Proteomic and Functional Analyses Reveal the Role of Chromatin Reader SFMBT1 in Regulating Epigenetic Silencing and the Myogenic Gene Program*

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Background: SFMBT1 is a poorly characterized chromatin reader.

Results: SFMBT1 is associated with multiple transcriptional corepressor complexes and required for MyoD-mediated transcriptional silencing.

Conclusion: Novel mechanisms of SFMBT1-mediated transcription repression and its regulation of myogenesis are identified.

Significance: Our findings contribute to the understanding of how histone modification language is interpreted to regulate gene expression and biological processes.

SFMBT1 belongs to the malignant brain tumor domain-containing chromatin reader family that recognizes repressive histone marks and represses transcription. The biological functions and molecular basis underlying SFMBT1-mediated transcriptional repression are poorly elucidated. Here, our proteomic analysis revealed that SFMBT1 is associated with multiple transcriptional corepressor complexes, including CtBP/LSD1/HDAC complexes, polycomb repressive complexes, and malignant brain tumor family proteins, that collectively contribute to SFMBT1 repressor activity. During myogenesis, Sfmbt1 represses myogenic differentiation of cultured and primary myoblasts. Mechanistically, Sfmbt1 interacts with MyoD and mediates epigenetic silencing of MyoD target genes via recruitment of its associated corepressors and subsequent induction of epigenetic modifications and chromatin compaction. Therefore, our study identified novel mechanisms accounting for SFMBT1-mediated transcription repression and revealed an essential role of Sfmbt1 in regulating MyoD-mediated transcriptional silencing that is required for the maintenance of undifferentiated states of myogenic progenitor cells.

Chromatin is composed of repetitive units called nucleosomes that consist of DNA (147 base pairs) wrapped around an octamer of histone proteins (two subunits of each of the histones H2A, H2B, H3, and H4) (1, 2). Posttranslational modifications of the N-terminal tails of histones affect chromatin structure, consequently serving as “histone marks” for transcriptional repression or activation (3, 4). One important type of histone mark is histone methylation, which is regulated by specific sets of proteins that carry out the addition, removal, or specific binding of methyl groups (4).

The MBT domain-containing proteins bind to low methylated histones, functioning as the chromatin “readers,” and have been linked to development and tumor suppression by genetic analyses (5). The MBT domain was first reported in Drosophila dl3mbt (6, 7). Mutations in the MBT domain of dl3mbt caused malignant overgrowth in the larval brain, implicating a tumor suppressing function of dl3mbt and the importance of the MBT domain (6, 7). In mammals, three different groups of MBT proteins have been identified, with hSCML2 and hSCMH1 containing two MBT domains; L3MBTL1, L3MBTL3, and L3MBTL4 containing three MBT domains; and L3MBTL2, SFMBT1, and SFMBT2 containing four MBT domains (5). The MBT proteins prevalently bind to mono- and dimethylated histone lysines in vitro and repress transcription via interaction with various repressors (5). Different MBT proteins have been identified in various protein complexes (5), suggesting that MBT proteins have distinct functional activities and modes of action in regulating chromatin.

In this study, we focused on a poorly characterized MBT protein, SFMBT1 (5cm-like with four mbt domains 1). Mammalian SFMBT1 contains four MBT repeat domains that are essential for mediating histone H3 N-terminal tail binding and transcriptional repression (8). In Drosophila, dSfmbt contains four MBT repeats and is a polycomb protein because dSfmbt knockout displays a classic polycomb phenotype with strong...
and widespread derepression of HOX genes (9). dSfmbt is part of the Pho repressive complex, which contains the DNA-binding protein Pho and recruits other polycomb proteins, mediating H3K27me3 for transcriptional silencing at target loci (10). The MBT repeat domain of dSfmbt preferentially binds to mono- or dimethylated lysine containing histone peptides (11). Although SFMBT1 was considered as a mammalian homologue for Drosophila dSfmbt in the literature, mammalian SFMBT1 and dSfmbt are probably not homologues, and SFMBT1 is likely unique to mammals on the basis of the following evidence: 1) SFMBT1 was not found in the mammalian Pho homolog YY1 protein complex in mammalian cells (12); 2) human SFMBT1 binds selectively with the N-terminal tail of histone H3 in a manner that appears to be independent of histone modification (8); and 3) SFMBT1 belongs to a different branch from dSfmbt on the basis of evolutionary relationship and domain organizations (5). Therefore, alternative mechanisms might account for the role of mammalian SFMBT1 in transcriptional regulation, and SFMBT1 might have unique functions. However, the molecular mechanisms underlying SFMBT1 transcriptional repression as well as its biological functions are unknown.

To gain unbiased biochemical insights into how SFMBT1 exerts its transcription repressor function, we performed affinity purification and MS analysis of the SFMBT1 protein complex. Our data revealed a novel biochemical connection of SFMBT1 with CtBP/LSD1/HDAC complexes, polycomb protein complexes (PRC), and other MBT proteins, suggesting functional cooperation of these corepressor proteins in establishing repressive chromatin states. We subsequently utilized a skeletal myogenesis model to investigate the biological functions of Sfmbt1 because epigenetic regulation has critical roles in the highly regulated myogenic process. Through gain of function and loss of function studies in combination with gene expression profiling studies, we found that Sfmbt1 critically regulates the myogenic programs through transcriptional silencing of the master regulator of myogenic process, MyoD.

EXPERIMENTAL PROCEDURES

**Plasmids**—pLKO.1-based lentiviral shRNA plasmids targeting mouse Sfmbt1 and MyoD genes were purchased from Open Biosystems. Human SFMBT1 truncation mutants (N: 1–473 aa, M: 494–699 aa, and C: 721–866 aa) were cloned to the pGex vector to generate GST fusion proteins. GFP-SFMBT1 was generated with the pEGFP-C3 vector. pQCXIP-FLAG-SFMBT1 was described previously (8). pMyog-luc was kindly provided by Dr. Stephen J. Tapscott (13). pCMV2-FLAG-L3MBTL3 was kindly provided by Dr. Toru Miyazaki (14). HA-tagged, full-length (FL) MyoD and truncated mutants (N: 1–66 aa, ΔN: 84–318 aa, C: 173–318 aa, and ΔC: 1–240 aa) were kindly provided by Drs. Serge A. Leibovitch and Slimane Ait-Si-Ali (15).

**Antibodies**—The antibodies were obtained from the following commercial sources: LSD1 (Abcam, catalog no. ab17721), anti-FLAG (M2, Sigma, catalog no. F-3165), anti-HA (Covance, catalog no. MMS-101P), GFP (Santa Cruz Biotechnology, catalog no. sc8334), CoREST (Millipore, catalog no. 07-455), BHC80 (Abcam, catalog no. ab41631), HDAC1 (Thermo, catalog no. PA1-860, and Santa Cruz Biotechnology, catalog no. sc7872), HDAC2 (Thermo, catalog no. PA1-861), EZH2 (Millipore, 07-400, and Active Motif, catalog no. 39875), RNF2 (Active Motif, catalog no. 39663), PHC1 (Active Motif, catalog no. 39723), SUZ12 (Millipore, catalog no. 07-379), β-actin (Sigma, catalog no. A5316), H3K4me2 (Millipore, catalog no. 07-030), H3K27me3 (Millipore, catalog no. 07-449), H3Ac (Millipore, catalog no. 06-599), H4Ac (Millipore, catalog no. 06-598), myosin (R & D Systems, catalog no. MF20), Myogenin (BD Biosciences, catalog no. 556358), and MyoD (BD Biosciences, catalog no. 556130).

**Cell Culture**—293FT and 293T cells were cultured in DMEM with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. C2C12 cells (ATCC) were cultured in DMEM with 20% heat-inactivated fetal bovine serum and penicillin/streptomycin. Primary myoblasts were isolated and maintained as described previously (16). Briefly, limb muscles from newborn mice were minced and then digested with collagenase/dispase at 37 °C for 30 min. The dissociated cells were collected and cultured in a collagen-coated dish with F-10-based primary myoblast selective growth medium (Ham F-10 nutrient mixture supplied with 20% FBS, 2.5 ng/ml bFGF/ml, 100 units/ml penicillin, and 100 μg/ml streptomycin). To selectively enrich myoblasts, myoblasts were physically dissociated from the culture dish during splitting, whereas the majority of fibroblasts remained attached. To further exclude the easily attaching fibroblasts, the dissociated cells were preplated to a fresh dish for 15 min, and then the unattached myoblasts were collected and cultured in a new dish. The selection process was repeated until a high purity of myoblasts was attained. For the myogenesis assay, myoblasts were grown to 80–90% confluence and induced for differentiation by switching from growth medium to differentiation medium (DMEM containing 2% horse serum).

**Nuclear Extract Preparation, Protein Complex Isolation, and Western Blot Analysis**—The detailed purification procedure was described previously (17). Briefly, nuclear extracts were prepared from 293T cells stably transduced with FLAG-SFMBT1. FLAG-SFMBT1 protein complexes were then purified using anti-FLAG M2 mAb-conjugated agarose beads (Sigma). After extensive washing, protein complexes were eluted using the elution buffer containing 50 μg/ml FLAG peptides at room temperature for 30 min. The eluted protein samples were separated on 4–12% NuPAGE Bis-tris gels (Invitrogen) and analyzed by LC-MS/MS. The procedures for Western blotting and immunoprecipitation studies were described previously (18).

**Viral Production, Titering and Transduction**—Viral production and transduction in cells was described previously (19). Stably transduced cells (polyclonal) were obtained and maintained by puromycin selection. To prepare concentrated viruses, supernatants containing lentiviruses were centrifuged at 25,000 rpm for 2 h at 4 °C using a SW28 rotor (Beckman). The pellets were suspended with PBS, aliquoted, and stored at −80 °C. The viral titers were determined following a procedure provided by Open Biosystems.

**Immunofluorescence Staining and Luciferase Assays**—Immunofluorescence staining and luciferase reporter assays were performed as described previously (18).
Epigenetic Regulation of Myogenesis by Sfmbt1

Microarray Analyses—Microarray experiments were performed using RNA isolated from the C2C12 scramble shRNA control and C2C12 Sfmbt1 knockdown. The total RNA was extracted using TRIzol reagents (Invitrogen) and purified using a Qiagen column with in-column DNase digestion. Hybridizations of Affymetrix mouse genome 430 2.0 arrays were performed at the Dana Farber Microarray core facility. The data were preprocessed and analyzed using dChip software. To reduce the false positives, probe sets defined as "Absent" detection calls crossing all samples were removed from the data analysis. Genes with a fold change ≥ 1.5 and p < 0.05 were considered differentially expressed.

GST Pull-down Assays—GST pull-down assays were performed as described previously (18). Briefly, bacterially expressed GST or GST-SFMBT1 fusion proteins were induced and purified with glutathione-Sepharose. Equivalent amounts of GST or GST-SFMBT1 immobilized on glutathione-Sepharose were then incubated with a nuclear extract of 293T transfected with HA-MyoD. After extensive washing, the pull-down HA-MyoD was detected by Western blot analysis.

Real-time RT-PCR—cDNA was generated with 2 μg of total RNA using a GeneAmp RNA PCR kit (Applied Biosystems). Real-time PCR was performed using the StepOne™ real-time PCR system (Applied Biosystems) with the SYBR Green PCR core reagents kit (Applied Biosystems). The sequences for the primers used are listed in supplemental Table S1.

ChIP—ChIP assays were performed as described previously (20). Briefly, cells were fixed with formaldehyde for 10 min at room temperature to cross-link the DNA to chromatin-associated protein complexes. The cross-linking was stopped with glycine, and cells were then collected and sonicated with a MISONIX S-4000 sonicator to shear DNA to lengths between 200 and 800 bp. The DNA-protein complexes were then immunoprecipitated with the indicated antibodies and washed extensively. The ChIP DNA was then purified and eluted with 100 μl of H2O. A total of 2.5 μl of ChIP DNA was used for real-time PCR analysis. The primer sequences are listed in supplemental Table S1.

DNase I Sensitivity Assay—A DNase I sensitivity assay was performed with RQ1 RNase-free DNase (Promega). Nuclei of 5 × 10^5 cells were treated with DNase at 37 °C for 30 min, and then the reactions were stopped with EDTA at a final concentration of 25 mM. Genomic DNA samples were purified by protease K digestion and phenol chloroform extraction. The purified DNA samples were analyzed by real-time PCR to measure the relative amount of uncut DNA around the promoter region of Myogenin.

Statistical Analysis of Experimental Data—An independent Student’s t test was used to analyze data from various experiments.

RESULTS

SFMBT1 Is Associated with Multiple Transcriptional Corepressor Complexes—To elucidate the mechanisms underlying transcriptional repression function of SFMBT1, we performed a proteomic analysis to identify SFMBT1-interacting partners in mammalian cells. The SFMBT1 protein complex was immunoprecipitated using anti-FLAG beads from nuclear extracts of FLAG-SFMBT1-expressing 293T cells followed by elution with FLAG peptides. The SFMBT1-associated proteins were next separated by SDS-PAGE (Fig. 1A) and subjected to MS analysis. We found that SFMBT1-associated proteins consist of distinct transcriptional corepressors, including 17 components of the CtBP/LSD1/HDAC protein complex, 11 members of PRC, and four MBT proteins (Fig. 1B and supplemental Fig. S1). We further validated the interaction of SFMBT1 with these corepressors identified from our MS analysis. As shown in Fig. 1C, we performed immunoprecipitation of FLAG-SFMBT1 followed by Western blotting and validated that LSD1 core complex components, including LSD1, CoREST, BHC80, HDAC1, and HDAC2, were present in SFMBT1 immunoprecipitates. Using similar assays, we observed that SFMBT1 interacted with PRC1 proteins PHC1 and RNF2 but not with PRC2 members EZH2 and SUZ12 (Fig. 1D), which is consistent with our MS data (B). Because our MS data also indicated an interaction between SFMBT1 and L3MBTL3, we performed coimmunoprecipitation and detected an interaction between GFP-SFMBT1 and FLAG-L3MBTL3 in mammalian cells (Fig. 1E).

Sfmbt1 Expression Is Down-regulated During Myogenic Differentiation—The biological functions of SFMBT1 are unknown. Previous observations that mouse Sfmbt1 is expressed in skeletal muscles (21) and that Drosophila dSfmbt is associated with the promoters of genes involved in muscle development in embryos and larvae (22) suggest a possible role of Sfmbt1 in regulating skeletal myogenesis. Importantly, epigenetic silencing is critical in restricting the expression of muscle genes in muscle progenitors and maintaining proper growth and differentiation (23–25). To investigate the function of Sfmbt1 in myogenesis, we utilized the C2C12 myoblast differentiation model and first investigated the pattern of Sfmbt1 expression during myogenic differentiation. We found that the Sfmbt1 transcript level was highest in undifferentiated myoblasts and reduced during the course of differentiation (Fig. 2A). Thus, these data suggest a possible role for Sfmbt1 in repressing myogenic differentiation.

SFMBT1 Overexpression Blocks and Sfmbt1 Depletion Enhances Myogenic Differentiation—We next investigated the respective functional consequences of SFMBT1 overexpression and Sfmbt1 depletion on myoblast proliferation and differentiation. First, C2C12 myoblasts were transduced with human FLAG-SFMBT1 retroviruses or empty vector control viruses followed by puromycin selection. SFMBT1 expression was confirmed by Western blot analysis (Fig. 2B). The transduced C2C12 myoblasts were induced to differentiate, and myotube formation and muscle-specific gene expression were then examined various days after differentiation. We found that ectopic SFMBT1 expression resulted in a block in myotube formation (Fig. 2C) and a decrease in expression of muscle differentiation markers (myosin and Myogenin) (D). Ectopic SFMBT1 expression appeared to have little effect on the prolif-
operation of undifferentiated myoblasts in growth medium (Fig. 2).

Thus, SFMBT1 represses myogenic differentiation, likely contributing to the maintenance of undifferentiated states of myoblasts.

Next, we knocked down Sfmbt1 expression in C2C12 myoblasts using lentiviral-mediated shRNA targeting Sfmbt1 (shSfmbt1) or scrambled shRNA as a control (Fig. 3A). When induced for differentiation, Sfmbt1 knockdown cells displayed a more differentiated phenotype than control cells, as shown by the formation of larger and more myotubes (Fig. 3B and supplemental Fig. S2) and earlier and more robust expression of myogenic transcription factor Myogenin and muscle gene Myosin (Fig. 3C). Moreover, Sfmbt1 knockdown significantly reduced the proliferation rate of C2C12 myoblasts cultured in growth medium (Fig. 3D). The shRNA knockdown effects were specific, as we observed similar effects with an independent shRNA construct targeting a different region of Sfmbt1 (supplemental Fig. S3). Importantly, we found that Sfmbt1 knockdown promoted differentiation and reduced proliferation of primary mouse myoblasts (Fig. 3, E–H, and supplemental Fig. S2). Therefore, our gain of function and loss of function studies (Figs. 2 and 3) strongly indicate that Sfmbt1 is required for proper proliferation of myogenic progenitor cells and prevention of premature differentiation.

Sfmbt1 Represses Transcription of Muscle Transcription Factors Such as Myogenin, Mef2C, and Muscle Structural Genes—To investigate the molecular mechanisms underlying Sfmbt1 regulation of myogenesis, we examined Sfmbt1-regulated genes in undifferentiated myoblasts. We performed a microarray analysis to compare gene expression between proliferating Sfmbt1 knockdown and control cells and identified a total of 206 up-regulated genes and 307 down-regulated genes with the criteria of fold change >1.5 and p < 0.05 (Fig. 4A and supplemental Table S2, S3). The up-regulated genes with highest fold changes in Sfmbt1 knockdown cells were numerous muscle differentiation genes whose expression is normally repressed in proliferating, undifferentiated muscle progenitors. These genes included muscle transcription factors, Myogenin and Mef2C, and many myofibrillar genes such as troponin, Tnnc, Tnnt, Tnni, Myosin light and heavy chains, and α-actin. Elevated expression levels of several typical muscle genes such as Myogenin (myog), Mef2c, Myh3, Mylf3, Gas2, and Tnnc2 in Sfmbt1 knockdown cells were confirmed by real-time RT-PCR (Fig. 4B). On the other hand, expression of the muscle stem cell factors Bmi1 (26) and Nestin (27) was decreased when Sfmbt1 was depleted, suggesting a role of Sfmbt1 in the maintenance of the muscle stem cell state. Our findings thus support that Sfmbt1 criti-
cally confers transcriptional silencing of muscle genes in myogenic progenitor cells.

**Sfmbt1 Interacts with the Muscle Transcription Factor MyoD**—
The myogenic transcription factor MyoD is associated with transcriptional corepressors to repress MyoD-mediated transcription in undifferentiated myogenic progenitors (25). Because Sfmbt1 depletion resulted in enhanced expression of important MyoD target genes such as *Myogenin* and *Mef2c* in myoblasts, we investigated a possible interaction between Sfmbt1 and MyoD. We compared the Sfmbt1 target gene set from our microarray data with the published MyoD ChIP-Sequence gene set (28) and found that about 42% of the up-regulated genes because of Sfmbt1 knockdown showed physical MyoD binding to their promoters (Fig. 4C). These data suggest that MyoD interacts with and recruits Sfmbt1 to MyoD target loci for transcriptional repression in myogenic progenitors.

To test this possibility, we determined a possible physical interaction between Sfmbt1 and MyoD. Our reciprocal coimmunoprecipitation assays using FLAG-SFMBT1-expressing C2C12 cells showed that FLAG-SFMBT1 is readily detectable in endogenous MyoD immunoprecipitates and endogenous MyoD in FLAG-SFMBT1 immunoprecipitates (Fig. 5A), indicating an interaction between SFMBT1 and MyoD. Moreover, our mapping studies using a series of SFMBT1 (GST fusion) and MyoD (HA-tagged) truncated mutants revealed that MyoD interacts with the N-terminal (four-MBT repeat domain) and middle region (DUF 3588, domain of unknown function 3588) of SFMBT1 (Fig. 5, B and C) and that the bHLH domain of MyoD is required for its interaction with SFMBT1 (D and E). Because the muscle transcription factor Myogenin is also a bHLH member, we performed coimmunoprecipitation and found that Sfmbt1 failed to interact with Myogenin (supplemental Fig. S4). Therefore, our data indicate that Sfmbt1 specifically interacts with MyoD and represses MyoD-dependent transcription.

**Sfmbt1 Recruits Multiple Transcriptional Corepressor Complexes to Mediate Epigenetic Silencing of MyoD Target Genes**—
One key MyoD target is the muscle transcription factor *Myogenin*, which is essential for muscle cell differentiation (29). Therefore, we focused on the *Myogenin* gene and investigated how Sfmbt1 regulates MyoD-mediated transcriptional repression. First, we confirmed that exogenous SFMBT1 repressed the activity of a *Myogenin* promoter luciferase reporter in a dose-dependent manner (Fig. 6A). Conversely, depletion of
endogenous Sfmbt1 in C2C12 resulted in increased Myogenin promoter activity (Fig. 6B). Consistent with the promoter assays, Sfmbt1 knockdown resulted in enhanced transcription levels of endogenous myogenin (Fig. 4B). Moreover, Sfmbt1-depleted C2C12 cells showed an increase in Myogenin protein 1 day after differentiation by Western blotting (Fig. 3C) and immunofluorescence staining (supplemental Fig. S5). Next, we carried out ChIP assays using FLAG-SFMBT1-expressing C2C12 cells and found that FLAG-SFMBT1 is enriched on the MyoD binding region of the Myogenin promoter, indicating that Sfmbt1 directly regulates MyoD target myogenin gene transcription (Fig. 6C). Moreover, the depletion of MyoD decreased the binding of SFMBT1 to the Myogenin promoter (Fig. 6, C and D), which suggests that SFMBT1 depends on MyoD to localize on the Myogenin promoter and repress muscle genes in the myoblasts. Therefore, our overall data indicate that Sfmbt1 is localized on the MyoD target Myogenin promoter and represses MyoD-dependent transcription, which is required for myogenic differentiation.

SFMBT1 is associated with multiple transcriptionally repressive complexes, including the CtBP/LSD1/HDAC complex, PRC, and MBT family proteins (Fig. 1). In addition, SFMBT1 interacts with MyoD and directly regulates MyoD target gene transcription (Figs. 4–6). Therefore, we further examined the effect of Sfmbt1 depletion on the recruitment of multiple corepressors to the MyoD target Myogenin promoter by ChIP assays. As shown in Fig. 6E, Sfmbt1 knockdown resulted in decreased binding of multiple transcription corepressors such as LSD1, HDAC1, EZH2, and RING2 to the Myogenin promoter region that spans the MyoD binding sites. These data thus indicate that Sfmbt1 is required for the recruitment of multiple transcriptional corepressor complexes to MyoD target loci.
Because the Sfmbt1-associated CtBP/LSD1/HDACs complex and PRC components have enzymatic activities to catalyze histone methylation/demethylation and deacetylation, we further investigated whether Sfmbt1 depletion affects the histone modification status on the MyoD target loci. Our ChIP assays showed that Sfmbt1 knockdown resulted in decreased levels of histone modifications. The comparisons of the up-regulated gene set because of Sfmbt1 knockdown, and the gene set showing MyoD binding in the promoter (28) reveals that about 42% of the Sfmbt1 target genes showed MyoD binding in the promoters.

**FIGURE 4.** Sfmbt1 target genes in C2C12 myoblasts have a significant overlap with direct MyoD target genes. A, a heat map showed changes in Sfmbt1 knockdown and control cell gene expression profiling on the basis of microarray analysis. B, several Sfmbt1 target genes were validated by quantitative RT-PCR. Data are presented as mean ± S.E. C, a significant subset of Sfmbt1 target genes showed MyoD binding on their promoters. The comparisons of the up-regulated gene set because of Sfmbt1 knockdown, and the gene set showing MyoD binding in the promoter (28) reveals that about 42% of the Sfmbt1 target genes showed MyoD binding in the promoters.

**FIGURE 5.** Sfmbt1 interacts with myogenic master transcription factor MyoD. A, FLAG-SFMBT1 interacted with endogenous MyoD in C2C12 by reciprocal IP. Nuclear extracts from FLAG-SFMBT1-expressing C2C12 cells were immunoprecipitated with anti-igG and anti-MyoD antibodies, respectively (left panel), or anti-igG and anti-FLAG antibodies, respectively (right panel). Western blotting was subsequently performed using the indicated antibodies. B, diagram of full-length and truncated mutants of SFMBT1. C, GST pull-down assays showed that MyoD binds to the N-terminal and central regions of the SFMBT1 protein. Various GST-SFMBT1 fusion proteins immobilized on glutathione-Sepharose were incubated with whole cell lysates of HA-MyoD-transfected 293T cells. The pull-down products were analyzed by Western blotting with anti-HA antibodies. D, the domain structure of MyoD and various truncated mutants are shown. E, the bHLH domain of MyoD interacted specifically with SFMBT1 by coimmunoprecipitation assays. 293T cells were cotransfected with FLAG-SFMBT1, and full-length (FL) or truncated mutants of HA-MyoD and whole cell lysates were harvested 48 h after transfection. SFMBT1 IP was performed with anti-FLAG-agaroses followed by Western blotting with HA antibodies.
the transcriptionally repressive histone mark H3K27me3 and increased levels of active histone marks, H3K4me2, AcH3, and AcH4 (Fig. 6F), in the MyoD-binding region of the Myogenin gene promoter.

Moreover, a protein complex analysis showed that SFMBT1 is associated with the MBT family proteins and PRC1 proteins that function in chromatin compaction, suggesting that the SFMBT1-associated complex might contribute to chromatin structure compaction on target promoters. Therefore, we compared chromatin accessibility flanking the transcription initiation site (TSS) of MyoD target gene Myogenin before and after SFMBT1 knockdown using a DNase I sensitivity assay. In brief, intact nuclei isolated from SFMBT1-depleted and control C2C12 cells were treated with DNase I at 37 °C for 30 min. Genomic DNA was then isolated and amplified with the primers spanning the TSS site of the Myogenin gene. The relative levels of uncut DNA around the TSS site of the myogenin gene were presented. *, p < 0.05; **, p < 0.01. H, proposed model of SFMBT1 in regulating MyoD-mediated epigenetic silencing of myogenesis.
Epigenetic Regulation of Myogenesis by Sfmbt1

DISCUSSION

The molecular mechanisms and biological functions of SFMBT1 were previously uncharacterized. In this study, through protein complex analysis, gene expression profiling, and myogenic differentiation assays, we identified novel mechanisms accounting for SFMBT1-mediated transcription repression and revealed a critical role of Sfmbt1 in regulating muscle cell growth and differentiation programs that contribute to the maintenance of undifferentiated states of myogenic progenitors.

Our unbiased proteomic analysis of the SFMBT1 protein complex and subsequent biochemical interaction validations strongly indicate that SFMBT1 is associated with multiple transcriptional corepressors, including the CtBP/LSD1/HDAC complex components, the PRC components, and other MBT proteins. Multiple studies have revealed functional interplay among these protein complexes in transcriptional repression. For instance, the CtBP/LSD1/HDAC complex components catalyze histone demethylation/deacetylation (30, 31) and recruit polycomb proteins to target promoters (32, 33). The PRC1 complex maintains transcriptional repression and compacts chromatin at target promoters (10). Moreover, the MBT protein Scm in the PRC1 complex recruits other components of PRC2 and PRC1 to mediate the histone modifications at target loci and repress transcription (34). In addition, the L3MBTL proteins function as chromatin readers and compact the chromatin (35, 36). Because these SFMBT1-associated proteins are critical regulators of histone modifications, polycomb protein recruitment, and chromatin compaction, our study thus identified a novel, central role of SFMBT1 in coordinating transcriptional repressive complexes in transcriptional repression.

It was shown previously that Drosophila dSfmbt binds to low methylated repressive histone marks in vitro (9, 11), yet human SFMBT1 was able to bind to unmodified H3 (8). In light of different associated protein complexes, it is possible that mammalian SFMBT1 might interact with histone marks differently from dSfmbt in terms of the requirement of post-translational modifications. Our data support that SFMBT1 might interact with histone tails and function as a repressive chromatin reader and recruit multiple corepressor complexes to regulate the histone modifications and chromatin compaction at target loci, subsequently repressing transcription.

The biological functions of SFMBT1 have not been elucidated. In this study, we investigated the function and mechanism of Sfmbt1-mediated transcriptional regulation in skeletal myogenesis, as current evidence strongly indicates that skeletal myogenesis is under epigenetic control (37). By utilizing the skeletal myogenesis model, we showed that 1) Sfmbt1 expression is down-regulated during myogenic differentiation, 2) overexpression of SFMBT1 in C2C12 myoblasts blocks the myogenic differentiation, and 3) Sfmbt1 knockdown promotes the myogenic differentiation in C2C12 and primary myoblasts and reduces proliferation. Overall, gain of function and loss of function of Sfmbt1 significantly impact growth and differentiation of myogenic cells in vitro. Therefore, our data suggest a critical role of Sfmbt1 in regulating skeletal myogenesis, likely via supporting muscle stem cell proliferation and preventing premature differentiation.

The mechanisms of polycomb and MBT protein recruitment to specific genomic loci and regulation of target gene expression in a mammalian system remains unclear (5, 10, 38). Many studies have revealed that transcription factors are crucial for polycomb and MBT protein recruitment to target promoters (34–36). It has been reported that the bHLH transcription factor MyoD recruits different epigenetic regulators to myogenic target loci and establishes spatiotemporal muscle-specific gene expression patterns in the myogenic developmental process (25). About 42% of the derepressed genes in Sfmbt1 knockdown C2C12 cells are direct MyoD targets (Fig. 4C), strongly suggesting that MyoD might recruit Sfmbt1 to target promoters to mediate the transcription silencing. This is supported by our findings that SFMBT1 interacts with MyoD and represses MyoD target Myogenin transcription (Figs. 5 and 6). Moreover, Sfmbt1 directly binds to the MyoD target Myogenin promoter and is required for the recruitment of its associated transcriptional repressors to the Myogenin promoter (Fig. 6). Accordingly, the transcriptional repressive histone mark H3K27me3 decreases and the active marks H3K4me2, AcH3, and AcH4 increase on the MyoD target promoter when Sfmbt1 is depleted. Our chromatin accessibility assay revealed that the chromatin structure in the promoter region of MyoD target Myogenin became more accessible in Sfmbt1 knockdown C2C12 cells, suggesting that Sfmbt1 is required for the chromatin compaction of MyoD binding regions. Therefore, our data indicated that Sfmbt1 is required for the recruitment of multiple repressive complexes to MyoD and maintenance of repressive histone marks and chromatin compaction on MyoD target promoters.

These findings thus allow us to propose a model in which the MBT domain-containing protein Sfmbt1 associates with multiple transcriptional repressive complexes and functions as a negative regulator of myogenic differentiation (Fig. 6H). In undifferentiated myoblasts, Sfmbt1 interacts with MyoD and recruits its associated repressors to MyoD to mediate histone modifications and chromatin compaction on MyoD target promoters, which excludes the binding of coactivators and results in epigenetic silencing of muscle genes and maintenance of the undifferentiated state of myogenic progenitor cells. When Sfmbt1 is depleted, transcriptional corepressors are no longer associated with MyoD, which facilitates transcriptional derepression of MyoD target genes and promotes myogenic differentiation. In summary, our study provides strong evidence that Sfmbt1 links functionally to Polycomb proteins, MBT proteins, and the LSD1 complex and critically regulates target gene transcription and, subsequently, cell growth and differentiation programs.

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REFERENCES

1. Luger, K., and Richmond, T. J. (1998) DNA binding within the nucleosome core. *Curr. Opin. Struct. Biol.* 8, 33–40

2. Luger, K., and Hansen, J. C. (2005) Nucleosome and chromatin fiber dynamics. *Curr. Opin. Struct. Biol.* 15, 188–196

3. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell* 128, 693–705

4. Ruthenburg, A. J., Li, H., Patel, D. J., and Allis, C. D. (2007) Multivalent engagement of chromatin modifiers by linked binding modules. *Nat. Rev. Mol. Cell Biol.* 8, 983–994

5. Bonasio, R., Lecona, E., and Reinberg, D. (2010) MBT domain proteins in development and disease. *Semin. Cell Dev. Biol.* 21, 221–230

6. Gateff, E., Löfler, T., and Wismar, J. (1993) A temperature-sensitive brain tumor suppressor mutation of *Drosophila melanogaster*. Developmental studies and molecular localization of the gene. *Mech. Dev.* 41, 15–31

7. Wismar, J., Löfler, T., Habscheidmichael, N., Vef, O., Geissen, M., Zirwes, R., Altmeyer, W., Sass, H., and Gateff, E. (1995) The *Drosophila melanogaster* tumor suppressor gene lethal3(malignant brain tumor encodes a proline-rich protein with a novel zinc finger. *Mech. Dev.* 53, 141–154

8. Wu, S., Trievel, R. C., and Rice, J. C. (2007) Human SFMBT is a transcriptional repressor protein that selectively binds the N-terminal of histone H3. *FEBS Lett.* 581, 3289–3296

9. Klymenko, T., Papp, B., Fischle, W., Köcher, T., Schelder, M., Fritsch, C., Wild, R., Wilm, M., and Müller, C. (2007) A Polycrom group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. *Genes Dev.* 20, 1110–1122

10. Simon, J. A., and Kingston, R. E. (2009) Mechanisms of polycrom gene silencing. Knowns and unknowns. *Nat. Rev. Mol. Cell Biol.* 10, 697–708

11. Klymenko, T., Papp, B., Fischle, W., Köcher, T., Schelder, M., Fritsch, C., Wild, R., Wilm, M., and Müller, C. (2006) A Polycrom group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. *Genes Dev.* 20, 1110–1122

12. Simon, J. A., and Kingston, R. E. (2009) Mechanisms of polycrom gene silencing. Knowns and unknowns. *Nat. Rev. Mol. Cell Biol.* 10, 697–708

13. Griffin, J. D. (2000) MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. * rdf. Protoc. Hum. Genet. Chapter 13, 13.4.1–13.4.19

14. Ogawa, H., Ishiguro, K., Gauthiez, S., Livingstone, D. M., and Nakatani, Y. (2002) A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G2 cells. *Science* 296, 1132–1136

15. Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000) MAML1, a human homologue of *Drosophila* mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* 26, 484–489

16. Shen, H., McElhinny, A. S., Cao, Y., Gao, P., Liu, J., Bronson, R., Griffin, J. D., and Wu, L. (2006) The Notch coactivator, MAML1, functions as a novel coactivator for MEF2C-mediated transcription and is required for normal myogenesis. *Genes Dev.* 20, 675–688

17. Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000) MAML1, a human homologue of *Drosophila* mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* 26, 484–489

18. Shen, H., McElhinny, A. S., Cao, Y., Gao, P., Liu, J., Bronson, R., Griffin, J. D., and Wu, L. (2006) The Notch coactivator, MAML1, functions as a novel coactivator for MEF2C-mediated transcription and is required for normal myogenesis. *Genes Dev.* 20, 675–688

19. Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S., and Griffin, J. D. (2000) MAML1, a human homologue of *Drosophila* mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* 26, 484–489