HDAC1 activates FoxO and is both sufficient and required for skeletal muscle atrophy

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SUMMARY

The Forkhead boxO (FoxO) transcription factors are activated and necessary for the muscle atrophy during several pathophysiological conditions, including muscle disuse and cancer cachexia. Yet, the mechanisms which lead to FoxO activation are not well defined. Recent data from our lab and others indicate that the activity of FoxO is repressed under basal conditions via reversible lysine acetylation, which becomes compromised during catabolic conditions. The purpose of the current study was therefore to determine the extent to which histone deacetylase (HDAC) proteins contribute to FoxO activation and induction of the muscle atrophy program. Through the use of various pharmacological inhibitors to block HDAC activity we demonstrate that class I HDACs are key regulators of FoxO and the muscle atrophy program during both nutrient deprivation and skeletal muscle disuse. Furthermore, we demonstrate through the use of WT and d.n. HDAC1 expression plasmids that HDAC1 is sufficient to activate FoxO and induce muscle fiber atrophy, in vivo, and is necessary for the muscle fiber atrophy associated with muscle disuse. The ability of HDAC1 to cause muscle atrophy required its deacetylase activity and was linked to its induction of several atrophy genes, including atrogin-1, which required FoxO3a deacetylation. Moreover, pharmacological inhibition of class I HDACs during muscle disuse via treatment with MS-275 significantly attenuated both disuse muscle fiber atrophy and contractile dysfunction. Together these data solidify the importance of class I HDACs in the muscle atrophy program and indicate that class I HDAC inhibitors are feasible countermeasures to impede muscle atrophy and weakness.
INTRODUCTION

Skeletal muscle atrophy and weakness accompany several pathophysiological conditions, including muscle disuse (D'Antona et al., 2003), aging (Gosselin et al., 1994; Larsson et al., 1997a; Larsson et al., 1997b; Lowe et al., 2001; Thompson and Brown, 1999), cancer (Roberts et al., 2013a; Roberts et al., 2013b) and chronic heart failure (Evans et al., 1995; Greutmann et al., 2011). The loss of skeletal muscle mass and impaired function during these conditions contribute to reduced physical performance and quality of life, prolonged hospital stays and enhanced mortality (Evans, 2010). Unfortunately, effective countermeasures to impede the loss of muscle mass and function during these often complex and overlapping conditions are limited, emphasizing the importance of research aimed at understanding the cellular mechanisms of muscle atrophy and dysfunction. Although the underlying cause of atrophy and weakness are unique to each atrophy condition, a common transcriptional program of increased atrophy gene (atrogene) expression occurs in multiple models of muscle atrophy (Lecker et al., 2004; Sacheck et al., 2007). Furthermore, the upstream transcription factors which induce these transcriptional changes also appear to be commonly involved during conditions of muscle atrophy. For example, the Forkhead Box O (FoxO) transcription factors are activated during multiple models of muscle atrophy, and are both sufficient and required for muscle atrophy (Sandri et al., 2004). Indeed, FoxO is necessary for the typical gene expression changes and muscle fiber atrophy associated with skeletal muscle disuse (Reed et al., 2011a; Senf et al., 2010), cancer cachexia (Reed et al., 2012) and sepsis (Reed et al., 2012) in vivo, as well as during dexamethasone treatment (Sandri et al., 2004) and nutrient deprivation (Raffaello et al., 2010) of skeletal myotubes. Given this importance of FoxO in the atrophy program, identifying mechanisms which regulate FoxO activation in skeletal muscle has tremendous potential for the development of therapeutics to preserve muscle mass and function across a wide-range of distinct and coinciding atrophy conditions.

We and others recently demonstrated that the cellular localization and activity of the FoxO transcription factors in skeletal muscle are regulated via acetylation (Bertaggia et al., 2012; Senf et al., 2011). We found that FoxO interacts with, and is acetylated by, the histone acetyltransferase (HAT) proteins p300/CBP. We also found that reducing HAT activity in skeletal muscle was sufficient to induce FoxO transcriptional activity, while increasing HAT activity prevented FoxO nuclear localization, transcriptional activity and target-gene
transcription in response to nutrient deprivation in C2C12 skeletal myotubes, and in whole muscle, in vivo, in response to muscle disuse (Senf et al., 2011). Work from Bertaggia et al. further demonstrated, through mutation of six FoxO3a lysine acetylation sites, that acetylation of FoxO3a indeed represses its transcriptional activity and promotes its cytosolic localization (Bertaggia et al., 2012). The authors also demonstrate that three days following denervation, the ratio of acetylated to total FoxO3a is acutely decreased in skeletal muscle which contributes to FoxO3a-dependent transcription of atrophy genes. Thereafter a progressive increase in FoxO3a acetylation was observed and attributed as a protective mechanism to promote FoxO3a cytosolic redistribution in an effort to turn off the atrophy program. These findings collectively indicate that decreased acetylation of FoxO3a in skeletal muscle is an important early mechanism controlling its ability to drive the atrophy program.

Post-translational modification of proteins through acetylation occurs via the enzymatic activity of HATs, while the removal of acetylated residues occurs through the opposing actions of histone deacetylases (HDACs). In skeletal muscle, HATs and HDACs are most well known for their regulation of muscle development and differentiation through the regulation of histone acetylation which leads to chromatin modification and transcriptional activation or repression (McKinsey et al., 2001). More recently, the class II HDACs, HDAC4 and HDAC5, were demonstrated to promote neurogenic atrophy through their transcriptional repression of Dach2, which normally acts to repress myogenin-dependent induction of atrophy related genes (Moresi et al., 2010). However, as previously mentioned, in addition to regulating gene transcription through histone acetylation, the catalytic activity of HATs and HDACs also regulate gene expression through altering the acetylation status and function of transcription factors such as FoxO. Yet limited information currently exists on the specific HDACs which regulate FoxO acetylation status in skeletal muscle during normal conditions and those which contribute to decreases in FoxO acetylation and activation during catabolic conditions.

The current study therefore aimed to determine whether the deacetylase activity of specific HDAC proteins contribute to FoxO activation and induction of the muscle atrophy program. Specifically, we determined the role of HDACs on FoxO activity and atrophy associated with nutrient deprivation and skeletal muscle disuse. To do this, we first used the global HDAC inhibitor Trichostatin A (TSA) to inhibit class I and class II HDACs in skeletal muscle cells and whole muscle, in vivo, to determine whether HDACs contribute to FoxO
activation and the atrophy program in response to nutrient deprivation. Based on these findings we subsequently determined whether class I or class II HDACs preferentially regulate FoxO activation, and then carried these findings over to the more physiologically relevant model of skeletal muscle disuse. Using a class I HDAC inhibitor and expression plasmids for specific class I HDACs we demonstrate the requirement of class I HDACs for FoxO activation, atrophy gene transcription, skeletal muscle atrophy and contractile dysfunction during muscle disuse. Furthermore, our findings pinpoint the class I HDAC, HDAC1, as a novel regulator of FoxO signaling in skeletal muscle that is both sufficient and required for skeletal muscle atrophy.
RESULTS

FoxO nuclear localization and activation in response to nutrient deprivation is mediated via HDAC activity.

To determine whether FoxO transcriptional activity in skeletal muscle is regulated by class I and II HDACs, we treated 3-day differentiated skeletal myotubes transfected with a FoxO-responsive reporter plasmid with TSA, which inhibits both class I and II HDACs. Myotubes were treated with TSA (or vehicle) during control conditions and during nutrient deprivation, which we and others have previously shown increases FoxO nuclear localization and transcriptional activity (Mammucari et al., 2007; Senf et al., 2011). As shown in Fig. 1A, TSA strongly repressed FoxO reporter activity in myotubes under normal conditions as well as during 18 hours of nutrient deprivation. These data indicate that class I and/or class II HDACs maintain basal levels of FoxO activity in skeletal muscle cells and facilitate FoxO activation in response to nutrient deprivation. Another mechanism to increase FoxO activity is to reduce the basal activity of Akt, which normally phosphorylates and retains FoxO transcription factors in the cytosol. We therefore transfected skeletal myoblasts with a FoxO-responsive reporter plasmid, plus a dominant negative (d.n.) Akt expression plasmid (or empty vector), to reduce endogenous Akt activity and increase FoxO activity. Following three days of differentiation we treated myotubes with TSA (or vehicle) for 24 hours to determine if TSA could reverse the d.n. Akt-induced increase in the FoxO reporter. As shown in Fig. 1B, d.n. Akt induced the FoxO-reporter by 50%, which was reversed in the presence of TSA. This therefore demonstrates that treatment with TSA can block FoxO activation even when signaling through Akt is depressed, and further suggests that TSA-mediated repression of FoxO is not dependent on Akt signaling.

We further determined whether inhibition of HDACs through TSA regulates FoxO nuclear localization. Skeletal myoblasts were transfected with FoxO3a-DsRed or FoxO1-GFP expression plasmids and, following three days of differentiation, myotubes were nutrient deprived in the presence of TSA or vehicle. The localization of ectopic FoxO3a-DsRed and FoxO1-GFP were visualized through fluorescent microscopy, and their average level of fluorescence in the nuclear and cytoplasmic compartments was measured (Fig. 1C). As depicted in the representative images, FoxO3a-DsRed (Fig. 1D,E) and FoxO1-GFP (Fig. 1F,G) are localized predominately to the cytoplasm during control conditions, but show increased localization to the nucleus in response to nutrient deprivation, which is confirmed by co-
fluorescence with DAPI-stained nuclei. In contrast, inhibition of class I and II HDACs through treatment with TSA prevented the increase in both FoxO3a-DsRed and FoxO1-GFP nuclear localization in response to nutrient deprivation.

To further determine whether inhibition of class I and II HDACs also prevents the increased gene expression of atrophy-related and FoxO target genes during nutrient deprivation, we performed qRT-PCR on 3-day differentiated myotubes following 6 hours of nutrient deprivation (or control conditions) in the presence or absence of TSA. As shown in Fig. 1G, TSA repressed the increase in the FoxO target genes atrogin-1/MAFbx, MuRF1 and Lc3, which play a role in degradation, as well as Gadd45a and p21 which are involved in growth arrest. Together these data indicate that class I and II HDACs regulate FoxO nuclear localization and transcriptional activation in response to nutrient deprivation, and are necessary for the increased transcription of several atrophy-related target genes.

Inhibition of class I and class II HDACs during nutrient deprivation, in vivo, prevents skeletal muscle fiber atrophy.

We next sought to carry over our findings to nutrient deprivation, in vivo. We therefore determined whether TSA could prevent the muscle fiber atrophy associated with nutrient deprivation in mice. Mice were injected intraperitoneally (i.p.) with either vehicle (sterile 1X PBS) or TSA, and were then assigned to a control group (fed) or a nutrient deprivation group. Following three days of nutrient deprivation, muscles from both groups were harvested. To ensure TSA was indeed altering protein acetylation in muscle, we examined the effect of TSA on the acetylation of a known class I HDAC target, histone H3, and a known class II HDAC target, α-tubulin. As shown in figure Fig. 2A, TSA treated muscles showed an increase in acetylated histone H3 and α-tubulin. To determine the effect of TSA on muscle fiber cross-sectional area (CSA), skeletal muscle cross-sections taken from plantaris muscles were incubated in wheat germ agglutinin to outline fiber membranes, and the average muscle fiber CSA was calculated for each group. Representative images of muscle cross-sections from each group are shown in Fig. 2B. In response to three days of nutrient deprivation, skeletal muscle fiber CSA decreased by 27% in vehicle-treated mice, which was completely prevented in nutrient deprived mice treated with TSA (Fig. 2C). These data therefore demonstrate that class I and/or class II HDACs are necessary for muscle fiber atrophy in response to nutrient deprivation, in vivo. Furthermore,
these data provide further physiological significance to our findings in skeletal muscle cells, which indicate that HDACs regulate the atrophy-associated transcription factor FoxO and promote atrophy gene transcription in response to nutrient deprivation.

**Class I HDACs preferentially regulate FoxO activation during nutrient deprivation.**

Because TSA inhibits class I and class II HDACs, which are each comprised of several distinct HDAC family members, the possible HDAC(s) which may regulate the FoxO transcription factors are numerous. We therefore sought to narrow down the potential HDAC proteins which may regulate FoxO, through determining whether class I or class II HDACs preferentially regulate FoxO activity. To do this we treated skeletal muscle myotubes transfected with a FoxO reporter with MC-1568 (class II HDAC inhibitor) or MS-275 (class I HDAC inhibitor) during control conditions and during 6 hours of nutrient deprivation. As shown in Fig. 3A, treatment with both MC-1568 and MS-275 reduced basal levels of FoxO reporter activity, though MS-275 reduced basal FoxO reporter activity to a greater magnitude. In contrast, during nutrient deprivation, inhibition of class II HDACs via MC-1568 reduced FoxO reporter activity only marginally, while inhibition of class I HDACs via MS-275 completely prevented FoxO reporter activation in response to nutrient deprivation. These data indicate that while both class I and class II HDACs regulate basal levels of FoxO activity in skeletal muscle cells, class I HDACs are necessary for FoxO activation in response to a catabolic stimulus.

**HDAC1 is sufficient to increase FoxO transcriptional activity**

Although MS-275 is a class I HDAC inhibitor it does not inhibit HDAC8 (Hu et al., 2003). We therefore screened the remaining class I HDACs, HDAC1, HDAC2 and HDAC3 to determine which of these proteins regulate FoxO activity. To do this we injected and electroporated whole rat soleus muscles, *in vivo*, with a FoxO-dependent luciferase reporter plasmid plus an empty vector, or expression plasmids for wild type (WT) or dominant negative HDAC1, 2 or 3. Despite successful overexpression of HDAC2 and HDAC3 (Figure 3C) neither regulated FoxO activity. However, WT HDAC1 was sufficient to increase FoxO transcriptional activity ~3-fold, which required its deacetylase activity (Fig. 3B). Since HDAC1 increases FoxO activity, and MS-275 (which preferentially inhibits HDAC1 (IC$_{50}$ = 300 nM (Hu et al., 2003))) prevents FoxO
activation, these findings together demonstrate that HDAC1 regulates FoxO signaling in skeletal muscle.

**HDAC1 is sufficient to induce muscle fiber atrophy, in vivo.**

Given our finding that overexpression of HDAC1 is sufficient to increase FoxO activity and that FoxO is sufficient to cause skeletal muscle fiber atrophy (Sandri et al., 2004) we subsequently hypothesized that HDAC1 may be sufficient to cause skeletal muscle fiber atrophy. In order to test this hypothesis we injected and electrotransfered rat soleus muscles with expression plasmids for GFP only, or GFP constructs also expressing WT HDAC1 or d.n. HDAC1 and harvested muscles 7 days later for CSA or gene expression analyses. As shown in the representative cross-sections in Fig. 3D, WT HDAC1-GFP expressing fibers were visually smaller than fibers expressing GFP alone or d.n. HDAC1-GFP. Quantification of the average CSA of transfected fibers revealed that fibers expressing WT HDAC1-GFP were 42% smaller than fibers expressing GFP alone (Fig. 3E), demonstrating that HDAC1 is sufficient to induce muscle fiber atrophy in the absence of any physiological stimulus. Furthermore, since the CSA of fibers expressing d.n. HDAC1-GFP was not different from those expressing GFP, this further demonstrates that HDAC1 causes muscle fiber atrophy through its deacetylase activity.

In order to determine whether the HDAC1-mediated increase in FoxO activity and muscle fiber atrophy is associated with the transcriptional activation of known atrophy-related FoxO target genes, we further measured the mRNA levels of *atrogin-1*, *MuRF1*, *Ctsl* (cathepsin L) and *Lc3*, which are elevated in skeletal muscle in response to multiple catabolic conditions and are involved in protein degradation (Mammucari et al., 2007; Sandri et al., 2004). Overexpression of WT HDAC1 was sufficient to induce the gene expression of *atrogin-1* (60%), *MuRF1* (45%), *Ctsl* (25%) and *Lc3* (25%), which required its deacetylase activity, since d.n. HDAC1 did not similarly increase the mRNA levels of these genes (Fig. 3F). Importantly, the transcriptional activity of the FoxO transcription factors can be regulated via direct lysine acetylation, which was recently demonstrated as a regulatory mechanism to inhibit FoxO in skeletal muscle (Bertaggia et al., 2012; Senf et al., 2011). Indeed, the ability of an Akt phosphorylation defective FoxO3a (FoxO3a-TM) to induce an atrogin-1 promoter construct and cause skeletal muscle fiber atrophy was recently shown by Bertaggia et al. to be reversed via mutation of six lysine acetylation sites within the FoxO3a-TM construct to mimic the acetylated
form (FoxO3a-TM-6KQ). We therefore next sought to determine whether HDAC1-induced transcription of *atrogin-1* was mediated through FoxO3a deacetylation at these specific lysine residues. To do this, we injected and electrotransfered muscles with an *atrogin-1* promoter reporter plasmid plus expression plasmids for WT HDAC1 (or GFP) plus empty vector, WT FoxO3a or FoxO3a 6KQ (which cannot be regulated via deacetylation at the indicated residues). If WT HDAC1 indeed interacts with and deacetylates endogenous FoxO3a to increase *atrogin-1* transcription, presence of the FoxO3a 6KQ should interfere with the ability of WT HDAC1 to induce the *atrogin-1* promoter. As shown in Fig. 3G, both WT HDAC1 and WT FoxO3a are sufficient to increase the *atrogin-1* reporter, and show an additive effect when co-expressed together. However, similar to the findings of Bertaggia et al, the FoxO3a 6KQ mutant is not sufficient to induce the atrogin-1 promoter (Bertaggia et al., 2012). Furthermore the FoxO3a 6KQ mutant significantly interferes with the ability of HDAC1 to increase the *atrogin-1* promoter, providing strong evidence that HDAC1 regulates atrogin-1 through deacetylation of FoxO3a at these specific lysine residues.

Given this finding we next determined whether we could detect changes in endogenous FoxO3a acetylation in muscles transfected with WT and d.n. HDAC1 expression plasmids. Total acetylated proteins were immunoprecipitated from muscles using an anti-acetyl-lysine antibody and subsequently immunoblotted for FoxO3a. As shown in Fig. 3H, muscles injected with d.n. HDAC1 demonstrated an increase in acetylated FoxO3a, which indicates that FoxO3a is indeed a target of HDAC1 deacetylase activity. In order to determine whether HDAC1 directly complexes with FoxO3a, we also immunoprecipitated HDAC1 from muscles and subsequently immunoblotted for FoxO3a, and found that these proteins co-precipitate. We repeated these experiments using a FoxO1 antibody and found that HDAC1 also complexes with FoxO1 and is a target of HDAC1 deacetylase activity (Fig. 3H). Since we and others have previously demonstrated that increases in FoxO1 and FoxO3a acetylation are associated with increases in their phosphorylation status (Bertaggia et al., 2012; Matsuzaki et al., 2005; Senf et al., 2011), we further measured the effect of HDAC1 on endogenous FoxO1 and FoxO3a phosphorylation. While WT HDAC1 had no visual effect on the basal levels of FoxO phosphorylation, d.n.HDAC1 modestly increased both phospho-FoxO1 and phospho-FoxO3a.

Although WT HDAC1 did not decrease the basal levels of FoxO phosphorylation or acetylation, WT HDAC1 was nonetheless sufficient to increase FoxO activity and induce muscle
atrophy. Therefore, we next determined whether HDAC1-induced muscle fiber atrophy required FoxO activation. To do this we co-injected WT HDAC1-GFP (or GFP) with DsRed or a d.n. FoxO-DsRed expression plasmid to inhibit FoxO-dependent transcription, and harvested muscles 7 days later for CSA analyses. Expression of d.n. FoxO-DsRed localized to the nucleus and induced significant muscle hypertrophy (both of which we have previously demonstrated (Reed et al., 2012; Reed et al., 2011b) while WT HDAC1-GFP induced significant myofiber atrophy (Fig. 3D & I). Interestingly, co-expression of d.n. FoxO-DsRed and WT HDAC1-GFP together did not significantly alter muscle fiber size (Fig. 3I & J). This finding indicates that HDAC1-induced muscle atrophy is counteracted by the hypertrophic and/or anti-atrophic effects of d.n. FoxO, and suggests that endogenous FoxO may mediate the atrophy induced by HDAC1. However, because HDAC1 also prevented the hypertrophy induced by d.n. FoxO, this further suggests that HDAC1 likely regulates myofiber size through additional pathways independent of FoxO.

**HDAC1 is required for muscle fiber atrophy, in vivo.**

Importantly, our studies thus far have focused on the ability of HDAC1 to induce the muscle atrophy program in the absence of a physiological atrophy stimulus. We therefore next sought to determine whether HDAC1 deacetylase activity mediates physiological muscle atrophy induced by muscle disuse. To test this we injected GFP or d.n. HDAC1-GFP into rat solei and cast-immobilized muscles for 7 days prior to cross-sectional area analyses. As shown in the representative muscle cross-sections of immobilized muscles in Fig. 4A, GFP-positive fibers were not visually different from non-transfected fibers. However, d.n. HDAC1-GFP positive fibers were visually larger than the surrounding non-transfected fibers in immobilized muscle. Measurement of the average fiber CSA in immobilized muscles demonstrates that fibers expressing d.n. HDAC1-GFP (2048 ± 154 µm²) are significantly larger (74%) than GFP-expressing fibers (1178 ± 16 µm²) (Fig. 4B). When calculated as a percentage of fiber CSA from muscles of weight bearing mice, immobilization caused a 55% decrease in fiber size in GFP-transfected fibers that was attenuated by 60% in fibers expressing d.n. HDAC1-GFP. Since d.n. HDAC1-GFP did not affect fiber CSA during weight bearing conditions, these data provide strong evidence that HDAC1 is necessary for the progression of disuse muscle atrophy, and that this is mediated through its deacetylase activity.
We next sought to determine if HDAC1 is necessary for FoxO activation and the increased transcription of atrophy-related FoxO target genes during muscle disuse. To do this we transfected rat solei with either expression plasmids for WT HDAC1-GFP, d.n. HDAC1-GFP or GFP, with a subset of rats also co-transfected with a FoxO responsive luciferase reporter plasmid. Rats were subsequently assigned to weight bearing or cast immobilized conditions for 3 days to induce muscle disuse after which muscles were harvested for measurement of luciferase activity or mRNA analysis. Measurement of FoxO-dependent luciferase activity in immobilized muscles revealed that WT HDAC1 potentiated the immobilization-induced increase in FoxO activity, while d.n. HDAC1 attenuated the increase in FoxO activity (Fig. 4C). Thus, this finding demonstrates that HDAC1 deacetylase activity is required for the normal increase in FoxO activity in response to muscle disuse.

As shown in Fig. 4D and as expected, cast-immobilization significantly increased the mRNA levels of atrogin-1, MuRF1, Ctsl and Lc3. Similar to our findings during weight bearing conditions, when normalized to the control group (weight bearing, GFP), overexpression of WT HDAC1 during immobilization further increased the mRNA levels of these atrophy genes, which required its deacetylase activity. Moreover, expression of d.n.HDAC1 during immobilization repressed the immobilization-induced increase in these atrophy genes. Since d.n. HDAC1 did not affect the expression of these genes in weight bearing muscle (Fig. 3E), these data indicate that HDAC1 is necessary for their gene induction in response to muscle disuse, and that this requires its deacetylase activity. Thus, based on these collective findings HDAC1 may mediate disuse muscle atrophy through deacetylating and activating FoxO to induce atrophy gene transcription. Importantly, since a reduction in FoxO phosphorylation is a widely used marker of FoxO activation during atrophy conditions, we further measured the effect of the HDAC constructs on endogenous FoxO phosphorylation during muscle disuse. As shown in Fig. 4E, overexpression of WT HDAC1 reduced the phosphorylation of both FoxO1 and FoxO3a, while overexpression of d.n. HDAC1 strongly increased their phosphorylation. Thus, HDAC1 may contribute to the muscle atrophy phenotype during muscle disuse through both deacetylating FoxO and reducing its sensitivity to phosphorylation. Moreover, based on additional data analyzing the total abundance and cellular localization of endogenous HDAC1, HDAC1 may shuttle out of the nucleus to exert its effect on FoxO within the cytoplasm. Indeed, although total protein levels of HDAC1 were unchanged, the relative abundance of HDAC1 in the nuclear fraction decreased,
while HDAC1 increased in the cytosol (Fig 4F). Thus, HDAC1-mediated deacetylation of FoxO in the cytosol may be an important signal that leads to decreased phosphorylation and nuclear localization of FoxO. However, this relocalization of HDAC1 to the cytosol during disuse may also lead to increased deacetylation of other HDAC1 substrates located in the cytosol, which could also contribute to the muscle atrophy phenotype.

**Inhibition of class I HDACs during skeletal muscle disuse prevents contractile dysfunction and reduces the extent of fiber atrophy.**

Since we found that HDAC1 deacetylase activity was an important regulator of the muscle atrophy program associated with muscle disuse, we next sought to determine whether disuse muscle atrophy and the associated muscle weakness could be prevented by treatment with MS-275. As mentioned above, MS-275 is a class I HDAC inhibitor that exerts strong preference towards HDAC1 (Hu et al., 2003). Mice were therefore injected i.p. with either vehicle or MS-275, and were assigned to a control (weight bearing) group or immobilized group. Mice continued to receive daily injections of MS-275 or vehicle, and after 10 days of immobilization soleus muscles were harvested from both groups. To confirm MS-275 was altering protein acetylation in muscle, we examined the effect of MS-275 on the acetylation of a known class I HDAC target, histone H3 and a known class II HDAC target, α-tubulin. Unlike TSA, which increased the acetylation of both histone H3 and α-tubulin (Fig. 2A), MS-275 only increased the acetylation of histone H3 (Fig. 5A). To determine the effect of MS-275 on soleus muscle fiber atrophy, sections were incubated in wheat germ agglutinin to outline fiber membranes and the average muscle fiber CSA was calculated for each group. Representative images of soleus muscle cross-sections from each group are shown in Fig. 5B. Following 10 days of cast immobilization, soleus muscle fiber CSA decreased 41% in vehicle treated mice, which was attenuated by 39% in immobilized mice treated with MS-275 (Fig. 5C).

To determine whether MS-275 could also protect against the muscle weakness induced by immobilization, we subsequently measured soleus muscle force production, *in vitro*, in a subset of mice. Following 10 days of immobilization absolute force in the soleus muscle was decreased 55 – 61% across all stimulation frequencies ≥ 50 (Hz), demonstrating both submaximal and maximal force deficits in response to muscle disuse (Fig. 5D). However solei from immobilized mice treated with MS-275 showed a 31 – 35% attenuation of the force...
deficits observed in both submaximal and maximal absolute force across all stimulation frequencies $\geq 80$ (Hz) (Fig. 5D&E). Since skeletal muscle force production is a function of both muscle mass and the intrinsic contractile properties of the muscle, we subsequently normalized force to muscle weight and plotted the specific force-frequency relationship. In vehicle treated mice, a $25 - 31\%$ decrease in submaximal and maximal specific force was apparent across all stimulation frequencies $\geq 80$ (Hz), indicating significant contractile dysfunction. However this decrease in specific force was completely prevented in mice treated with MS-275 (Fig. 5F&G). Depressions in muscle force that are evident following normalization to muscle mass indicate impairments in contractile function. Therefore, our finding that MS-275 completely prevented the decrease in specific force in 10-day immobilized muscles suggests that class I HDACs contribute to contractile dysfunction during disuse. There are several potential mechanisms that may contribute to contractile dysfunction during muscle disuse, including (but not limited to) shifts in myosin isoforms (Caiozzo et al., 1998; Caiozzo et al., 1996; Campione et al., 1993; Fitts et al., 2000), alterations in calcium release and sensitivity (Fraysse et al., 2003), and the preferential degradation of myosin heavy chain (MHC) (Derde et al., 2012; Ochala et al., 2011) which is mediated through the FoxO-target gene MuRF1 (Clarke et al., 2007). Since we found that HDAC1 was necessary for both FoxO activation and MuRF1 gene expression, and MS-275 preferentially inhibits HDAC1, we hypothesized that the preservation of specific force may be related to the sparing of MHC. Thus, we isolated myofibrillar proteins from gastrocnemius muscles of control and 10-day immobilized mice treated with MS-275 or vehicle and measured the relative levels of MHC and actin from equal amounts of protein lysate. As shown in Fig. 5H, cast immobilization resulted in a significant reduction in the relative abundance of MHC, which was prevented in immobilized mice treated with MS-275. Although the levels of actin showed a slight decrease in content in response to immobilization, this difference was not statistically significant, and was unchanged by treatment with MS-275. Given that the ratio of myosin to actin can dictate contractile function, the sparing of myosin by MS-275 during immobilization could explain, in part, the protection from contractile dysfunction. In summary these findings collectively demonstrate that class I HDACs are critical regulators of the muscle atrophy program and contribute to both muscle fiber atrophy and contractile dysfunction during disuse.
DISCUSSION

The results of this study demonstrate that class I HDACs, and specifically HDAC1, are necessary for the muscle atrophy and contractile dysfunction associated with skeletal muscle disuse. We show that HDAC1-dependent atrophy during disuse requires its deacetylase activity, and is mediated, in part, through its activation of FoxO and the expression of several atrophy-related genes. Moreover, overexpression of HDAC1 in skeletal muscle was also sufficient to cause significant muscle atrophy in the absence of any physiological atrophy stimulus. Together these findings solidify the importance of HDAC1 in the regulation of the muscle atrophy program, and indicate that therapeutics targeting HDAC1 could be feasible countermeasures to impede muscle atrophy. Furthermore, since inhibition of class I HDACs during muscle disuse also rescued the decrease in skeletal muscle specific force, these data also suggest that targeting class I HDACs may preserve muscle function not only through sparing of muscle fiber size, but through additional mechanisms that directly regulate contractile function.

The class I HDAC proteins include HDACs 1, 2, 3 and 8. The class I HDAC inhibitor used in the current study, MS-275, inhibits the catalytic activity of HDAC1, 2 and 3, but has the greatest inhibitory effect on HDAC1 (Dokmanovic et al., 2007; Hu et al., 2003; Kennedy et al., 2013). From our experiments using both MS-275 and HDAC1, HDAC2 and HDAC3 expression plasmids, our findings pinpoint HDAC1 as a primary regulator of FoxO in skeletal muscle and as a key regulator of the atrophy program. However, since HDAC1 and HDAC2 are often found in complex together, HDAC1 may work in conjunction with HDAC2 to regulate FoxO activity. HDAC1/2 are generally thought of as global transcriptional repressors due to their role in the deacetylation of histones, which limits accessibility to gene promoters. However, gene array analyses of skeletal muscle from HDAC1/2 double knockout (dKO) mice show only modest changes in global gene expression when compared to muscles from control mice (Moresi et al., 2012). Based on this finding the authors concluded that the functions of HDAC1/2 in skeletal muscle are likely more specific than global transcriptional repression. In fact, they found that HDAC1/2 were necessary for the maintenance of normal skeletal muscle structure and function. This finding was linked to HDAC1/2-dependent induction of several autophagic genes, including atg5, gaparapl1, Lc3 and p62, and it regulation of autophagic flux. While flux through autophagy is required for cellular homeostasis during normal conditions, increased autophagic flux during catabolic conditions contributes to the muscle atrophy process (Mammucari et al.,
2007; Masiero and Sandri, 2010). Although we did not focus on autophagy in the current manuscript, our findings that HDAC1 is both sufficient and required for physiological muscle atrophy could be related to its role in the induction of autophagy. In relation to this, FoxO3a also induces autophagy and muscle atrophy, (Mammucari et al., 2007; Zhao et al., 2007), and we found that HDAC1 is both sufficient and required for FoxO activation. Thus, it seems plausible that the induction of atrophy by HDAC1 may involve FoxO-dependent induction of autophagy. In support of this, we found that HDAC1 was both sufficient and required for the induction of Lc3, which is a known FoxO target gene involved in autophagy. However, HDAC1 was also necessary for the increased gene expression of other FoxO target genes involved in the ubiquitin proteasome pathway (Atrogin-1 and MuRF1) and in the inhibition of protein synthesis (4E-BP1). Therefore, HDAC1 could promote muscle atrophy through increasing FoxO-dependent transcription of target genes involved in several different pathways which lead to increased protein turnover.

Recent data demonstrate that decreased acetylation of FoxO3a during atrophy conditions is a critical mechanism which activates FoxO3a-dependent transcription, and its ability to induce muscle fiber atrophy (Bertaggia et al., 2012; Senf et al., 2011). However, until now, the specific proteins regulating FoxO3a deacetylation in skeletal muscle were unknown. Our findings indicate that HDAC1 directly deacetylates FoxO, and is necessary for FoxO activation in response to skeletal muscle disuse. Interestingly, since we found that endogenous HDAC1 re-localizes from the nucleus to the cytosol in response to muscle disuse, we hypothesize that HDAC1 may deacetylate FoxO in the cytosolic compartment to facilitate the nuclear localization and transcriptional activation of FoxO.

Although this is the first evidence to support class I HDACs as activators of FoxO in skeletal muscle and in the induction of muscle atrophy, class I HDACs have previously been identified as therapeutic targets for muscular dystrophy (Colussi et al., 2008; Consalvi et al., 2011; Minetti et al., 2006). Class I HDACs associate with MyoD and repress MyoD-dependent transcription of target genes involved in satellite cell-mediated myofiber growth and regeneration (Puri et al., 2001), which is the rationale for the use of HDAC inhibitors in muscle dystrophy. Minetti et al. demonstrated that in mdx mice, inhibition of class I HDACs through MS-275 reduced muscle fibrosis and cellular infiltrate, increased muscle fiber CSA and enhanced the time to exhaustion during an exercise performance test (Minetti et al., 2006). These findings
were associated with the induction of follistatin, which is a MyoD target gene that promotes myoblast fusion and hypernucleation of myofibers through its negative regulation of myostatin. Interestingly, myostatin is elevated in some models of disuse muscle atrophy, though the importance of myostatin for disuse atrophy is controversial, with evidence to support (Murphy et al., 2011) and refute (Hamrick et al., 2007) its involvement. Therefore, although we did not measure follistatin levels in the current study, increased transcription of follistatin and subsequent repression of myostatin signaling following inhibition of class I HDACs could also be involved in the attenuation of disuse muscle fiber atrophy and weakness in the current study.

In conclusion, our data pinpoints HDAC1 as a primary regulator of FoxO in skeletal muscle that is both sufficient and required for skeletal muscle atrophy. Importantly, our findings also demonstrate that during muscle disuse, class I HDACs are necessary for not only fiber atrophy and the associated muscle weakness, but also contribute to additional cellular processes which cause contractile dysfunction independent from the loss of muscle mass. These findings collectively indicate that class I HDAC inhibitors are feasible countermeasures to inhibit muscle atrophy and weakness that may be effective in multiple conditions of muscle atrophy.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats weighing ~200 g, and C57BL/6 mice weighing ~20g, were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were maintained in a temperature controlled environment with a 12-h light/dark cycle, and provided a standard diet and water ad libitum. The University of Florida Institutional Animal Care and Use Committee approved all animal procedures.

Animal Models

The hind limbs of rats were bilaterally cast-immobilized 4 days following plasmid injection for either 3 or 7 days to induce muscle disuse and has been previously detailed (Senf et al., 2008). For experiments using MS-275, mice were treated (daily) with i.p. injections of either MS-275 (Selleckchem, Houston, TX, USA, 5mg/kg) or vehicle (1% DMSO) beginning 1 day prior to the 10 day period of weight bearing conditions or bilateral hind limb cast-
immobilization. For nutrient deprivation experiments, mice in the fed and nutrient deprived (ND) groups were treated (daily) with TSA (Sigma-Aldrich, St. Louis, MO, USA, 0.6mg/kg) or vehicle (sterile 1X PBS) via i.p. injection beginning 1 day prior to the 3-day period of control (fed) conditions or withholding of food (ND).

**Cell Culture Experiments**

C2C12 mouse skeletal muscle myoblasts were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in high glucose DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and antibiotics. Myoblasts were passaged at 70% confluency and were seeded on 0.1% gelatin coated tissue culture plates. Transient transfection was performed with FuGENE® HD Transfection Reagent (Promega Corp, Madison, WI, USA) when cells were at a density of 80% confluency. Twenty four hours following transfection, myotube differentiation was induced by replacing the growth medium with differentiation medium containing 2% horse serum and antibiotics in high glucose DMEM (Cellgro, Manassas, VA, USA). Medium was replenished every two days. Cells were maintained at 37°C with 5% CO₂ and 95% air. Three- to five-day-differentiated myotubes were used for experiments. For treatment with HDAC inhibitors, three day differentiated myotubes were either treated with vehicle (1% DMSO), 50 nM of TSA (Sigma-Aldrich), 5 µM of MC1568 or 5 µM of MS-275 (Selleckchem) for 18 hrs (reporter experiments) or 6 hrs (gene expression and localization experiments) in differentiation medium or HBSS (nutrient deprivation) as previously described (Senf et al., 2011). The relative localization of ectopic FoxO3a-DsRed and FoxO1-GFP was determined through calculating the average fluorescence (Mean Grey Value) in DAPI-stained (nuclear) regions versus non DAPI-stained (cytosolic) areas of transfected myofibers using ImageJ software and calculating the ratio of nuclear/cytosolic mean fluorescence.

**Plasmids**

The FoxO reporter plasmid containing three Forkhead-responsive element (3xFHRE) was obtained from Addgene (plasmid 1789) and was deposited by Dr. Michael Greenberg (Harvard University, Boston, MA, USA) and has been previously described (Brunet et al., 1999). The WT HDAC1 expression plasmid was obtained from Addgene (plasmid 11054) and was deposited by Dr. Ramesh Shivdasani (Harvard University, Boston, MA, USA) and has been previously
described (Tou et al., 2004). The d.n. HDAC1 expression plasmid was a gift from Dr. Yi Qiu (University of Florida, Gainesville, FL USA) and has previously been described (Qiu et al., 2011). The d.n. HDAC1 contains a histidine to alanine substitution at amino acid 141 which renders the enzyme catalytically inactive, yet retains its ability to interact with binding partners and deacetylase targets (Hassig et al., 1998; Mal et al., 2001). This deacetylase mutant is commonly referred to as a dominant negative due to its ability to block endogenous HDAC1-mediated events, which is presumed to occur through outcompeting endogenous HDAC1 for binding in protein complexes (Ito et al., 2002; Lei et al., 2010; Mal et al., 2001). The WT and d.n. HDAC 2 and 3 expression plasmids were gifts from Dr. Ed Seto (Moffit Cancer Center, Tampa, FL) and have been previously described (Juan et al., 2000; Qiu et al., 2011). The FoxO3a-6KQ construct was created via mutagenesis of FoxO3a-TM-6KQ [which was a gift from Dr. Marco Sandri (Venetian Institute of Molecular Medicine, Padova, Italy) and has been previously described (Bertaggia et al., 2012)] such that the Akt phosphorylation sites were restored. The d.n. Akt, atrogin-1-GL2, and d.n. FoxO-DsRed constructs have also been previously used and described (Reed et al., 2011a; Senf et al., 2008; Senf et al., 2011). pRL-TK-Renilla was purchased from Promega (Madison, WI, USA). Plasmid DNA was amplified and isolated from bacterial cultures using Endotoxin-Free Maxi or Mega Prep Kits (Qiagen, Valencia, CA, USA) and re-suspended in sterile filtered PBS for in vivo transfections, or Tris-EDTA (TE) buffer for transfections in culture as described previously (Senf and Judge, 2012).

**In vivo plasmid delivery**

Rats were acutely anesthetized and a small incision was made on the lateral side of the lower leg to expose the soleus muscle. Each solei was injected with 50 µl of sterile 1X PBS containing 10 µg of expression plasmid and/or 40 µg of reporter plasmid, followed by electroporation at 75 V/cm using an electric pulse generator (Electro Square Porator ECM 830: BTX, Hawthorne, NY, USA) as previously described (Senf et al., 2008).

**In vitro muscle contractile properties**

The methods and solutions used for the measurements of soleus muscle function have been previously described (Ferreira et al., 2010; Roberts et al., 2013b). Upon muscle removal, one end of the soleus was tied to a Dual-Mode Muscle Lever System (300C-LR, Aurora...
Scientific Inc, Aurora, Canada) and the other end was secured to a glass rod using 4.0 silk sutures. Following 20 minutes of thermo-equilibration, the soleus was placed at optimal length (Lo) and force-frequency measurements began. The soleus was stimulated with a supramaximal current (600-800 mA) with 0.25 ms pulses delivered through a stimulator (701C, Aurora Scientific Inc.) and a train duration of 500 ms. All data were recorded and analyzed using commercial software (DMC and DMA, Aurora Scientific Inc.).

**Histochemistry and CSA analyses**

To measure the muscle fiber cross-sectional area (CSA) 10 µm sections were taken from the midbelly of the soleus muscle using a Microm HM 550 cryostat (Microm International, Walldorf, Germany). Sections were incubated with Alexa Fluor 350-conjugated wheat germ agglutinin (Invitrogen) for 2 h and subsequently washed in PBS. Areas containing transfected fibers in muscle cross-sections were visualized and images captured using a Leica DM5000B microscope (Leica Microsystems, Wetzlar, Germany) and the Leica application suite, version 3.5.0 software. This software was also used to trace and measure muscle fiber CSA.

**Reporter Assays**

For reporter experiments tissue was harvested in Passive Lysis Buffer and a Modulus single tube multimode reader (Promega) was used to determine luciferase activity. For *in vitro* experiments, luciferase activity was determined by normalizing firefly luciferase activity to pRL-TK-Renilla luciferase activity using a Dual-Luciferase Reporter Assay (Promega) (Senf et al., 2008).

**RNA Isolation and qRT-PCR**

RNA was isolated from skeletal myotubes and skeletal muscle tissue using a TRIzol-based method as previously described (Senf et al., 2011). cDNA was generated from 1 µg of RNA using an Ambion® RETROscript® First Strand Synthesis Kit (Life Technologies, Grand Island, NY, USA) and was used as a template for quantitative RT-PCR, using a 7300 real-time PCR system (Applied Biosystems, Austin, TX, USA). Primers used for qRT-PCR were purchased from Applied Biosystems. Primers used for C2C12 muscle cells: *atrogin-1/MAFbx* (*Fbxo32, GenBank NM_026346.2*), *MuRF1* (*Trim63, GenBank NM_001039048.2*), *Gadd45a*
Primers used for rat muscle tissue: atrogin-1/MAFbx (GeneBank NM_133521.1), MuRF1 (GeneBank NM_080903.1), Gadd45a (GeneBank NM_024127.2), p21 (GeneBank NM_001106498.1), Lc3 (GeneBank NM_012823.1), Ctsl (GeneBank NM_001912.4), and 18s (GeneBank X03205.1). Quantification of gene expression was performed using the relative standard curve method and all data was normalized to the absolute control group and subsequently normalized to the gene expression of either Mrpl32 (C2C12s) or 18S (whole muscle).

**Western blotting and immunoprecipitation assays.**

Skeletal muscles were homogenized and processed for western blot analyses as previously performed and described (Senf et al., 2008). Primary antibodies for Acetyl-Histone H3 (#9677, Cell Signaling Technology, Boston, MA); Acetyl-α-tubulin (#3971, Cell Signaling Technology); Anti-α-tubulin (T6199, Sigma-Aldrich, St. Luis, MO, USA); FoxO1 (#9454, Cell Signaling Technology); phosphor-FoxO1 (Ser256) (#9461, Cell Signaling Technology); FoxO3a (SC-11351, Santa Cruz Biotechnology, Santa Cruz, CA, USA); phosphor-FoxO3a (Ser253) (#9466, Cell Signaling Technology); Histone H1 (SC-8030, Santa Cruz Biotechnology); SOD-1 (SC-11407, Santa Cruz Biotechnology); HDAC1 (SC-7872, Santa Cruz Biotechnology); HDAC2 (SC-7899); HDAC3 (SC-11417); Anti-acetyl-lysine (#05-515, Millipore, Billerica, MA, USA) were used per the manufacturer’s directions. Nuclear and cytosolic fractions were separated as previously described (Senf et al., 2009), and confirmation of successful fractionation was confirmed via Western blot for histone-H1 and SOD1. For the immunoprecipitation assays, 500 µg of protein were incubated overnight with either 4 µg of Anti-acetyl-lysine antibody, HDAC1 antibody or a non-specific IgG control antibody (#2729, Cell Signaling Technology) using the Catch and Release v2.0 Reversible Immunoprecipitation System (#17-500, Millipore) as used and described previously (Senf et al., 2011). The following day immunoprecipitated proteins were washed, eluted in denaturing buffer and boiled prior to western blot analyses.

**Contractile protein analyses**

For optimal extraction and processing of MHC and actin, muscles were homogenized in a
high-salt lysis buffer as previously shown (Cosper and Leinwand, 2012; Roberts et al., 2013b). Samples were mixed in Laemmli buffer (Bio-Rad, Hercules, CA, USA) and boiled, then 0.6µg of protein was loaded into 10% polyacrylamide gel (Criterion precast gels; Bio-Rad) and run at 200V for 50 minutes at 4°C. Coomassie brilliant blue (Thermo Fisher Scientific, Waltham, MA, USA) staining was used for protein visualization and an Odyssey Infrared Imaging system (LI-COR, Lincoln, NE, USA) was used to quantify the optical density of MHC. Levels of actin were determined using standard western blot analysis as previously described (Senf et al., 2008). The primary antibody for actin (1:1000, JLA20; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) was used according to the manufacturer’s directions. The Li-Cor Odyssey fluorescent detection system was used for visualization of actin following incubation with Alexa Fluor 680 fluorescent dye-conjugated secondary antibody (1:10,000, Invitrogen).

Statistical Analyses
All data were analyzed using a two-way ANOVA followed by Bonferroni post hoc comparisons or, when appropriate, a Student’s t-test (GraphPad Software, San Diego, CA, USA). All data are expressed as means ± s.e.m., and significance was established at p<0.05.

ACKNOWLEDGEMENTS
We thank the laboratory of Dr. Marco Sandri for providing the FoxO3a-TM-6KQ construct and Dr. Dan Ryder for his contribution in construct mutagenesis back to the FoxO3a-6KQ. This work was supported by U.S. National Institute of Arthritis and Musculoskeletal and Skin Diseases grants R01AR060209 (to A.R.J.) and R00HL098453 (to L.F.F.).
References


FIGURE LEGENDS

Figure 1. Inhibition of class I and II HDACs via TSA blocks induction of the muscle atrophy program. (A) FoxO-dependent luciferase reporter activity normalized to renilla luciferase from 3-day differentiated skeletal myotubes treated with TSA or vehicle during 18 hours of nutrient deprivation or control conditions. (B) FoxO-dependent luciferase reporter activity normalized to renilla luciferase from 4-day differentiated skeletal myotubes transfected as myoblasts with a d.n. Akt expression plasmid (or empty vector), and treated with TSA or vehicle 24 hours prior to harvest. (C-G) Three-day differentiated myotubes expressing ectopic FoxO3a-DsRed or FoxO1-GFP were nutrient deprived for 6 hours in the presence of TSA (or vehicle) and their cellular localization subsequently determined via fluorescent microscopy following fixation and incubation with DAPI to label cell nuclei. The mean fluorescence of FoxO3a-DsRed and FoxO1-GFP in nuclear and cytoplasmic compartments were calculated for each condition and are expressed as a ratio to indicate relative localization (C). Representative images from each condition are shown in D-G. (H) The relative mRNA levels of FoxO target genes, atrogin-1, MuRF1, Gadd45a, p21, Lc3 and 4e-bp1 in 3-day differentiated myotubes following 6 hours of nutrient deprivation (or control conditions) in the presence of TSA or vehicle. All data represent n = 3 and are reported as means ± s.e.m., normalized to the absolute control group. Significance was established at P < 0.05. *Significantly different from absolute control group. †Significantly different from vehicle within respective treatment group.

Figure 2. TSA treatment prevents skeletal muscle atrophy, in vivo. Mice were treated with TSA or vehicle (sterile PBS) for 3 days during normal (fed) conditions or during nutrient deprivation. (A) Representative Western blots for acetylated histone H3, acetylated α-tubulin and α-tubulin, as a control, from vehicle and TSA treated muscles. (B) Representative muscle cross sections incubated with wheat germ agglutinin to allow for visualization of muscle fiber membranes (blue). (C) Average plantaris muscle fiber CSA from all groups. Bars represent mean ± s.e.m for 6 muscles/group. Significance was established at P < 0.05. *Significantly different from absolute control group.

Figure 3. HDAC1 deacetylase activity is sufficient to cause skeletal muscle atrophy. (A and B) FoxO-dependent luciferase reporter activity (A) normalized to renilla from 3-day
differentiated skeletal myotubes treated with vehicle, MC-1568 (class II HDAC inhibitor) or MS-275 (class I HDAC inhibitor) for 6 hours during control condition or during nutrient deprivation and (B) from soleus muscles of rats transfected with either GFP, WT or d.n HDAC1, HDAC2 or HDAC3 expression plasmids. (C) Overexpression of HDAC2 and HDAC3, confirmed via Western blot. (D-F) Soleus muscles were transfected with GFP, WT HDAC1-GFP or d.n. HDAC1-GFP expression plasmids and harvested 7 days following transfection for (D) visualization of muscle fiber CSA, (E) measurement of mean fiber CSA, and (F) quantification of the relative mRNA levels of atrogin-1, MuRF1, Ctsl and Lc3 normalized to 18s. (G) Relative luciferase activity from soleus muscles transfected with a luciferase reporter plasmid driven by the atrogin-1 promoter, plus expression plasmids for GFP or WT HDAC1 plus empty vector (EV), WT FoxO3a or FoxO3a 6KQ. (H) The ability of HDAC1 to interact with and regulate the acetylation of endogenous FoxO1 and FoxO3a was determined in soleus muscles injected with GFP, WT HDAC1-GFP or d.n. HDAC1-GFP expression plasmids. Equal amounts of protein extract were incubated with either an acetyl-lysine antibody to immunoprecipitate (IP) total acetylated proteins, a HDAC1 antibody to IP HDAC1 protein complexes, or IgG as a negative control. Precipitated proteins were subjected to PAGE and immunoblotted for either FoxO3a or FoxO1. Experiments were independently repeated three times. Western blots were also performed on equal amounts of whole muscle lysates from the same samples using antibodies for phosphorylated and total FoxO1 and FoxO3a. (I) Representative muscle cross-sections and (J) mean muscle fiber CSA of soleus muscles co-transfected with expression plasmids for DsRed + GFP, DsRed + WT HDAC1-GFP, d.n. FoxO + GFP or d.n FoxO + WT HDAC1-GFP. Scale bars = 50 µm. All data represent mean ± s.e.m. for 6 muscles/group. Significance was established at P < 0.05. *Significantly different from absolute control group. †Significantly different from control within respective treatment group. aSignificantly different from EV + WT HDAC1.

Figure 4. HDAC1 deacetylase activity is required for skeletal muscle atrophy. (A) Representative muscle cross-sections and (B) mean fiber CSA from 7-day immobilized soleus muscles injected with GFP or d.n. HDAC1-GFP. The dashed line represents the average CSA of weight bearing GFP-transfected fibers. Scale bars = 50 µm. (C) Relative luciferase activity driven by a FoxO-dependent reporter plasmid and (D) relative mRNA levels of atrogin-1,
MuRF1, Ctsl and Lc3 normalized to 18s from the soleus of 3-day immobilized rats transfected with GFP, WT or d.n. HDAC1-GFP expression plasmids. All data from immobilized muscles are normalized to data collected from the absolute control group (weight bearing, GFP) to reflect the raw data. All data represent mean ± s.e.m. for 6 muscles/group. Significance was established at P < 0.05. *Significantly different from absolute control group. †Significantly different from control within respective treatment group. (E) Phosphorylated and total FoxO1 and FoxO3a protein levels from 7-day immobilized soleus muscles injected with GFP, WT or d.n. HDAC1-GFP as measured via Western blot. (F) Endogenous HDAC1 protein levels in whole muscle lysate or cytosolic and nuclear fractions in weight bearing and immobilized muscles were measured via Western blot. Included are Western blots for Histone H1 and SOD1 on nuclear and cytosolic fractions, demonstrating successful separation of the nuclear and cytosolic fractions.

Figure 5. **MS-275 prevents contractile dysfunction and attenuates muscle fiber atrophy associated with muscle disuse.** Mice were treated with MS-275 or vehicle for 10 days during normal weight bearing conditions or 10 days of muscle disuse induced via hind limb cast-immobilization. (A) Representative Western blots for acetylated histone H3, acetylated α-tubulin and α-tubulin (as loading control), from vehicle and MS-275 treated muscles (B) Representative soleus muscle cross sections incubated with wheat germ agglutinin to allow for visualization of muscle fiber membranes (blue). Scale bar = 50 µm. (C) The average muscle fiber CSA from each group. (D-G) Muscle force-generating capacity of soleus muscles from each group, including (D) absolute force-frequency relationship, (E) maximal absolute force, (F) specific force frequency relationship (force normalized to muscle weight), and (G) maximal specific force. (H) The relative protein content of myosin heavy chain was determined via polyacrylamide gel electrophoresis. All data represent mean ± s.e.m. for 6 muscles/group. Significance was established at P < 0.05. *Significantly different from vehicle treated, weight bearing group. †Significantly different from vehicle within respective treatment group.
Figure 1

(A) 3xFHRE reporter (Relative luciferase activity) for Control and Nutrient deprived conditions. Bars represent Vehicle and TSA treatments.

(B) 3xFHRE reporter (Relative luciferase activity) for Control and d.n. Akt conditions. Bars represent Vehicle and TSA treatments.

(C) Nuclear/Cytoplasmic Ratio for Control and Nutrient deprived conditions. Bars represent Vehicle and TSA treatments for FoxO3a and FoxO1.

(D-G) Immunofluorescence images showing the localization of Atrogin-1, MuRF1, Gadd45a, p21, Lc3, and 4e-bp1 under Control and Nutrient deprivation conditions.

(H) Relative mRNA levels normalized to Mrpl32 for Vehicle and TSA treatments for Atrogin-1, MuRF1, Gadd45a, p21, Lc3, and 4e-bp1 under Control and Nutrient deprivation conditions.
Figure 2

B

Vehicle
TSA

Fed
Nutrient deprived

Ac-Histone H3
Ac-α-tubulin
α-tubulin

C

Fed
Nutrient deprived

Muscle fiber CSA (μm²)

Vehicle    TSA

*
**Figure 3**

**A**

3xFHRE reporter (Relative luciferase activity)

- Control
- Nutrient deprived

Vehicle, MC-1568, MS-275

**B**

3xFHRE reporter (Relative luciferase activity)

- GFP
- WT
- d.n.

HDAC1, HDAC2, HDAC3

**C**

- WT, d.n.

IB: HDAC2
IB: α-tubulin
IB: HDAC3
IB: α-tubulin

**D**

GFP
WT HDAC1-GFP
d.n. HDAC1-GFP

**E**

Muscle fiber CSA (μm²)

- GFP
- WT HDAC1
- d.n. HDAC1

**F**

Relative mRNA level (normalized to 18S)

- Atrogin-1
- MuRF1
- Ctsl
- Lc3

**G**

Atrogin-1 promoter reporter (Relative luciferase activity)

- GFP
- WT HDAC1

**H**

IP: Ac-k
IP: HDAC1
IP: α-tubulin

**I**

DsRed
d.n. FoxO-DsRed

**J**

Muscle fiber CSA (μm²)

- GFP
- WT HDAC1

DsRed, d.n. FoxO-DsRed

- d.n. WT HDAC1

IB: α-tubulin
Figure 4

A) GFP and d.n.HDAC1-GFP

B) Muscle fiber CSA (μm²)

C) 3xFHRE reporter (Relative luciferase activity)

D) Relative mRNA level (normalized to 18S)

E) Whole cell Lysate

F) Weight Bearing  Imm

HDAC1
α-tubulin
HDAC1
Histone H1
SOD1
HDAC1
Histone H1
SOD1
Figure 5

A

<table>
<thead>
<tr>
<th>Vehicle</th>
<th>MS-275</th>
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<td>[Image of Western Blots]</td>
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- Ac-Histone H3
- Ac-α-tubulin
- α-tubulin

B

- Vehicle
- MS-275

- Weight Bearing Immobilized

- [Images of Immunofluorescence Staining]

C

- Muscle Fiber CSA (μm²)

- [Graph showing comparison between Vehicle and MS-275]

D

- Weight Bearing
- Weight Bearing + MS-275
- Immobilized
- Immobilized + MS-275

Graph showing the relationship between Frequency (Hz) and Absolute force (mN) for Vehicle and MS-275.

E

- Absolute force (mN)

- Graph showing comparison between Vehicle and MS-275.

F

- Specific Force (N/g)

- Graph showing comparison between Vehicle and MS-275.

G

- Specific Force (N/g)

- Graph showing comparison between Vehicle and MS-275.

H

- MHC Band Intensity

- [Graph showing comparison between Vehicle and MS-275]