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The methyltransferase SMYD3 mediates the recruitment of transcriptional cofactors at the myostatin and c-Met genes and regulates skeletal muscle atrophy

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Elucidating the epigenetic mechanisms underlying muscle mass determination and skeletal muscle wasting holds the potential of identifying molecular pathways that constitute possible drug targets. Here, we report that the methyltransferase SMYD3 modulates myostatin and c-Met transcription in primary skeletal muscle cells and C2C12 myogenic cells. SMYD3 targets the myostatin and c-Met genes and participates in the recruitment of the bromodomain protein BRD4 to their regulatory regions through protein–protein interaction. By recruiting BRD4, SMYD3 favors chromatin engagement of the pause–release factor p-TEFb (positive transcription elongation factor) and elongation of Ser2-phosphorylated RNA polymerase II (PolIISer2P). Reducing SMYD3 decreases myostatin and c-Met transcription, thus protecting from glucocorticoid-induced myotube atrophy. Supporting functional relevance of the SMYD3/BRD4 interaction, BRD4 pharmacological blockade by the small molecule JQ1 prevents dexamethasone-induced myostatin and atrogene up-regulation and spares myotube atrophy. Importantly, in a mouse model of dexamethasone-induced skeletal muscle atrophy, SMYD3 depletion prevents muscle loss and fiber size decrease. These findings reveal a mechanistic link between SMYD3/BRD4-dependent transcriptional regulation, muscle mass determination, and skeletal muscle atrophy and further encourage testing of small molecules targeting specific epigenetic regulators in animal models of muscle wasting.

[Keywords: SMYD3; BRD4; muscle atrophy; myostatin]

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Establishment and maintenance of skeletal muscle mass is determined by the dynamic balance between anabolic and catabolic pathways that promote increased synthesis of muscle proteins or their degradation, respectively (Glass 2010a). In physiological conditions, extracellular cues mediated by insulin growth factors, myostatin, and cytokines continuously fine-tune the rate of protein synthesis and degradation. Skeletal muscle atrophy is the unfavorable outcome of an imbalance in the two opposing signaling networks and occurs in a wide variety of settings, such as disuse, denervation, cancer cachexia, sarcopenia, and glucocorticoid administration. Signaling molecules such as myostatin, catabolic steroids, and proinflammatory cytokines have been identified as factors contributing to the atrophic process in skeletal muscle (Glass 2010b). However, the molecular mechanisms that control the expression of these key regulators of muscle mass are poorly understood. Cellular pathways activated by these molecules converge on transcription factors that are modified following catabolic/anabolic responses and translate extracellular signals into transcriptional programs. It is now recognized that transcription factors cooperate with chromatin modifiers to establish and maintain transcription networks throughout development, tissue homeostasis, and disease (Portela and Esteller 2010; Braun and Gautel 2011; Greer and Shi 2012). While the contribution of epigenetic modifiers and chromatin remodeling factors to skeletal muscle commitment and differentiation has been well documented (Perdiguerro et al. 2009; Albini and Puri 2010; Sartorelli...
and Juan 2011), their role in skeletal muscle atrophy has only recently begun to be investigated (Moresi et al. 2010) and remains largely unknown. Identifying epigenetic mechanisms underlying skeletal muscle atrophy is of interest because the reversibility and plasticity of epigenetic modifications can be exploited to design novel strategies for therapeutic intervention. Relevant for therapeutic purposes are recent reports on the beneficial effects of blocking the myostatin/activin signaling pathways in a mouse model of cancer cachexia (Benny Klimek et al. 2010; Zhou et al. 2010) and to counter muscle wasting in muscular dystrophies (Bartoli et al. 2007; Qiao et al. 2008; Wagner et al. 2008; Krivickas et al. 2009; Qiao et al. 2009).

Members of the SMYD family of histone methyltransferases are potential regulators of skeletal muscle mass, as they control gene expression in cardiac and skeletal muscle owing to their restricted or more abundant expression in myogenic tissues (Gottlieb et al. 2002; Brown et al. 2006; Thompson and Travers 2008). To date, five members of the SMYD family have been identified: SMYD1–5 (Brown et al. 2006). They share a distinctive architecture of their SET domain, which is split into two parts by a MYND [myeloid translocation protein 8, nervy, DEAF-1] domain followed by a cysteine-rich post-SET domain. The MYND domain is a cysteine-rich structure that mediates protein–protein interactions and DNA binding [Xu et al. 2011] and is present in proteins implicated in embryonic development and cancer progression. SMYD3 has been reported to promote H3K4me3 and, more recently, H4K5me1 (Hamamoto et al. 2004; Van Aller et al. 2012). However, the relevance of SMYD3-dependent epigenetic modifications to gene expression regulation has not been addressed.

Here, we report that SMYD3 regulates expression of myostatin, a member of the TGF-β family and a negative regulator of skeletal muscle growth during embryonic development (Amthor et al. 2006; Manceau et al. 2008) and muscle homeostasis (Trendelenburg et al. 2009). In primary mouse myoblasts and C2C12 myoblasts, numerous studies have provided evidence that myostatin signals via the canonical Smad2/3 pathway and inhibits myogenesis progression through down-regulation of differentiation-related genes such as MyoD and myogenin as well as their downstream targets [Langley et al. 2002; Rios et al. 2002; Jouliou et al. 2003; Yang et al. 2006; Huang et al. 2007]. In addition, myostatin plays a distinct role in adult myofibers, where it regulates muscle mass and promotes the activation of the FoxO1/3 pathway, leading to atrogene transcription and muscle mass loss in conditions related to muscle wasting (Glass 2010b; Braun and Gautel 2011). SMYD3 also controls transcription of c-Met, the hepatocyte growth factor receptor [Zou et al. 2009], involved in migration of muscle satellite cells and muscle atrophy (Anastasi et al. 1997; Gal Levi et al. 1998; Crepaldi et al. 2007).

Our findings reveal that SMYD3 favors chromatin recruitment of the bromodomain protein BRD4 and the positive transcription elongation factor complex [p-TEFb], thus facilitating early steps of transcriptional elongation. Consistently, the BRD4 inhibitor JQ1 negatively regulates expression of myostatin and atrophy-related genes and counteracts dexamethasone [Dex]-induced atrophy in cultured myotubes. Importantly, SMYD3 reduction blunted Dex-induced muscle atrophy in the animal. Thus, we identified SMYD3 as an effector of skeletal muscle atrophy and have begun to clarify the molecular mechanisms involved in its proatrophic function.

Results

**SMYD3 determines myotube size through regulation of myostatin expression**

Given its function in regulating zebrafish skeletal myogenesis [Fujii et al. 2011], we investigated the role of SMYD3 in a cell culture model of mammalian myogenesis. SMYD3 expression was reduced in C2C12 cells by transducing them with a specific shRNA. SMYD3-depleted C2C12 myotubes showed a higher percentage of myosin heavy chain (MHC)-positive multinucleated myotubes [Fig. 1A,B,D, Supplemental Fig. S1A–C] and simultaneously revealed an increase in average myotube diameter ($P$-value 0.043) [Fig. 1C]. Analogously, mouse primary skeletal muscle cells transfected with a chemically modified siRNA targeting SMYD3 showed increased ability to form MHC-positive multinucleated myotubes [Fig. 1E]. Formation of C2C12 myotubes with increased MHC reactivity and diameter was prevented by retroviral expression of human SMYD3 [hSMYD3], which is refractory to murine shRNA interference [Supplemental Fig. S1D–F], excluding off-target effects of the Sh-SMYD3. Increased fusion index and myotube diameter upon SMYD3 reduction may be the result of myotube hyperplasia, hypertrophy, or a combination of the two processes.

With the aim of defining the molecular pathways leading to increased myotube size in Sh-SMYD3 cells, we chose to investigate the impact of SMYD3 depletion on Akt/protein kinase B [Akt]. This choice was motivated by observations indicating that Akt activation represents a critical event during muscle hypertrophy [Bodine et al. 2001] and that constitutive Akt activation in adult skeletal muscle is sufficient to induce muscle hypertrophy [Lai et al. 2004]. While total Akt levels were not appreciably affected, Ser473-phosphorylated (activated) Akt was increased in both Sh-SMYD3 C2C12 myoblasts [Fig. 1F]. Several lines of evidence have linked the Akt pathway to myostatin, a crucial negative regulator of muscle mass in vertebrates. Hypertrophic skeletal muscles of myostatin knockout mice display higher activation of Akt signaling [Morisette et al. 2009; Lipina et al. 2010], and in vitro studies have documented negative modulation of the Akt signaling pathway upon myostatin delivery [McFarlane et al. 2006; Morisette et al. 2009; Trendelenburg et al. 2009]. Based on Akt activation and the observation that the Sh-SMYD3 myotube phenotype is reminiscent of that caused by myostatin knockdown [Morisette et al. 2009; Trendelenburg et al. 2009], we evaluated whether SMYD3 depletion may
affect myostatin transcription. As shown in Figure 1G, myostatin transcripts were significantly reduced in mouse primary skeletal muscle cells depleted for SMYD3 in both proliferating and differentiating conditions (Fig. 1G). Similarly, myostatin protein and mRNA levels were substantially diminished in Sh-SMYD3 C2C12 myoblasts and myotubes (Fig. 1H; Supplemental Fig. S1I). Culturing Sh-SMYD3 C2C12 cells in the presence of recombinant myostatin [at final concentration of 100 ng/mL] reduced MHC expression and diameter, thus rendering them comparable in size with the control (Fig. 1I). Taken together, these data suggest that SMYD3 regulates myostatin mRNA and protein levels in both C2C12 and primary skeletal muscle cells and controls the size of C2C12 cells in a myostatin-dependent fashion by influencing either hypertrophy or hyperplasia.

**Figure 1.** SMYD3 depletion promotes myotube hypertrophy and affects myostatin expression levels. (A) MHC immunostaining (green) of C2C12 cells transduced with control or Sh-SMYD3 retroviruses and induced to differentiate for 48 h. Nuclei were visualized with DAPI (blue). Error bars represent standard deviation (SD) \( n = 3 \). (\*\*) \( P \)-value < 0.001; (\*) \( P \)-value < 0.05. Bar, 50 μm. (B) Mean myotube diameter of Sh-SMYD3 and Sh-Scramble myotubes was quantified. Error bars represent SD \( n = 3 \). (\*) \( P \)-value < 0.05. (C) Immunoblot of SMYD3, MHC, and tubulin in C2C12 transduced with control or Sh-SMYD3 retroviruses at different stages of differentiation. (D) Primary skeletal muscle cells were isolated from 18-d-old mice and transfected with a double-strand RNAi oligonucleotide carrying a sequence targeting SMYD3 or its scramble sequence, and, after 24 h, cells were induced to differentiate for 36 h before immunostaining with MHC antibody (green). Nuclei were visualized by DAPI staining (blue). Bar, 100 μm. (E) Protein levels of SMYD3, phosphorylated Akt-Ser473, and total Akt were quantified by Western blot using whole-cell C2C12 extracts transduced with Sh-SMYD3 and Sh-Scramble retroviruses. The ratio of phospho-Akt/total Akt is provided after quantification of band intensity. (F) Myostatin and SMYD3 in whole-cell extract of C2C12 cells transduced with Sh-SMYD3 and control retroviruses, cultured in growth medium, and induced to differentiate for 20 h. Vinculin served as a loading control. (G) C2C12 cells were transduced with Sh-SMYD3 or Sh-Scramble retroviruses and allowed to differentiate for 48 h in the presence of recombinant myostatin [100 ng/mL]. Cells were fixed with 4% paraformaldehyde and immunostained with antibodies against MHC (green). Nuclei were visualized by DAPI staining (blue). Bar 50 μm. (H) Mean myotube diameter of Sh-SMYD3 and Sh-Scramble myotubes treated with recombinant myostatin or vehicle, as in I. Error bars represent SD \( n = 3 \). (\*) \( P \)-value < 0.05

**SMYD3 is recruited to regulatory regions of the myostatin and c-Met genes and favors engagement of Ser2-phosphorylated RNA polymerase II (PolII)**

Inspection of the myostatin gene sequence revealed the presence of two putative SMYD3-binding motifs within the first and second intron and of a third motif positioned within the 3' untranslated region (UTR) (Fig. 2A). Employ-
ing chromatin immunoprecipitation (ChIP) assays, we could not detect significant recruitment of SMYD3 to the consensus sites situated within either the second intron (probe 4) or the 3' UTR of the myostatin gene (probe 5) (Fig. 2B). However, SMYD3 enrichment was observed at a region encompassing an evolutionarily conserved SMYD3 consensus site in the first intron (probe 3). In addition, SMYD3 binding was detected at the myostatin promoter (probe 1), which, in contrast, does not contain canonical SMYD3 consensus motifs. A chromatin region located halfway between the myostatin promoter and the first intron was also enriched for SMYD3 binding (probe 2). These findings are consistent with a direct and indirect modality of SMYD3 chromatin recruitment (Kim et al. 2009). To further characterize the SMYD3-bound myostatin regions, we employed p300 and histone H3K4me1 antibodies in ChIP to identify potential enhancer regions (Heintzman et al. 2007). The myostatin first intron (probe 3) was enriched for both p300 and H3K4me1, whereas the 3' UTR (probe 5) was not significantly enriched for either mark (Supplemental Fig. S2A,B). As expected, the SMYD3-bound promoter region (probe 1) was occupied by p300 but was not significantly enriched for H3K4me1 (Supplemental Fig. S2A,B). These results indicate that the first intron of myostatin may host an active enhancer (Creyghton et al. 2010; Rada-Iglesias et al. 2011). The engagement of SMYD3 at regulatory regions and the reduced myostatin transcription upon Sh-SMYD3 interference prompted us to evaluate potential effects of SMYD3 on RNA PolII recruitment. PolII phosphorylation on the Ser5 C-terminal domain (CTD) is a highlight of transcriptional initiation, while PolII phosphorylation of Ser2 is a signature of transcription elongation (Brookes and Pombo 2009). By ChIP, Ser5-phosphorylated RNA PolII (PolII Ser5P) recruitment was moderately increased at the myostatin promoter and regions encompassing probes 2 and 3 in SMYD3-depleted cells compared with control cells. In contrast, PolII Ser2P engagement was decreased to background levels when SMYD3 was knocked down. PolII Ser2 and PolII Ser5 were unchanged at control active (NF-YA)
and silent β-globin promoters (Fig. 2C). To extend our findings to other SMYD3 targets, we chose to analyze c-Met, whose transcription is regulated by SMYD3 (Zou et al. 2009). c-Met plays a role in embryonic myogenesis and skeletal muscle regeneration (Anastasi et al. 1997; Gal Levi et al. 1998), and similar to myostatin, conditional activation of c-Met in skeletal muscle induces atrophy (Crepaldi et al. 2007). In both C2C12 myoblasts (Fig. 2D) and primary skeletal muscle cells (Supplemental Fig. S2C), c-Met transcripts decreased when SMYD3 was reduced. Previous studies have implicated the c-Met promoter in SMYD3-mediated regulation of the c-Met gene (Zou et al. 2009). Inspection of the c-Met promoter sequence revealed the presence of a SMYD3-binding site. Consistently, ChIP experiments documented SMYD3 enrichment at this region (Fig. 2E). Chromatin recruitment of both SMYD3 and PolII-Ser2P were reduced following SMYD3 depletion, while PolII-Ser5P was unaltered (Fig. 2E). Collectively, these data suggest that SMYD3 does not affect assembly of the RNA PolII preinitiation complex but is rather involved in the chromatin recruitment of elongating PolII-Ser2P at both the myostatin and c-Met genes.

**SMYD3 regulates myostatin transcription by favoring engagement of the bromodomain protein BRD4 and the p-TEFb-CDK9 subunit**

The p-TEFb complex (CycT1/CDK9) mediates PolII-Ser2 phosphorylation during the early elongation steps and can be recruited to promoter regions and the gene body by the bromodomain protein BRD4 (Brés et al. 2008). We therefore asked whether recruitment of BRD4 and p-TEFb was itself affected by SMYD3. Antibodies against the p-TEFb subunit CDK9 and BRD4 were employed in ChIP, which revealed that recruitment of both CDK9 and BRD4 was impaired upon SMYD3 depletion at the promoter and first intron of the myostatin gene (Fig. 3A) as well as the promoter of the c-Met gene (Fig. 3B). CDK9 and BRD4 proteins were not affected by SMYD3 reduction (Supplemental Fig. S3A). To evaluate whether SMYD3 may

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**Figure 3.** SMYD3 interacts with BRD4 and recruits BRD4 and pTEFb to the myostatin regulatory regions. (A) ChIP assay was performed with normal rabbit IgG and antibodies recognizing BRD4 and CDK9 on chromatin derived from control and Sh-SMYD3 proliferating C2C12 myoblasts. The location of the myostatin probe sets is indicated in Figure 2A. The NF-YA promoter region was amplified as a control gene. n = 3; (* P < 0.05. (B) ChIP, performed as in A, was analyzed for the c-Met promoter. (C) Protein complexes were immunoprecipitated from HEK293T cells overexpressing Flag-SMYD3 with an antibody recognizing either IgG or Flag. The immunoblot was detected with antibodies recognizing BRD4 and the Flag epitope. (D) Immunoprecipitation assay of nuclear extracts (NEs) obtained from Sh-Scramble cells. Protein complexes were immunoprecipitated using either IgG or an antibody against BRD4. Sh-SMYD3 NEs were loaded after immunoprecipitated samples. The Western blot was detected with antibodies against BRD4 and SMYD3. (E) In vitro reconstituted nucleosomes assembled at the myostatin promoter were incubated with nuclear extracts derived from C2C12 infected with either Sh-Scramble or Sh-SMYD3 retroviral supernatants. Biotinylated nucleosomes were captured with streptavidin, and interacting proteins were analyzed by immunoblot with the indicated antibodies.
associate with BRD4, extracts from HEK293T cells transiently transfected with Flag-SMYD3 plasmid were immunoprecipitated with anti-Flag-agarose beads, and the precipitated material was immunoblotted with an antibody recognizing BRD4. The outcome of this experiment indicated that endogenous BRD4 interacted with ectopically expressed SMYD3 [Fig. 3C]. As a control, we showed that the unrelated Flag-tagged Ezh2 protein was not able to associate with BRD4 [Supplemental Fig. S3B]. In addition, endogenous SMYD3 associated with BRD4 in C2C12 whole-cell extracts [Fig. 3D]. In immunoprecipitation experiments performed with HEK293T extracts overexpressing Flag-SMYD3, we also uncovered an interaction between SMYD3 and both PolII Ser5P and PolII Ser2P [Supplemental Fig. S3C,D]. These results not only confirmed a previous report describing an interaction between SMYD3 and RNA PolII [Hamamoto et al. 2004], but also revealed an association between SMYD3 and PolII isoforms that participate in the early steps of transcription.

In addition, endogenous SMYD3 associated with BRD4 in C2C12 whole-cell extracts [Fig. 3D]. We next tested the ability of the reconstituted bromodomain-containing (BD1 and BD2 [BRD4_1–470]) to associate with His-SMYD3, while the remaining BRD4 regions failed to do so [Fig. 4A, anti-His, middle panel]. We also generated four Flag-tagged SMYD3 deletion constructs and tested their capability to associate with His-BRD4 [Fig. 4B]. The region encompassing the first 96 amino acids of SMYD3 mediated the interaction with BRD4. Conversely, the N-terminal SMYD3 mutants [SMYD3_111–428 and SMYD3_219–428] did not associate with BRD4 [Fig. 4B, anti-His, middle panel]. Interestingly, the N-terminal region of SMYD3 encompasses the MYND domain, which is a protein–protein interaction module [Sirinupong et al. 2010].

To investigate whether the SMYD3 N-terminal region is relevant for SMYD3 function, we performed rescue experiments in Sh-SMYD3 and control cells [Supplemental Fig. S4A] with a retrovirus carrying either full-length or a SMYD3 mutant incapable of interacting with BRD4 [SMYD3_111–428]. While SMYD3 wild type could rescue the phenotype of Sh-SMYD3 cells, the SMYD3_111–428 mutant [containing the methylase SET domain] did not revert Sh-SMYD3 cell hypertrophy [Fig. 4C,D, Supplemental Fig. S4B–D]. These results suggest that the sole SET methyltransferase domain of SMYD3 is not sufficient to induce the atrophic response. We conclude that the N-terminal region of SMYD3 mediates BRD4 interaction and is crucial in regulating myotube size.

**SMYD3 expression is increased in Dex-induced atrophy of C2C12 myotubes**

The experiments presented above indicate a role of SMYD3 in regulating skeletal muscle cell differentiation and myotube size. To directly test whether SMYD3 may be involved in skeletal muscle atrophy, we employed a cell culture model where differentiated C2C12 myotubes are treated with the synthetic glucocorticoid Dex, leading to phenotypic reduction of myotube size [Clarke et al. 2007]. C2C12 cells were allowed to form myotubes and were then treated with Dex [100 μM] for 24 h. Transcript levels of SMYD3 increased following Dex treatment, while mRNA levels of other SMYD family members were unaffected except for a modest SMYD4 increase [Fig. 5A]. SMYD3 protein levels were also increased by Dex [Fig. 5B]. As expected, expression of myostatin and the atrogenes atrogin-1 [Fig. 5B,C] and MuRF1 was induced by Dex treatment [Fig. 5C].

**SMYD3 inhibition or BRD4 pharmacological blockade prevents myostatin and atrogene induction and counteracts Dex-induced atrophy in cultured myotubes**

The critical function exerted by myostatin in skeletal muscle atrophy [Ma et al. 2003; Glass 2010a] further prompted us to evaluate SMYD3's role in a setting of skeletal muscle atrophy. As previously described [Ma et al. 2001; McFarlane et al. 2006] and as reported in Figure 5, myostatin levels were induced by Dex treatment, and this phenomenon was accompanied by up-regulation of the...
Figure 4. The N-terminal regions of BRD4 and SMYD3 mediate protein interaction. (A, top panel) Schematic diagram of BRD4 constructs. Protein complexes were immunoprecipitated with M2-Flag beads or IgG from HEK293T cells cotransfected with wild-type Flag-SMYD3 and His-tagged BRD4 deletion mutants. Inputs are shown in the bottom panel. Immunoblotting was performed with HRP-conjugated antibodies recognizing the His and the Flag epitope for BRD4 and SMYD3, respectively. (B, top panel) Schematic diagram of SMYD3 constructs. Total extracts of HEK293 overexpressing the full-length His-BRD4 and SMYD3 truncated mutants were immunoprecipitated with M2-Flag beads or IgG, and the immunoblots were detected using anti-His and anti-Flag antibodies for BRD4 and SMYD3, respectively. Inputs are shown in the bottom panel. Immunoblotting was performed with HRP-conjugated antibodies recognizing the His and the Flag epitope for BRD4 and SMYD3, respectively. (C) MHC immunostaining (green) of Sh-Scramble and Sh-SMYD3 C2C12 cells transduced with empty vector or SMYD3 and SMYD3_111-428 retroviruses induced to differentiate for 48 h. Nuclei were visualized with DAPI (blue). Bar, 50 μm. (D) Immunoblot of MHC, Flag, and GAPDH of Sh-Scramble and Sh-SMYD3 C2C12 cells transduced with empty vector or SMYD3 and SMYD3_111-428 retroviruses induced to differentiate for 48 h. Numbers represent quantification of MHC band intensity.

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atrophy-related genes atrogin-1 and MuRF1. However, the transcript levels of myostatin, atrogin-1, and MuRF1 failed to increase in C2C12 cells depleted of SMYD3 following Dex treatment (Fig. 6A). These data suggested that SMYD3 is involved in the transcriptional up-regulation of myostatin and atrophy-related genes in Dex-induced skeletal muscle atrophy.

To evaluate whether the role of BRD4 in regulating myostatin transcription described in Figure 3 is functionally relevant also for myostatin expression during muscle atrophy, we employed the recently developed small molecule JQ1. JQ1 is a selective inhibitor of bromodomain and extraterminal (BET) family members; it occupies the acetyl-binding pockets of bromodomains and determines BRD4 dissociation from chromatin and loss of downstream signaling events to PolII (Delmore et al. 2011). We reasoned that JQ1 could interfere with myostatin transcription and hence may counteract skeletal muscle atrophy induced by Dex. Indeed, C2C12 myotubes treated with Dex and exposed to JQ1 (50 nM) showed decreased levels of myostatin, MuRF1, and atrogin-1 and increased MHC expression (Fig. 6B,C). Myoblasts cultured in the presence of JQ1 and prompted to differentiate gave rise to myotubes with larger diameter and increased levels of MHC when compared with control cells (Fig. 6C–F) and were resistant to the Dex-induced reduction of cell diameter (Fig. 6D,E). The BRD4 target c-myc has been previously shown to be down-regulated by JQ1 administration in several cellular models and served as an internal control (Supplemental Fig. S5A). These in vitro results indicate that pharmacological BRD4 inhibition prevents transcription of myostatin and atrophy-related genes, thus effectively counteracting Dex-induced myotube atrophy.

**SMYD3 depletion prevents Dex-induced in vivo skeletal muscle atrophy**

To determine whether SMYD3 is implicated in muscle atrophy in vivo, we induced skeletal muscle atrophy by Dex administration in C57BL/6 mice (n = 8 saline, n = 9 Dex) and reduced SMYD3 levels by electroporation with a plasmid carrying a shRNA targeting SMYD3 in the tibialis anterior (TA). A scrambled sequence was injected in the contralateral TA muscle to serve as a control. In agreement with SMYD3’s expression profile in C2C12 myotubes treated with Dex (Fig. 5A), endogenous SMYD3 mRNA levels were also increased by Dex exposure in TAs (Fig. 7A), while SMYD1, SMYD2, SMYD4, and SMYD5 transcripts were unaltered or negatively affected by Dex administration (Supplemental Fig. S6). As expected,
Smyd3 mRNA levels were reduced in muscle electroporated with Sh-Smyd3 [Fig. 7A], and Dex delivery reduced mouse body mass by 14.8% [Fig. 7B]. Notably, we found that SMYD3 knockdown increased the weight of TA muscles following either saline or Dex administration, and muscle mass sparing was significant only in Dex-treated mice ($P = 0.047$) [Fig. 7C]. These results suggest that SMYD3 plays a role in muscle mass control in the animal. Next, we stained cross-sections of TA muscles with an antibody recognizing laminin and measured cross-sectional areas in the four animal groups ($n=200$ fibers per muscle). As shown in Figure 7, D and E, median fiber size was higher in fibers electroporated with the Sh-SMYD3 plasmid than in fibers expressing the control scramble shRNA in both control (4521 ± 171 vs. 3896 ± 146 arbitrary units) and Dex-treated (3824 ± 128 vs. 3344 ± 111 arbitrary units) mice. Furthermore, fibers of bigger size were prevalent in TA muscles deficient of SMYD3 in either saline- or Dex-treated animals. The myofiber size of TA muscles depleted of SMYD3 and treated with Dex (3824 ± 128) was comparable with that of control myofibers treated with saline solution (3896 ± 146), thus suggesting that SMYD3 depletion prevents myofiber size reduction induced by the atrophic agent Dex.

Discussion

Our findings reveal a previously unappreciated role of SMYD3 in regulating skeletal muscle mass maintenance and glucocorticoid-induced skeletal muscle atrophy. SMYD3 function in cell culture and the animal is explained at least in part by its transcriptional regulation of myostatin, a critical negative regulator of cell differentiation and muscle mass [Lee 2004].

Myostatin plays a prominent role in muscle mass maintenance and several conditions related to muscle wasting, such as glucocorticoid-induced skeletal muscle atrophy. Glucocorticoids have broad-spectrum immunosuppressive and anti-inflammatory effects and are thought to be important regulators of muscle mass in catabolic diseases [Hanaoka et al. 2012]. Nevertheless, extended oversecretion or exogenous administration of glucocorticoids has catabolic effects on skeletal muscle,
which are ascribed to myostatin up-regulation (Ma et al. 2003; Hanaoka et al. 2012). In addition to its role in atrophy, myostatin up-regulation is a hallmark of different forms of muscle wasting, such as sarcopenia (McKay et al. 2012), cancer cachexia (Fearon et al. 2012; Lokireddy et al. 2012), and muscle disuse (Reardon et al. 2001). Strategies aiming at muscle size increase and strength through myostatin blockade hold promise for application in pathological conditions associated with progressive and irreversible muscle loss, such as cancer and muscular dystrophies (Kemaladewi et al. 2011; Pistilli et al. 2011). In addition to its role in regulating muscle mass, myostatin is expressed in developing somites and modulates muscle size during embryonic development by shifting precursor skeletal muscle cell fate toward myogenic differentiation instead of cell proliferation (Manceau et al. 2008). Furthermore, myostatin is also expressed in quiescent satellite cells (McCroskery et al. 2003).

Our experiments provide evidence for a role of SMYD3 in regulating processes involved in transcriptional elongation of the myostatin and c-Met genes in skeletal muscle cells. By recruiting a complex containing the acetyl-lysine recognition domain bromodomain BRD4 protein and pause–release factor pTEFb, SMYD3 promotes PolII Ser2P chromatin engagement. Control of transcriptional elongation is emerging as a major means of gene regulation in development and tissue homeostasis (Zhou et al. 2012). Whereas a plethora of transcription factors promotes the assembly of a preinitiation complex (Juven-Gershon and Kadonaga 2010), growing evidence indicates that a subset of transcriptional activators, such as c-Myc (Rahl et al. 2010) and NF-κB (Barboric et al. 2001), promotes early elongation steps (Levine 2011). The availability of selective small molecules targeting proteins implicated in regulating transcriptional initiation and elongation makes this process amenable to pharmacological intervention (Delmore et al. 2011). We successfully employed the bromodomain inhibitor JQ1 in proof-of-concept experiments to reduce glucocorticoid-induced myostatin and atrophy-related gene expression and prevent myotube atrophy. We cannot exclude that other BET proteins might be affected, even though, at the molarity employed here (50 nM), JQ1 is expected to preferentially inhibit BRD3 and BRD4 (Filippakopoulos et al. 2010). These results lend further support to, and expand the potential application of, muscle atrophy and wasting of

Figure 7. SMYD3 depletion prevents Dex-induced muscle atrophy in vivo. [A] Total RNA was extracted from fixed TA muscles, and SMYD3 transcript levels were measured by qRT–PCR. Relative expression was normalized against GAPDH. Error bars represent SD (n = 4). [*]P-value < 0.05. [B] Body mass of saline- and Dex-treated mice (n Sal = 8, n Dex = 9). [C] Muscle mass of control and Sh-SMYD3 TA electroporated muscles from either saline- or Dex-treated mice. [D] TA muscle fiber cross-sectional area (CSA) from control and Sh-SMYD3 electroporated muscles from saline-treated mice (values are median ± 95% CI). [E] TA muscle fiber CSA from control and Sh-SMYD3 electroporated muscles from Dex-treated mice (values are median ± 95% CI). (*) P < 0.05 versus relative control group; (#) P < 0.05 versus saline group.

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bromodomain inhibitors as therapeutic agents [Delmore et al. 2011; Zuber et al. 2011; Matzuk et al. 2012]. SMYD3’s abnormal overexpression in certain tumors [Hamamoto et al. 2004; Van Aller et al. 2012] has been linked to uncontrolled expression of genes that promote cancer progression and invasion. Interestingly, the recent reports that BRD4 plays a pivotal role in acute myeloid leukemia maintenance and multiple myeloma [Delmore et al. 2011; Zuber et al. 2011] raise the possibility that SMYD3 may collaborate with BRD4 in regulating growth-promoting genes by dynamics similar to the ones we describe here. BRD4 is a bromodomain protein that regulates a broad range of cellular processes and is ubiquitously expressed [Belkina and Denis 2012]. Conversely, SMYD3 appears to be more abundantly expressed in skeletal muscle compared with other tissues [Hamamoto et al. 2004]. The preferential tissue distribution of SMYD3 renders it an attractive target for the development of epigenetic drugs with increased specificity for the treatment of muscle diseases.

Materials and methods

Plasmids construction

Plasmids were constructed as detailed in the Supplemental Material.

Cell cultures and satellite cell isolation

C2C12 cells and HEK293T cells were obtained from American Type Culture Collection. Mouse C2C12 myoblasts were cultured in DMEM without Na-Pyruvate (Invitrogen) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U of penicillin, and 0.1 mg/mL streptomycin at 37°C and 5% CO2 and induced to differentiate by switching the medium to DM medium, which is DMEM [Invitrogen] supplemented with 2% horse serum [Invitrogen], 10% calf serum, transferrin, and selenium [Invitrogen].

For myostatin treatment, C2C12 was induced to differentiate in DM supplemented with recombinant myostatin [R&D Systems] at a final concentration of 100 ng/mL. C2C12 was allowed to differentiate in DM for 36 h, and then forming myotubes were treated with 100 µM Dex (Sigma-Aldrich) for 24 h before RNA extraction. C2C12 was treated with 50 nM JQ1 (Cayman Chemical) for 12 h and allowed to differentiate, maintaining JQ1 in differentiation medium. After 24 h in differentiation medium, forming myotubes were treated with 100 µM Dex or vehicle for an additional 24 h.

Satellite cells were isolated from muscles of 18-d-old mice as describe in Joe et al. [2010] with minor modifications. Isolated cells were resuspended in growth medium composed of F-10 HAM (Sigma), 20% FBS, 1% chicken embryo extract (SeraLab), 2.5 mg/mL human fibroblast growth factor (Cell Signaling), 2 mM L-glutamine, 100 U of penicillin, and 0.1 mg/mL streptomycin. Cells were preplated overnight to eliminate contaminating fibroblasts, and the nonadherent cells were plated in differentiation medium on Matrigel-coated plates (ECM gel, Sigma). After 2–3 d of culture, cells were used for experiments.

siRNA transfection and retroviral transduction

Retroviral transduction and primary skeletal muscle stem cell transfections were performed as described [see the Supplemental Material; Caretti et al. 2004].
Interacting proteins were eluted by boiling the samples and were analyzed by immunoblot.

Statistical methods
Statistical significance was determined by Student’s t-test ([*] P-value < 0.05, [**] P-value < 0.001).

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