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Histone Methyltransferase SETD3 Regulates Muscle Differentiation*

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Histone lysine methylation, as one of the most important factors in transcriptional regulation, is associated with a various physiological conditions. Using a bioinformatics search, we identified and subsequently cloned mouse SET domain containing 3 (SETD3) with SET (Su(var)3–9, Enhancer-of-zeste and Trithorax) and Rubis-subs-bind domains. SETD3 is a novel histone H3K4 and H3K36 methyltransferase with transcriptional activation activity. SETD3 is expressed abundantly in muscular tissues and, when overexpressed, activates transcription of muscle-related genes, *myogenin*, *muscle creatine kinase* (*MCK*), and *myogenic factor 6* (*Myf6*), thereby inducing muscle cell differentiation. Conversely, knockdown of SETD3 by shRNA significantly retards muscle cell differentiation. In this study, SETD3 was recruited to the myogenin gene promoter along with MyoD where it activated transcription. Together, these data indicate that SETD3 is a H3K4/K36 methyltransferase and plays an important role in the transcriptional regulation of muscle cell differentiation.

The conformational structure or molecular charge of the histone core complex can be modified via methylation of the lysine/arginine residue in the histone tail, which affects gene expression and heterochromatin formation (1). Arginine methylation is mediated by PRDM family proteins, which are characterized by the presence of a PR (PRD1-BF1 and RIZ homology) domain at their N terminus, whereas lysine is methylated by histone methyltransferase (HMTase),[4] which commonly harbors the SET (Su(var)3–9, Enhancer-of-zeste and Trithorax) domain (2). By forming complexes with a broad variety of transcription factors, HMTases perform an important role in the regulation of gene expression, stem cell renewal, reproductive organ maturation, and tumorigenesis in mammals (2–5). Additionally, HMTases have been confirmed as crucial to myofibril organization (6), intestinal and pancreatic differentiation (7), and neurogenesis (8) in zebrafish.

Muscle differentiation requires sequences of harmonized steps after the commitment of mesodermal progenitor cells to the muscular lineage (9). Under the regulation of diverse modifiers, myoblasts fuse with other neighboring myoblasts to generate multinucleated myotubes (10). During differentiation, the cell cycle is withdrawn and muscle-specific transcription factors activated. Mesodermal precursor cells with muscular lineages are differentiated into skeletal muscle or smooth muscle via the interplay of muscle-specific factors, including MyoD, myogenin, myogenic factor 5 (Myf5), muscle regulatory factor 4 (MRF4), and myocyte enhancer factor-2 (MEF2) (10).

Histone modification enzymes have been implicated in muscle cell differentiation through the regulation of muscle-specific gene expression (11, 12). Chromatin modification enzymes such as histone acetyltransferases, deacetylases (HDACs), and chromatin remodeling factors have recently been reported to regulate MyoD activity during muscle differentiation. For example, the histone acetyltransferases p300 and p300/CBP-associated factor (PCAF) acetylate histones H3, H4, and MyoD, with both proteins synergistically activating the transcription of MyoD target genes via recruitment of chromatin-remodeling complexes. Before skeletal muscle differentiation, HDACs such as HDAC1 and Sir2 located on chromatin inhibit gene activation (13). These enzymes play roles as positive or negative regulators of MyoD and control MyoD activity by balancing epigenetic status through post-translational modifications (14, 15). Recent work has demonstrated that Suv39h1, which methylates histone H3K9 for silencing of myogenin gene expression, provides a checkpoint between proliferation and differentiation through association with MyoD (16). Moreover, H3K27 methylation by Ezh2 represses muscle gene expression as well as differentiation through recruitment of the transcriptional regulator YY1 (17). The reversible methylation status of histones also constitutes a critical regulatory mechanism in muscle-related gene activation or repression (18–20). Histone demethylase LSD1 plays a key role in muscle differentiation by controlling epigenetic marks in the myogenin and myosin heavy chain gene promoters (21). Jmjd1a demethylates H3K9 in the myosin heavy chain gene promoter and enhances muscle differentiation by interacting with myocardin factors (22).

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[4] The abbreviations used are: HMTase, histone methyltransferase; HDAC, histone deacethyltransferase; MCK, muscle creatine kinase; Myf, myogenic factor; SET, Su(var)3–9, Enhancer-of-zeste and Trithorax.

In this study, we report and describe a novel SET domain-harboring protein SETD3 with HMTase activity. SETD3 was expressed predominantly in muscle cells and exerted general transcriptional stimulatory effects deriving from H3K4 and H3K36 methylation. Accordingly, we suggest that SETD3 plays an important role in muscle differentiation by regulating muscle-specific factors, most notably myogenin, through interaction with MyoD.

EXPERIMENTAL PROCEDURES

Bioinformatics—To identify a novel SET domain-harboring protein, we used the EnsMart protein mining program. We used the Pfam ID filter (00865) and the novel protein option. Candidate genes were blasted to the Pfam site to calculate the probabilities of the SET domain and other structures.

Plasmid Constructs—The full-length open reading frame of SETD3 along with truncated SETD3 constructs (SETD3-N, SETD3-C) were PCR-amplified from a mouse cDNA library (Clontech). The PCR products were subcloned into *pGEX-4T1* (Amersham Biosciences), *pCMX-GAL4*, or *His/V5*-tagged *pcDNA3.1* (Invitrogen) expression vectors. The shRNA against mouse SETD3 (RMM1766-96884536) was purchased from OpenBiosystems. The full-length open reading frame of zebrafish SETD3 cDNA was also purchased from OpenBiosystems. The PCR products were subcloned into HA/myc/His-tagged pcDNA6 (Invitrogen). The siRNAs against mouse MyoD (sc-35991) were purchased from Santa Cruz Biotechnology.

Cell Cultures—NIH3T3 (mouse embryo fibroblast), H9c2 (rat cardiomyoblast), and C2C12 (mouse skeletal myoblast) cells were obtained from the Korean Cell Line Bank. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Welgene) containing 10% fetal bovine serum (FBS), or 15% for C2C12 cells, in growth medium (GM) and then induced to differentiate in DMEM supplemented with 2% horse serum or 1% FBS (differentiation medium, DM). To establish the cell line stably expressing knocked down endogenous *SETD3*, C2C12 and H9c2 cells were transfected with *sh-SETD3* using Lipofectamine 2000 (Invitrogen), after which the stably transfected clones were selected.

In Vitro HMTase Assays—HMTase assay was conducted overnight at 30 °C in a 35-μl reaction mixture containing 1 μg/μl core histones from calf thymus (Roche Applied Science) as a substrate for enzyme activity, 100 nCi of *S*-adenosyl-[*methyl*-^{14}C]-L-methionine (SAM; Amersham Biosciences), GST-SETD3, and GST in 5 × HMTase assay buffer (50 mM Tris, pH 8.5, 20 mM KCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1.25 M sucrose) (23). Peptides (H3N1 (ARTKQT), H3N2 (TARKST), H3N3 (AARKSA), H3N4 (STGGVK), and H4N1 (RHRKVA)) were synthesized based on the amino acid sequences of histones H3 and H4 (Peptron). Proteins/peptides were filtered with p81 filter paper (Millipore) and washed three times in cold 10% TCA and 95% ethanol for 5 min at room temperature. The filters were then air-dried, and 5 ml of Ultima Gold (PerkinElmer Life Sciences) was added followed by quantification of [^{14}C]SAM using a scintillation counter (PerkinElmer Life Sciences).

LC-MS Spectrometry—Synthetic peptides (H3N1, H3N2, and H3N4) were used as substrates in the HMTase assay with GST-SETD3. The reaction was halted by precipitation with 10% TCA followed by centrifugation for 10 min at 4000 rpm. The methylated peptides in the supernatants were analyzed via LC-MS at the Korea Basic Science Institute.

Western Blot Analysis—The transfected cells were lysed and loaded onto 14% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed overnight with anti-H3K4-me2 (1:2000), anti-H3K9-me2 (1:2000), anti-H3K27-me2 (1:2000), and anti-H3K36-me2 (1:2000) antibodies (Millipore) at 4 °C. The blots were finally incubated with HRP-conjugated goat anti-rabbit antibodies (Santa Cruz Biotechnology) and detected using an ECL system (Santa Cruz Biotechnology).

Transfection and Luciferase Assay—NIH3T3 and C2C12 cells were seeded in 48-well plates and transfected with Lipofectamine 2000 (Invitrogen) along with *MH100-GAL4-SV40-Luc* (100 ng), *pcDNA3.1-SETD3* (100 ng, 200 ng, 250 ng), *pCMX-GAL4-SETD3* (100 ng, 200 ng, 250 ng), or *sh-SETD3* (100 ng, 200 ng). Luciferase activity was measured by adding 20 μl of luciferin as a substrate to 80 μl of cell lysates using a Glomax luminescence luminometer (Promega). The level of β-galactosidase activity and protein concentration were used for normalization of reporter luciferase and β-galactosidase activities, respectively. Each value is the mean of quadruplicate determinations of a single assay. The results shown are representative of at least three independent experiments.

Real-time PCR Analysis of Muscle Target Genes—H9c2 and C2C12 cells were transfected with *SETD3* or *sh-SETD3*, after which total RNA was isolated using RNAiso Plus (TaKaRa). The cDNA was generated using 1 μg of RNA with oligo(dT) and reverse transcriptase (Enzynomics). The primer sequences of the muscle genes are provided in Table 1. The amplification reaction was performed under the following conditions: 35 cycles of denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C. Disassociation curves were generated after each PCR run to ensure that a single product of the appropriate length was amplified. The mean threshold cycle (C_t) ± S.E. were calculated from individual C_t values obtained from triplicates per stage. The normalized mean C_t was estimated as ΔC_t by subtracting the mean C_t of *GAPDH* from that of the muscle genes. The value $\Delta\Delta C_t$ was calculated as the difference between control ΔCt and values obtained for each sample. The *n*-fold change in gene expression relative to untreated control was calculated as $2^{-\Delta\Delta Ct}$.

Chromatin Immunoprecipitation Assays—C2C12 cells were differentiated and harvested after 4 days. The resultant cells were then treated with 1% formaldehyde for 10 min in medium at 37 °C, followed by the addition of 125 mM glycine for 5 min at room temperature, after which the cells were scraped into SDS lysis buffer. The sonicated chromatin was immunoprecipitated with specific antibodies and subsequently recovered with protein A/G-agarose beads (GeneDEPOT). After reversing the cross-links, chromatin was subjected to proteinase K digestion and the DNA purified. Then, a 326-bp fragment corresponding to nucleotides −227 to +109 of the mouse myogenin gene proximal promoter along with a 304-bp fragment correspond-

TABLE 1
Oligonucleotides used for RT-PCR

Target mRNA	Organism	Primer sequence[a]
SETD3	*Mus musculus*	F: 5′-GATGGGAAAAGAGAAGATTAC-3′ R: 5′-ACTGTGGATCACAAACTCTGC-3′
	Rattus norvegicus	F: 5′-AGTACATTGAGCGCCTACAGGCCTT-3′ R: 5′-ACCCAGCCTGACAGACAATCTCAGTT-3′
Myogenin	*Mus musculus*	F: 5′-AGTACATTGAGCGCCTACAGGCCTT-3′ R: 5′-ACCCAGCCTGACAGACAATCTCAGTT-3′
	Rattus norvegicus	F: 5′-AGGAGAGAAAGATGGAGTCCAGAG-3′ R: 5′-TAACAAAAGAAGTCACCCCAAGAG-3′
MCK	*Mus musculus*	F: 5′-CCGTTCGGCAACACCCACAACA-3′ R: 5′-TCCAGGTCGTCTCCACCCTTGA-3′
	Rattus norvegicus	F: 5′-ACTACAAGCCTCAGGAGGAGTA-3′ R: 5′-CTTATCGCGAAGCTTATTGTAG-3′
Myf6	*Mus musculus*	F: 5′-ACTAAAGAAAATCAACGAAGCCTTTGA-3′ R: 5′-AAATACTGTCCACGATGGAAGAAA-3′
	Rattus norvegicus	F: 5′-CTTAAGAAAATCAACGAAGCCTTTGA-3′ R: 5′-AAATACTGTCCACGATGGAAGAAA-3′
GAPDH	*Mus musculus*	F: 5′-GCATGGCCTTCCGTGTTCCT-3′ R: 5′-CCCTGTTGCTGTAGCCGTATTCAT-3′
	Rattus norvegicus	F: 5′-TGCACCACCAACTGCTTAG-3′ R: 5′-GATGCAGGGATGATGTTC-3′

[a] F, forward; R, reverse.

ing to nucleotides −2180 to −1876 of the mouse myogenin gene distal promoter were PCR-amplified. The primer sequences were as follows: myogenin gene promoter (forward, 5′-AGGGTGGGGTGGGGGCAAAA-3′; reverse, 5′-CGGGGGCTCGAAGCCCTGAA-3′). Myogenin gene distal promoter region (forward, 5′-GCCTCAGTGTGTCCCAATTT-3′; reverse, 5′-TCTGTCCTGCTATCCCTGCT-3′). The amplification reaction was performed under the following conditions: 35 cycles of denaturation at 94 °C, annealing at 58 °C, and extension at 72 °C. Disassociation curves were generated after each PCR run to ensure that a single product of appropriate length was amplified. The mean $C_t \pm$ S.E. were calculated from individual C_t values obtained from triplicate determinations per stage. The normalized mean C_t was estimated as ΔC_t by subtracting the mean C_t of input from that of myogenin gene.

Immunoprecipitation—For the MyoD interaction assays, the transfected cells were lysed for 1 h in lysis buffer at 4 °C. After the cells were immunoprecipitated with anti-SETD3 antibodies overnight at 4 °C, protein A/G-agarose beads (GeneDEPOT) were added for 3 h with rotation at 4 °C. The bound proteins were then analyzed via immunoblotting with anti-GFP (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-MyoD (Millipore), and anti-IgG (Santa Cruz Biotechnology) antibodies. To generate SETD3 rabbit polyclonal antibody, we used TFDGKREDYFPDL (amino acids 70–82 of mouse SETD3) as immunogens.

In Vitro Transcription and Translation Reactions—For *in vitro* transcription and translation, ^{35}S-labeled SETD3 proteins were constructed using a coupled transcription and translation (TNT) system (Promega). In brief, 1 μg of DNA was added directly to TNT rabbit reticulocyte lysates, and then permitted to react for 2 h at 30 °C. GST and GST-MyoD purified proteins were incubated overnight with 10 μl of radioactive TNT reaction in reaction buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Triton X-100). Beads were washed five times with wash buffer (50 mM Tris, pH 7.6, 300 mM NaCl, 0.5% Triton X-100) and resuspended, after which the proteins were resolved by SDS-PAGE and visualized by autoradiography.

Immunocytochemistry—C2C12 cells were seeded in 4-well chamber slides and transiently co-transfected with *pEGFP-SETD3* and *pcDNA6-MyoD*. After 2 days, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. After blocking with 1% BSA, cells were incubated with anti-MyoD antibody, followed by incubation with Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories). Then slides were then mounted with GEL/MOUNT (Biomeda), and resulting images were examined by confocal laser scanning microscopy (Zeiss LSM5 Exiter).

RESULTS

Bioinformatics Screening and Cloning—SETD3 was predicted to be located on the 14th chromosome in human (ENSG00000183576) and on the 12th chromosome in mouse (ENSMUSG00000056770). The predicted full-length SETD3 was 594 amino acids, and several shorter transcript isoforms were also predicted. In addition to the SET domain, SETD3 was shown to harbor the Rubis-subs-bind domain, also referred to as the Rubisco LSMT substrate binding domain (Fig. 1*A*). This structure plays an important role in binding to the N-terminal tails of histones H3 and H4 (24). No other short variants included the Rubis-subs-bind domain, suggesting weaker substrate binding affinity compared with that of full-length SETD3. We cloned the full-length variant mouse SETD3, and *SETD3* deletion mutants were constructed that lacked either a SET domain (SETD3-C) or Rubis-subs-bind domain (SETD3-N) (Fig. 1*A*).

Tissue Distribution of SETD3—Initially, we evaluated the pattern of SETD3 expression in the tissues of 8-week-old mice using SETD3 antibodies. SETD3 was prominently expressed in the heart and skeletal muscles and was also detected weakly in the stomach, small intestine, and colon (Fig. 1*B*), thereby suggesting that SETD3 may perform a function in the muscle environment. Shorter isoforms were also detected in the lung and skeletal muscles, but the expression levels were relatively low.

Novel HMTase Activity of SETD3—To determine whether or not SETD3 might have HMTase activity, we conducted an *in vitro* HMTase assay with GST-SETD3 protein. As shown in Fig.

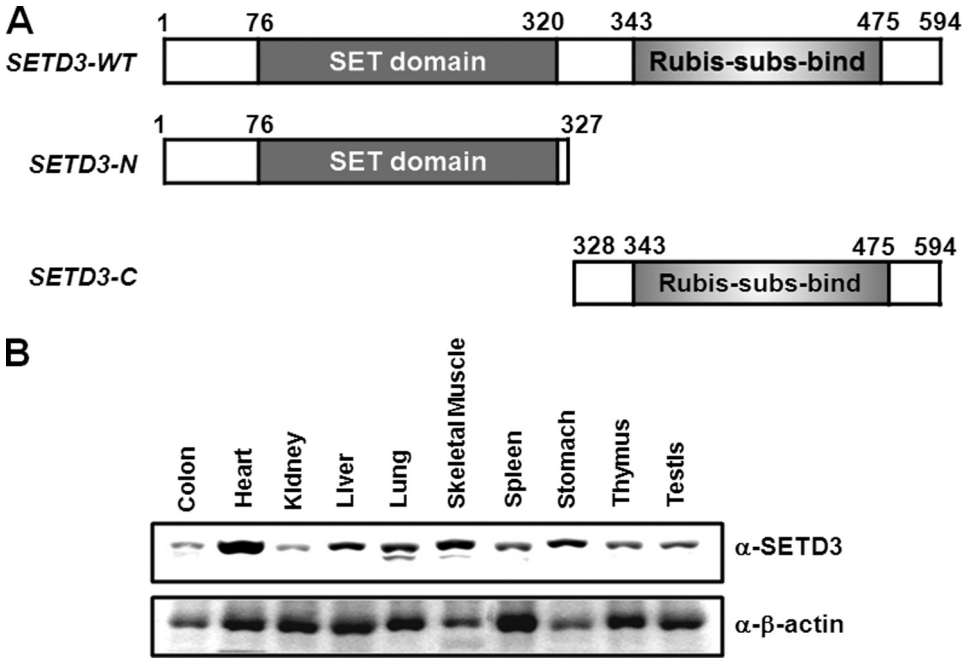

FIGURE 1. **Domain structure of SETD3 and tissue distribution.** *A*, schematic representation of *SETD3* deletion mutants. Full-length *SETD3* containing SET and Rubis-subs-bind domains is shown. *B*, mouse adult tissue blot hybridized with anti-SETD3 antibodies. β-Actin was used as a control.

2*A*, we were able to detect the HMTase activity of SETD3 on core histones; this activity was determined to be dose-dependent. The SET domain lacking SETD3-C completely lost HMTase activity. However, the Rubis-subs domain deletion mutant SETD3-N demonstrated weak HMTase activity, suggesting that the Rubis-subs-bind domain is required for HMTase activity (Fig. 2*A*). We next performed an HMTase assay with histone H3 and H4 peptides wherein each six amino acid-peptide harbored only one lysine residue. SETD3 demonstrated profound methylation activity toward the histone H3 peptides, H3N1 and H3N4, indicating that histones H3K4 and H3K36 are the primary methylation target residues (Fig. 2*B*). To further characterize the lysine specificity of SETD3, the histone peptides were analyzed via LC-MS spectrometry after *in vitro* HMTase assay. We detected mono- and dimethylated H3N1 peptides as well as mono- and dimethylated H3N4 peptides. The molecular mass of unmethylated H3N1 peptide was determined to be 775.3 Da, whereas the mono- and dimethylated peptides evidenced peaks at 791.1 and 806.2 Da, respectively. For the H3N4 peptides (532.2 Da), we observed both a monomethylated peak at 548.2 Da as well as a weak dimethylated peak at 563.1 Da (Fig. 2*C*). By way of contrast, the H3N2 (H3K9) peptides evidenced no peak changes (data not shown). We further evaluated the methylation specificity of SETD3 toward H3K4 and H3K36 *in vivo* by conducting Western blotting using specific antibodies in SETD3-overexpressing cells. Following SETD3 overexpression, the nucleosome extracts were probed with dimethylated lysine 4, 9, 27, and 36 H3 antibodies. Consistent with our mass spectrometry results, the dimethylation of H3K4 and H3K36 increased in the SETD3-overexpressing cells to a level relative to that of mock-transfected cells (Fig. 2*D*). Deletion of the SET domain (SETD3-C) but not Rubis-subs-bind domain (SETD3-N) failed to dimethylate H3K4 and H3K36 (Fig. 2*E*). Collectively, these findings demonstrate that SETD3 exerts specific HMTase activity in the mono- and dimethylation of H3K4 and H3K36 under both *in vitro* and *in vivo* conditions.

SETD3 Induces Transcriptional Activation—It was noted previously that histone H3K4 HMTase SMYD1 functions as a transcription activator, playing an essential role in myogenesis and cardiogenesis in different species (6, 25–27). To determine whether or not methylation of H3K4 and H3K36 by SETD3 can be attributed to a general transcription activation effect similar to that of SMYD1, we conducted a transient transfection assay using the *CMX-GAL4-SV40* reporter system. The transfection of *pcDNA3.1-SETD3* into NIH3T3 and C2C12 skeletal myoblast cells resulted in the activation of luciferase activity in a dose-dependent manner, inducing up to a 3-fold increase in basal transcription (Fig. 3, *A* and *C*). A reduction in transcription with *sh-SETD3* confirmed the role of SETD3 in *SV40* transactivation (Fig. 3*C*). To evaluate the direct effects of SETD3 on transcriptional regulation, we fused SETD3 to the DNA binding domain of GAL4 and conducted a transfection assay with a GAL4-dependent luciferase reporter. The GAL4-SETD3 fusion protein activated basal transcription of the reporter gene, showing that the direct recruitment of GAL4-SETD3 to the GAL4 binding site-containing promoter could mediate transcriptional activation (Figs. 3, *B* and *D*). These findings suggest that SETD3 may activate the transcription of target genes in muscle cell differentiation.

SETD3 Induces Muscle Differentiation—Next, we attempted to investigate the *in vivo* function of SETD3 in muscle cells because SETD3 was expressed abundantly in skeletal muscle tissues (Fig. 1*B*). The differentiation of C2C12 cells was induced by incubation for 12 days with 1% FBS, and the level of SETD3 expression was monitored. SETD3 expression gradually increased for up to 12 days during muscle cell differentiation (Fig. 4*A*). To determine whether or not SETD3 activates known

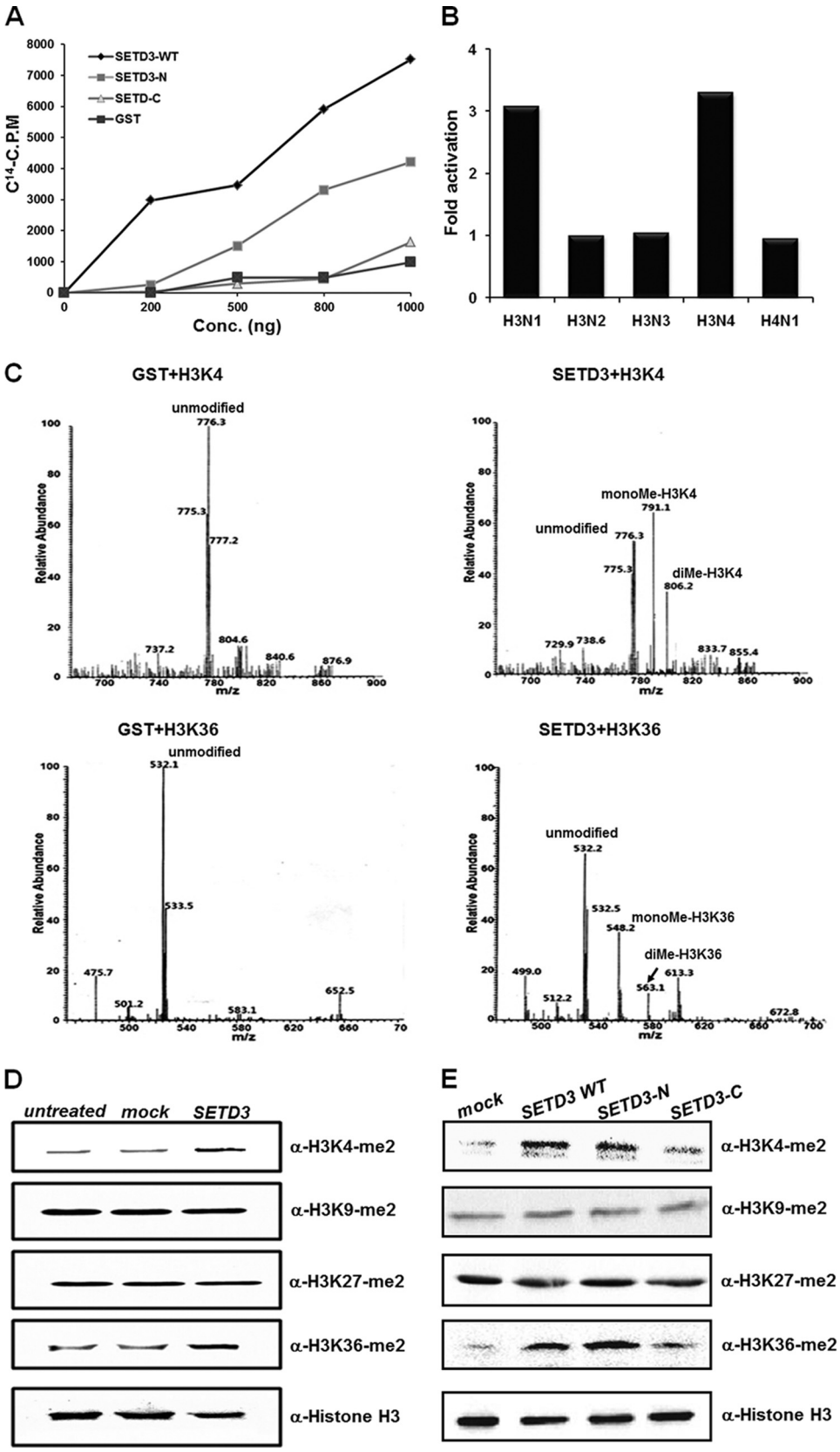

FIGURE 2. **SETD3 is a novel histone H3-specific methyltransferase.** *A* and *B*, scintillation counting for HMTase activity of SETD3 deletion mutants. Core histones (*A*) and synthesized histone peptides (*B*) were used as substrates in the HMTase assay. Methylation levels were quantified via filter binding assay, and the data are expressed as raw counts per minute (C.P.M.) incorporated. *C*, methylated peptide samples analyzed by LC-MS. *D* and *E*, extracts of cells transfected with the indicated SETD3 and deletion mutant constructs Western blotted with anti-H3K4-me2, anti-H3K9-me2, anti-H3K27-me2, anti-H3K36-me2, and anti-histone H3 antibodies.

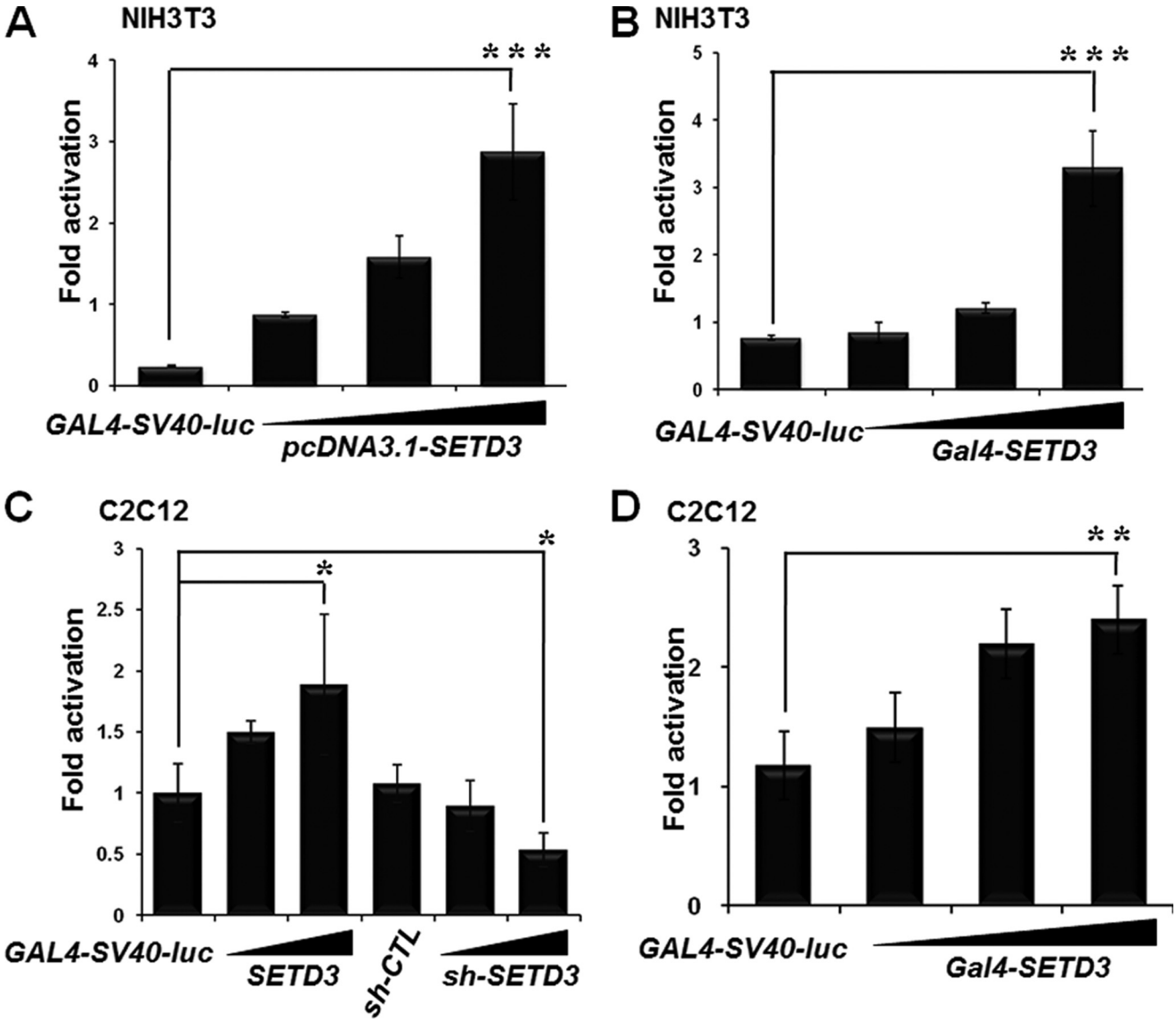

FIGURE 3. **SETD3 induces transcriptional activation.** *A* and *C*, *pcDNA3.1-SETD3*, *sh-SETD3*. *B* and *D*, GAL4 DNA-binding domain-tagged SETD3 constructs employed for the reporter luciferase assay. NIH3T3 cells (*A* and *B*) and C2C12 cells (*C* and *D*) were transiently transfected with *GAL4-SV40-luc* and increasing concentrations of SETD3. The amount of DNA in the transfection was maintained at a constant level using *pcDNA3.1* empty vector. Exactly 48 h after transfection, cells were lysed with lysis buffer and assayed for luciferase assay. Levels of β-galactosidase activity and protein concentration were employed for normalization of reporter luciferase and β-galactosidase activities, respectively. Each value is the mean of quadruplicate determinations of a single assay. The results are shown as means ± S.D. (*error bars*); $n = 5$. *Asterisks* indicate statistical significance (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$) compared with reporter plasmid alone or *sh-CTL*.

muscle cell differentiation markers, we transiently overexpressed SETD3 and analyzed gene expression via real-time PCR. When SETD3 was overexpressed under growth conditions, the levels of muscle differentiation markers such as myogenin, *MCK*, and *Myf6* increased dramatically in H9c2 cells, and MCK was increased in C2C12 cells (Fig. 4*B*). To investigate further whether or not SETD3 affects muscle gene expression, we stably knocked down SETD3 using shRNA. Significant reduction in the expression of muscle differentiation marker genes (myogenin, *MCK*, *Myf5*, and *Myf6*) was observed when SETD3 was stably knocked down by shRNA in H9c2 and C2C12 cells (Fig. 4*C*). *MyoD* and Desmin gene expression was also decreased in SETD3-knocked down C2C12 cells (data not shown). Our findings demonstrate that SETD3 activates the transcription of muscle differentiation target genes and induces muscle differentiation. The endogenous role of SETD3 was further corroborated by the observation that three independent *sh-SETD3* clones all blocked muscle differentiation (Fig. 4*D*). The morphology of sh-SETD3-treated cells showed no indication of cell elongation, which is the result of myoblast fusion and multinucleation (data not shown). This suggests that SETD3 knockdown-mediated inhibition of myogenesis is partly due to the blockage of myoblast fusion.

SETD3 Regulates Myogenin Transcription through Interaction with MyoD—MyoD functions as a myogenesis regulator and controls the expression of muscle cell differentiation marker genes. To determine whether or not SETD3 can regulate the transcription of a target gene, myogenin, we examined its promoter activity using a luciferase reporter system. First, we co-transfected the myogenin promoter reporter gene and

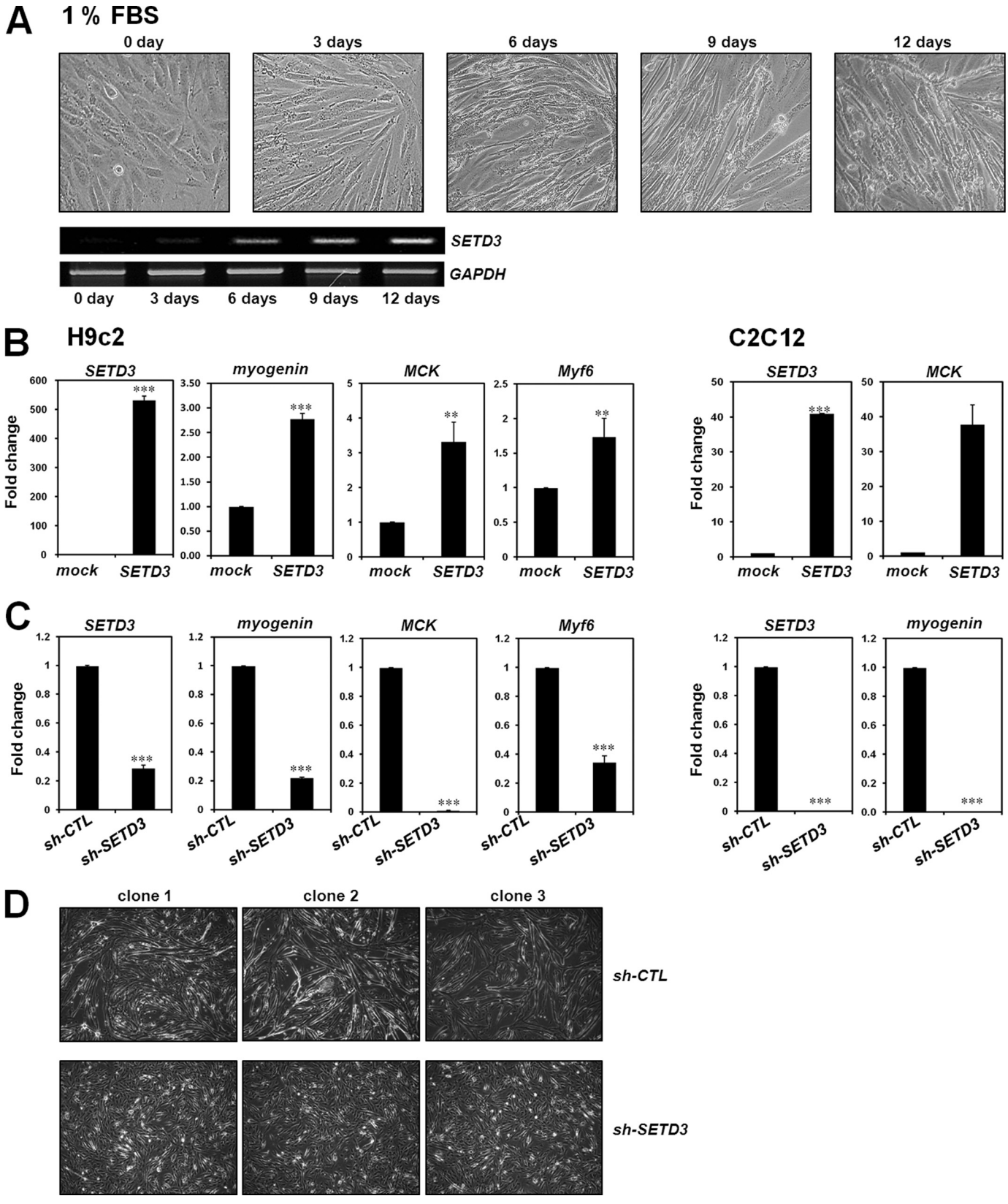

FIGURE 4. **SETD3 promotes muscle cell differentiation.** *A*, phase-contrast images at indicated time points (days) of C2C12 cells (*upper panel*) and the expression levels of *SETD3* detected via RT-PCR (*lower panel*). GAPDH was used as a control. *B*, H9c2 and C2C12 cells transfected with *pcDNA3.1-V5-SETD3* under GM conditions. Expression levels of myogenin, *MCK*, and *Myf6* in H9c2 and C2C12 cells were determined via real-time PCR. *C*, expression levels of myogenin, *MCK*, and *Myf6* in H9c2 and C2C12 cells with stably knocked-down SETD3 determined by real-time PCR. *D*, phase-contrast images taken 3 days after transfection of *sh-SETD3* into H9c2 cells.

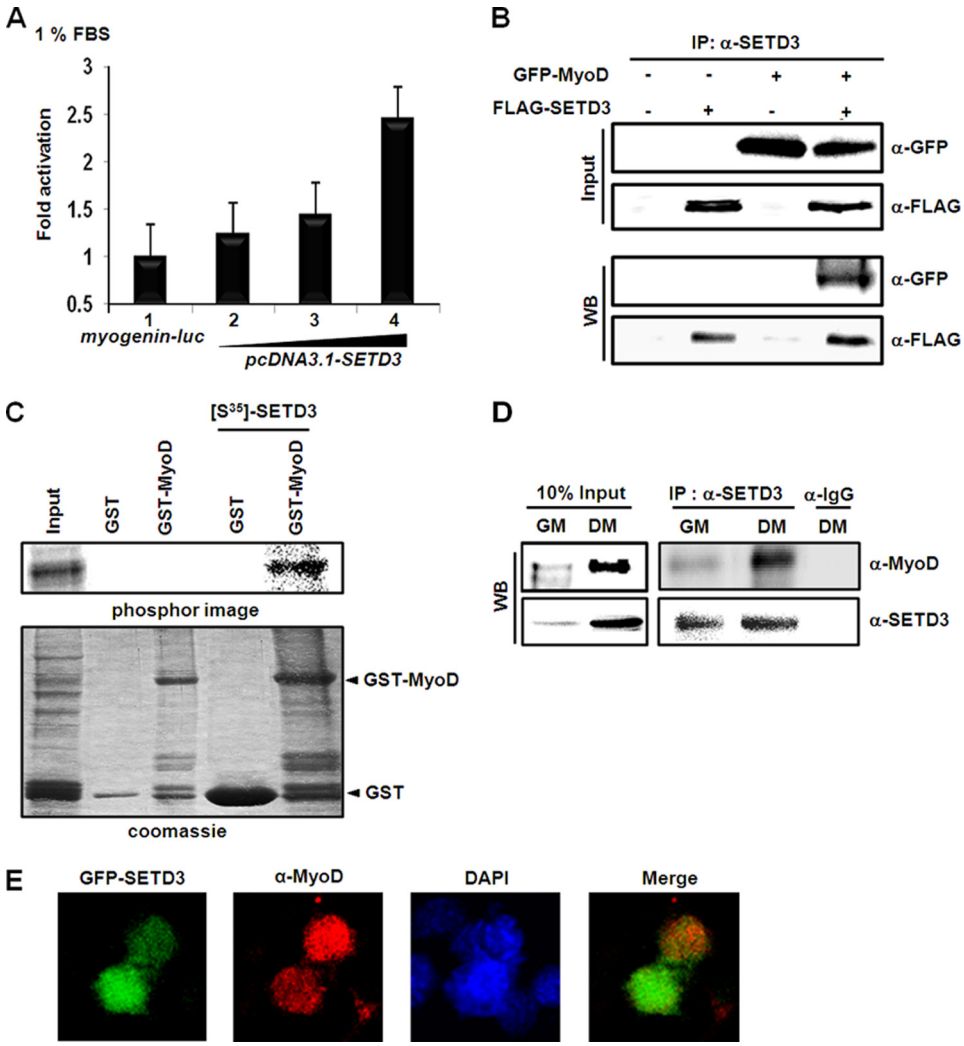

FIGURE 5. **SETD3 interacts with MyoD and is recruited to myogenin gene promoter.** *A*, C2C12 cells were transfected with the *pGL3*-myogenin promoter (100 ng) or *pcDNA3.1-SETD3* (none, 100, 300 ng). *B*, C2C12 cells were co-transfected with FLAG-tagged *SETD3* and GFP-tagged *MyoD* constructs as indicated, and SETD3 immunoprecipitates were analyzed via immunoblotting with anti-FLAG and anti-GFP antibodies. *C*, SETD3 was *in vitro* transcribed/translated and then incubated with purified GST-MyoD and GST bound to beads. GST and GST-MyoD were incubated without ^{35}S-labeled SETD3 (*second* and *third lanes*). Amounts of GST-MyoD proteins were determined via Coomassie staining. Pulled-down proteins were analyzed via SDS-PAGE with a PhosphorImager. *D*, physical interaction of endogenous proteins in C2C12 cells under GM and DM conditions is shown. *E*, C2C12 cells co-transfected with *pEGFP-SETD3* and *pcDNA6-MyoD* were immunostained with antibodies against MyoD. DAPI staining represents nucleus. *F*, schematic diagram shows primer pairs in ChIP assay and real-time PCR analysis (*upper panel*). ChIP analyses were conducted using control IgG, anti-SETD3, and anti-MyoD antibodies prior to or after 48 h of differentiation of C2C12 cells. Immunoprecipitated DNA fragments were amplified via real-time PCR from the myogenin gene promoter region. Recruitment of MyoD and SETD3 to the myogenin promoter region and distal promoter region was normalized to input. *G*, recruitment of MyoD and SETD3 to the myogenin promoter and distal region was analyzed via ChIP assay and real-time PCR using *si-MyoD*-treated C2C12 cells. *H*, ChIP assay and real-time PCR were used to measure the methylation status of histones H3K4-me2, H3K9-me2, H3K27-me2, and H3K36-me2 in the myogenin promoter region and distal promoter region prior to and after 48 h of differentiation of C2C12 cells under DM conditions. All data are expressed as means ± S.D. (*error bars*); $n = 3$. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

SETD3 into C2C12 cells. As the concentration of SETD3 increased, the luciferase activity increased up to a level of 2.5-fold (Fig. 5*A*). Previous research suggested that MyoD regulates myogenesis via interaction with HMTase Suv39h1 by methylating H3K9 on the myogenin gene promoter (16). Thus, we decided to test the interaction of SETD3 with MyoD during muscle differentiation. Initially, we evaluated the interaction between SETD3 and MyoD via immunoprecipitation (IP) assay. We overexpressed *FLAG-SETD3* and *GFP-MyoD* in C2C12 cells and conducted IPs using anti-SETD3 antibodies. Immunoblotting with anti-GFP antibodies confirmed the interaction between SETD3 and MyoD (Fig. 5*B*). To confirm further the direct interaction between SETD3 and MyoD, *in vitro* transcription was conducted, after which translated SETD3 was incubated with purified GST-MyoD. As anticipated, SETD3 interacted with GST-MyoD but not with GST, further verifying direct interaction between SETD3 and MyoD (Fig. 5*C*). We further analyzed the association between endogenous MyoD and SETD3 upon induction of muscle differentiation. Physical interaction of endogenous SETD3 and MyoD was confirmed in differentiated C2C12 cells (Fig. 5*D*). To determine whether or not SETD3 localized to the nucleus, we monitored SETD3 and MyoD expression by immunofluorescence analysis. As expected, overexpressed SETD3 and MyoD were present predominantly in the nucleus (Fig. 5*E*). To establish further the mechanism underlying SETD3-mediated transcriptional acti-

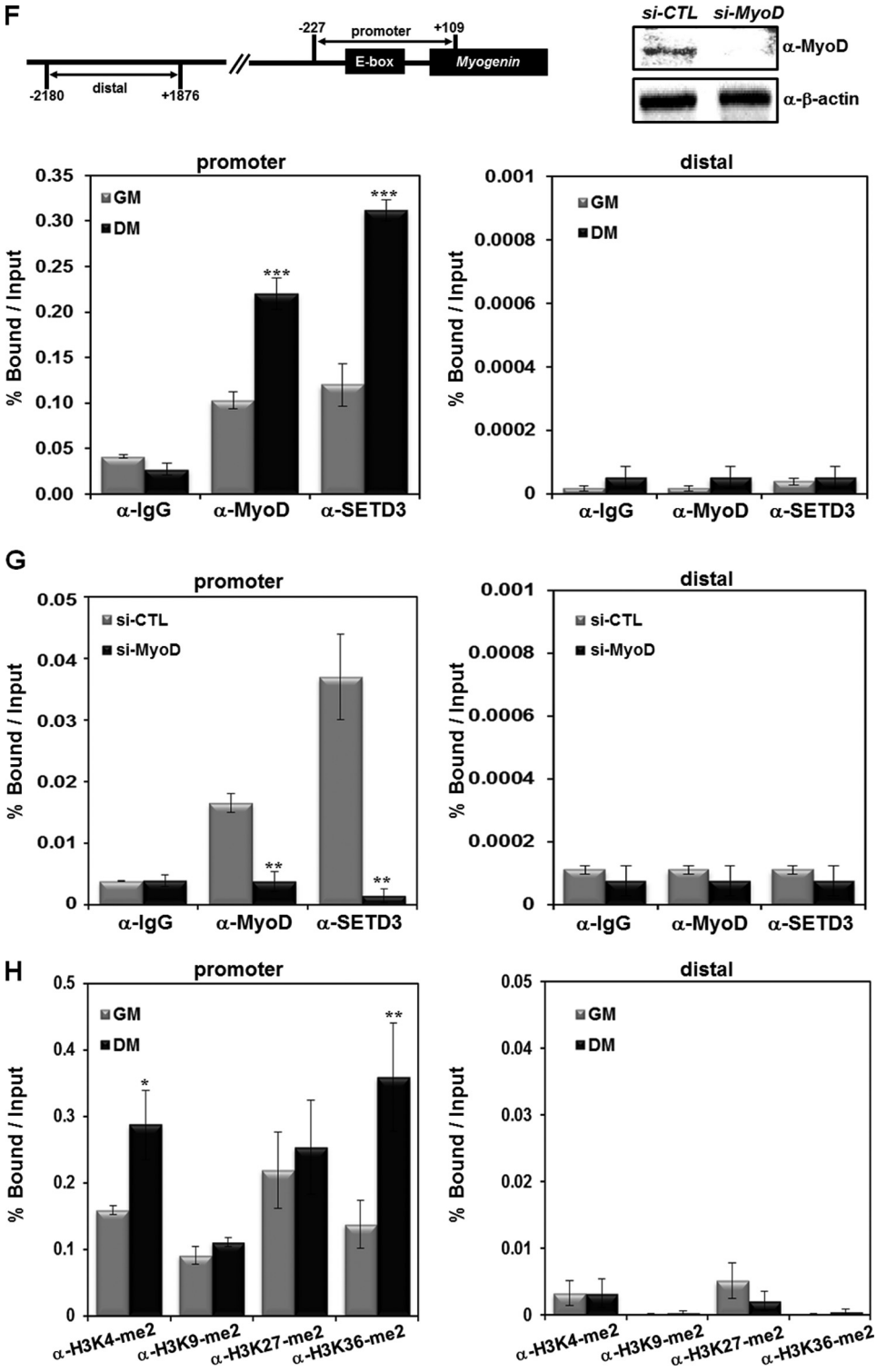

FIGURE 5—*continued*

vation of the myogenin gene promoter, we conducted ChIP analysis with real-time PCR and investigated the occupancy of the myogenin gene promoter region. IP was conducted on both undifferentiated (GM) and differentiated cells (DM). The immunoprecipitated DNA fragments were amplified via real-time PCR from the promoter region (positions −227 to +109; E-box containing) and distal promoter region (positions −2180 to −1876) of the myogenin gene. Compared with the results under GM conditions, recruitment of MyoD and SETD3 to the myogenin gene promoter region increased under DM conditions (Fig. 5*F*). The depletion of MyoD by *si-MyoD* in C2C12 cells also resulted in failed recruitment of SETD3 and MyoD to the myogenin gene promoter under DM conditions (Fig. 5*G*). Additionally, the levels of dimethylated H3K4 and dimethylated H3K36 increased up to 1.8-fold and 2.6-fold, respectively, under DM conditions, whereas the level of dimethylated

H3K9 did not increase (Fig. 5*H*). On the contrary, no changes were detected in MyoD and SETD3 recruitment, and hence histone methylation in the distal promoter region of the myogenin gene remained the same (Fig. 5, *F*, *G*, and *H*). Our findings collectively indicate that SETD3 elevates the transcription of myogenin via interaction with MyoD, and recruitment of SETD3 to the myogenin promoter further modifies the methylation status of H3K4 and K36.

DISCUSSION

In this study, we conducted a bioinformatics search for uncharacterized proteins containing the SET domain and identified a 594 amino acids protein that we named SETD3, which harbors a SET domain and Rubis-subs-bind domain. SETD3 is highly expressed in mouse muscle tissues, which suggests that it may have muscle-related functions *in vivo*.

Because a variety of histone methylations have been identified as important regulatory markers for gene expression, we next attempted to identify the specific HMTase activity of SETD3 and evaluate its effects on the transcriptional regulation of target genes. Using different *in vitro* and *in vivo* HMTase assays, we determined that SETD3 evidences H3K4 and H3K36 methyltransferase activity. The methylation of H3K4 and H3K36 is linked with transcriptional activation (28). Consistent with the results of other studies, H3K4 and H3K36-methylating SETD3 was found to activate the basal transcription of reporter genes.

Skeletal myogenesis is a developmental process that requires delicate balance between transcriptional activation and repression. It has been reported in previous studies that H3K4 HMTase SMYD1 performs a critical function in heart and skeletal muscle development (6, 25). In particular, the results of SMYD1 knockdown studies using zebrafish embryos suggest that HMTase activity is required for myofiber maturation (6, 25). SETD3 overexpression induces muscle cell differentiation as well as the expression of muscle-specific genes such as *MCK*, myogenin, and *Myf6*. Consistent results were obtained when we evaluated the regulatory role of SETD3 in the transcription of the MyoD target gene, myogenin. Per the results, the H3K4- and H3K36-methylating activity of SETD3 induced activation of the muscle-specific marker myogenin as well as muscle cell differentiation. We further determined that SETD3 and MyoD interacted each other, synergistically activating myogenin expression after recruitment to the target gene promoter.

A recent report suggesting that SMYD1 expression is regulated by serum response factor as well as myogenin further suggests the role of a complex transcriptional network in myogenesis (26). Amino acid sequence alignment between SMYD1 and SETD3 SET domains shows very low similarity (15%) between these two methyltransferases. Further research will therefore be necessary to identify the transcriptional regulatory mechanism of SETD3 in myogenesis.

Collectively, our findings provide evidence demonstrating that HMTase SETD3 plays a role in muscle differentiation via interaction with MyoD and recruitment to the target gene *myogenin* promoter. Our results linking H3K4/K36 methylation by SETD3 with muscle cell differentiation provide insights into yet another role of histone modification-mediated transcriptional regulation in muscle development.

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Histone Methyltransferase SETD3 Regulates Muscle Differentiation

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