distinct pattern where more cytosolic staining of HDAC4 was observed in the slow/oxidative fiber-enriched soleus (Sol) muscle as opposed to the predominantly nuclear localization of HDAC4 in the adjacent fast/glycolytic fiber-rich gastrocnemius (GA) muscle. This result indicates that HDAC4 mainly resides in the cytosol of slow/oxidative fibers, which would activate MEF2-dependent transcription.

HDAC4 phosphorylation and cytosolic accumulation are induced by transgenes that activate slow/oxidative fibers

To investigate if differential HDAC4 phosphorylation is a characteristic feature of different myofibers, we examined muscles from transgenic mice expressing a MCK-driven, constitutively-activated calcineurin (CN) transgene, which induces slow/oxidative fiber formation in normally fast/glycolytic fiber-dominant muscles (Chin et al., 1998). As shown in Fig. 2A, while HDAC4 was more abundantly phosphorylated on S467, a target of CaMKII (Bacs et al., 2006), in Sol muscle than in fast-type muscles in control mice, HDAC4 phosphorylation was markedly induced in all fast fibers analyzed, including plantaris (PL), tibialis anterior (TA), and white vastus (WV) muscle from CN-transgenic mice. This result indicates that HDAC4 phosphorylation positively correlates with the slow/oxidative fibers compared to fast/glycolytic fibers.

Constitutively-active CaMK also induces slow/oxidative fibers (Wu et al., 2002). CaMKII has been shown to bind and phosphorylate HDAC4, resulting in its cytoplasmic localization (Bacs et al., 2006). KN-62, a CaMK inhibitor, can block cytoplasmic translocation of HDAC4 induced by electrical stimulation in isolated myofibers (Liu et al., 2005). We confirmed that HDAC4 phosphorylation (S467) was indeed increased by CaMKII activation in cultured cells (Fig. 2B). To investigate whether CaMKII activation promotes HDAC4 phosphorylation and cytoplasmic translocation in skeletal muscle, we electroporated a constitutively active CaMKII-T287D plasmid into wild-type mouse TA muscle. Increased accumulation of phospho-HDAC4 was observed in CaMKII-T287D positive myofibers by immuno-blotting

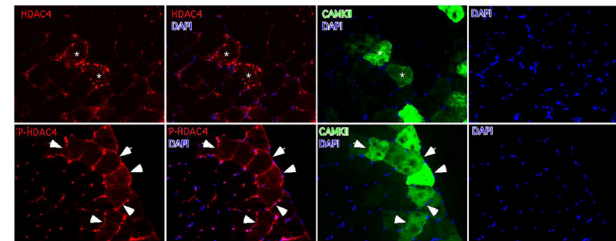
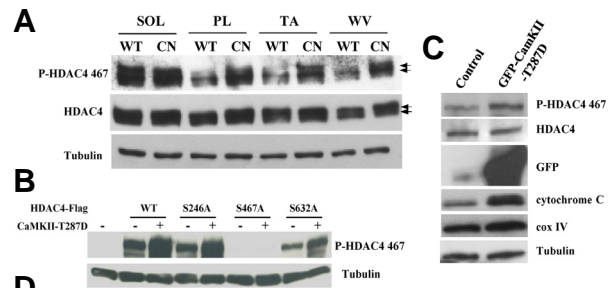


Fig. 2. Increased phosphorylation and cytoplasmic accumulation of HDAC4 by calcineurin or CaMKII activation. (A) Increased phosphorylation of HDAC4 was observed in calcineurin (CN) transgenic mouse muscles that acquired a fast-to-slow fiber type conversion. Muscles were collected from control and CN mice and muscle lysates from slow Sol and fast fibers including plantaris (PL), tibialis anterior (TA), and white vastus (WV) were analyzed by Western blot analysis with the indicated antibodies. (B) Increased HDAC4 phosphorylation at Ser-467 was confirmed by constitutively active CaMKII-T287D expression in Cos-7 cells. (C) Increased HDAC4 phosphorylation at Ser-467 correlated with expression of oxidative gene markers in TA muscle expressing CaMKII-T287D. Muscle lysates were subjected to Western blot analysis using the indicated antibodies. (D) Sub-cellular localization of HDAC4 and phospho-HDAC4 was determined by immunostaining analysis of frozen cross-sections from GFP-CaMKII-T287D-transfected TA muscles. The asterisk indicates CAMKII transfected fibers with peripheral HDAC4 accumulation similar to phospho-HDAC4. The arrowheads depict clear regions of membranous and cytoplasmic phospho-HDAC4 accumulation in CAMKII transfected fibers in contrast to neighboring untransfected fibers. Note the overall increased phosphorylation of HDAC4 with specific immunoreactivity along the muscle sarcolemmal membrane.

(Fig. 2C) and immuno-staining (Fig. 2D). Importantly, an enrichment of phospho-HDAC4 along the muscle periphery became apparent in CaMKII-T287D-positive myofibers (Fig. 2D, arrows), suggesting an increase in HDAC4 nuclear export. Indeed, mitochondrial oxidative markers, including Cytochrome C and Cox IV, were induced in CaMKII-T287D-positive myofibers (Fig. 2C). In untransfected myofibers, HDAC4 signal was more concentrated to the nuclei (Fig. 2D). Together, these results indicate that phosphorylation increases HDAC4 cytoplasmic relocation and oxidative gene expression in skeletal muscle.

HDAC4 represses slow/oxidative fiber gene expression

Nuclear HDAC4-enriched fast fibers suggested that it might repress the MEF2-dependent slow/oxidative fiber gene expression. To test this possibility, C2C12 myoblasts stably expressing a HDAC4 siRNA with or without re-expressing an HDAC4

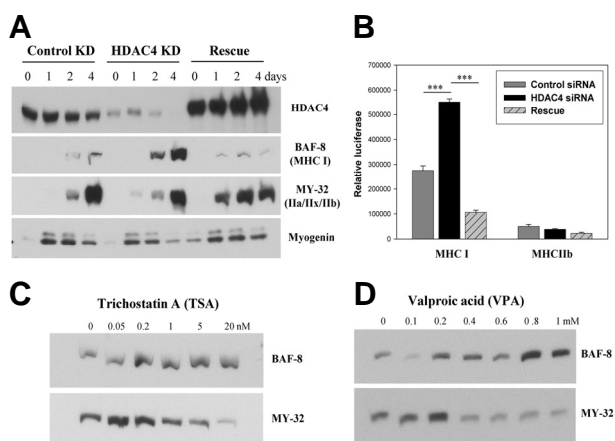


Fig. 3. HDAC4 inhibits slow fiber MHC expression in cultured myotubes. (A) C2C12 myoblast controls or those stably expressing an HDAC4 siRNA (KD) or re-expressing HDAC4 wild type (Rescue) were differentiated for indicated time points and cell lysates were subjected to Western blot analysis using indicated antibodies against slow and fast myosin heavy chains (MHC), I and II, respectively. (B) C2C12 myotubes stably expressing an HDAC4 siRNA or re-expressing HDAC4 wild type plasmid were analyzed for promoter-driven luciferase activities using MHC I and II-specific reporter constructs. Columns, mean; Bars, SD ($n = 3$). $***P < 0.001$ versus control siRNA. (C,D) Expression of slow and fast MHC I and II in C2C12 myotubes treated with HDAC4 inhibitors. C2C12 myotubes were treated overnight with the indicated concentrations of trichostatin A (TSA) or valproic acid (VPA) in a dose-dependent manner. Cell lysates were subjected to Western analysis using the indicated antibodies.

plasmid were differentiated into myotubes, and the expression of slow-fiber MHC I and fast fiber MHC II was determined. As shown in Fig. 3A, HDAC4 knockdown (KD) or HDAC4 re-expression (Rescue) did not have a significant effect on C2C12 differentiation as indicated by myogenin induction, thus suggesting that modulating HDAC4 expression does not alter the differentiation program per se, but rather specifically regulates MEF2 target gene expression (Cohen et al., 2009; Zhao et al., 2001). However, the expression of slow-fiber MHC I (BAF-8) was elevated by HDAC4 KD and suppressed by the re-expression of HDAC4. Consistent with this finding, HDAC4 KD significantly increased promoter activity of MHC I, whereas HDAC4 re-expression suppressed this activity (Fig. 3B). HDAC4 expression levels did not have a significant effect on MHC II expression (Fig. 3A, MY-32) or its promoter activity (Fig. 3B). These results indicate a preferential effect of HDAC4 in preventing slow fiber-specific MHC I expression in C2C12 cells. We next investigated whether HDAC inhibitors, valproic acid (VPA) and trichostatin A (TSA), alone can differentially affect MHC I expression. Consistent with HDAC4 being a negative regulator of MHC I, a dose-dependent increase in MHC I was observed in VPA- and TSA-treated C2C12 cells (Figs. 3C and 3D). Interestingly, HDAC inhibitors decreased MHC II expression. These findings reveal that HDAC inhibitors can affect fiber type-specific MHC expression in cultured muscle cells.

Since MEF2 and PGC-1 α expression positively regulates oxidative gene expression (Lin et al., 2002; Potthoff et al., 2007b), we next examined whether HDAC4 regulates PGC-1 α

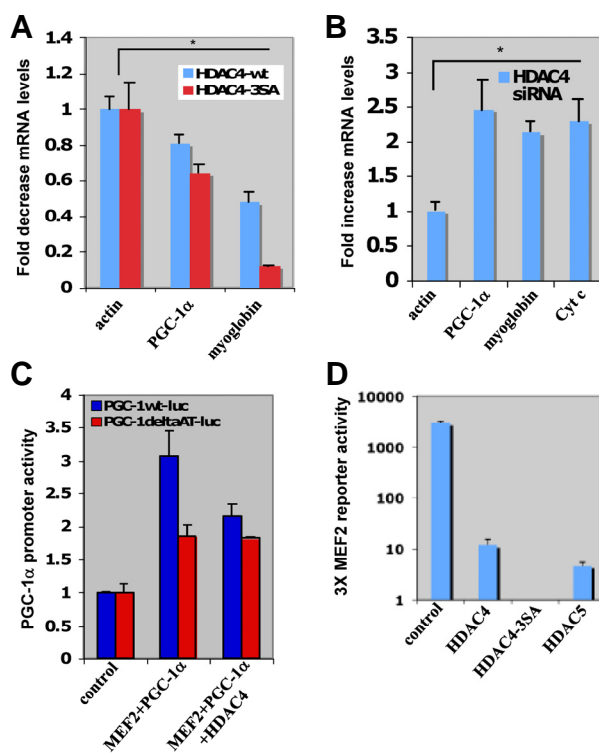


Fig. 4. HDAC4 inhibits MEF2-dependent expression of PGC-1 α and related oxidative genes. (A) Tibialis muscles (TA) were electroporated with expression plasmids containing control, wild type HDAC4, or the nuclear-localized HDAC4-3SA mutant and oxidative gene expression was evaluated by RNA analysis of isolated TA muscle lysates by real-time RT-PCR. Oxidative gene expression was determined using gene-specific primers detecting PGC-1 α , and myoglobin, all of which are up-regulated in slow-fibers. Values represent fold repression relative to actin levels. Columns, mean; bars, SEM ($n = 3$). $*P < 0.05$. (B) RNA analysis similar to panel A was performed to monitor oxidative gene expression after HDAC4 siRNA knockdown in TA muscles. Columns, mean; bars, SEM ($n = 3$). $*P < 0.05$. (C) PGC-1 α promoter luciferase activities were evaluated in transfected myotubes using wild-type PGC-1 α or a mutant promoter containing deletion of AT-rich elements harboring MEF2-binding sites (deltaMEF2). HDAC4 suppressed PGC-1 α promoter activity in a MEF2-dependent manner. (D) MEF2 promoter activity was examined in transfected myotubes using wild-type HDAC4, HDAC4-3SA mutant, or HDAC5.

gene expression. To this end, we electroporated wild type and constitutively nuclear HDAC4-3SA mutant (Zhao et al., 2001) into TA muscles. As shown in Fig. 4A, PGC-1 α expression was modestly repressed by wild-type HDAC4, while more prominent repression was observed by the HDAC4-3SA mutant. The relatively modest effect of ectopic wild-type HDAC4 could be due to endogenous HDAC4 enrichment in the nuclei of TA muscle. Supporting a repressive activity of HDAC4 on PGC-1 α -dependent oxidative programming, knockdown of HDAC4 by a siRNA in TA muscle led to elevated expression of PGC-1 α , myoglobin and Cytochrome C (Fig. 4B). Furthermore, HDAC4 repressed PGC-1 α promoter activity in a MEF2-dependent manner, as deletion of AT-rich MEF2-binding elements prevented HDAC4-mediated repression of PGC-1 α (Fig. 4C). Con-

firming that HDAC4-mediated transcriptional repression occurs via a nuclear mechanism, both wild-type HDAC4 and most robustly, the nuclear-targeted HDAC4-3SA mutant, strongly suppressed MEF2 promoter activity (Fig. 4D). Collectively, these results indicate that HDAC4 establishes fast fiber gene expression via repression of MEF2-dependent PGC-1 α and related oxidative genes.

DISCUSSION

In this study, we provide evidence that nuclear HDAC4 is involved in the repression of oxidative gene expression that specifies slow fiber fate. Although it was previously reported that HDAC4 levels were markedly reduced in slow/oxidative fibers (Potthoff et al., 2007b), our results showed that HDAC4 was more abundant in the slow/oxidative Sol muscle compared to the fast/glycolytic muscles (Figs. 1 and 2A). These findings are consistent with a previous report (Liu et al., 2005), which demonstrated HDAC4 cytoplasmic translocation, and not degradation, in response to slow fiber patterns of electrical stimulation. The basis of this discrepancy is not known, but could possibly be due to different experimental conditions such as stringency of muscle lysis buffer. In slow Sol muscle, cytoplasmic HDAC4 was dominant, in contrast to fast fiber-rich GA muscle (Fig. 1). Accordingly, the more cytoplasmic localization of HDAC4 would permit more active MEF2-dependent oxidative gene expression. Indeed, conversion of fast/glycolytic fibers to more oxidative fibers by calcineurin transgene expression led to an increase in HDAC4 phosphorylation (Fig. 2A). Further supporting a role of HDAC4 phosphorylation in specifying the oxidative fiber phenotype, we also observed that CaMKII activation in TA muscle caused the induction of several oxidative markers, which correlated with HDAC4 phosphorylation and subsequent cytoplasmic translocation (Figs. 2C and 2D). These findings are also in agreement with a previous *in vitro* analysis showing that slow-twitch fiber activation by electrical stimulation of myofibers causes cytoplasmic translocation of HDAC4 leading to MEF2 activation (Liu et al., 2005). Thus, our results suggest that dynamic HDAC4 phosphorylation and subcellular relocalization might be involved in maintaining and reprogramming muscle fiber functionality in adult muscle.

Multiple signaling networks collaborate to ensure proper fiber-type specification based on the physiological demand of the muscle. Further studies would be required to determine the role of HDAC4 in adult muscle remodeling. It is important to point out that inactivation of HDAC4 alone is not capable of "switching" fiber-types *in vivo* (Potthoff et al., 2007b), which reflects the functional redundancy of class IIa HDACs. Indeed, the closely-related HDAC5 can also suppress MEF2-dependent PGC-1 α expression and is subjected to phosphorylation-dependent nuclear export (Czubryt et al., 2003; Vega et al., 2004). Thus, HDAC4 likely collaborates with other class IIa members in fiber type specification and oxidative gene expression. Consistent with this possibility, we noticed that pan-HDAC inhibitor treatment causes a reduction of MHC II fiber specific MHC and concurrent induction of MHC I fiber MHC in C2C12 myotubes (Figs. 3C and 3D). Indeed, HDAC inhibitor treatment *in vivo* using a mouse model of muscular dystrophy was previously shown to prevent muscle degeneration associated with fast fiber conversion (Minetti et al., 2006). Given that exercise tends to increase slow/oxidative fibers, whether HDAC inhibitors can similarly promote a slow/oxidative fiber transition *in vivo* and improve muscle functionality profile requires future study.

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