Histone Methyltransferase SETD3 Regulates Muscle Differentiation*

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Gwang Hyeon Eom†1, Kee-Beom Kim§1, Jin Hee Kim‡1, Ji-Young Kim¶1, Ju-Ryung Kim‡, Hae Jin Kee‡, Dong-Wook Kim‡, Nakwon Choe§, Hye-Jeong Park¶, Hye-Ju Son¶, Seok-Yong Choi‡, Hyun Kook¶2, and Sang-Beom Seo‡3

From the †Department of Life Science, College of Natural Sciences, Chung-Ang University, Seoul 156-756, Republic of Korea, the §Department of Biomedical Sciences, Chonnam National University Medical School, Gwangju 501-746, Republic of Korea, and the ¶Department of Biology, Chosun University, Gwangju 510-759, Republic of Korea

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-specific genes. SETD3 is expressed abundantly in muscular tissues and, when overexpressed, activates transcription of muscle-specific genes. 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.

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 During differentiation, the cell cycle is withdrawn and muscle-specific transcription factors activated.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, deactylases (HDACs), and chromatin remodeling factors have recently been reported to regulate MyoD activity during muscle differentiation. For example, the histone acetyltransferases p300 and p300/CREB-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).
SETD3 Induces Muscle Differentiation

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 Ensembl 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 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 34734 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-meo2 (1:2000), anti-H3K9-meo2 (1:2000), anti-H3K27-meo2 (1:2000), and anti-H3K36-meo2 (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).

 Luciferase Assays—NIH3T3 and C2C12 cells were seeded in 48-well plates and transfected with Lipo-fectamine 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, 250 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 (Enzymomics). 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 (Ct) ± S.E. were calculated from individual Ct, values obtained from triplicates per stage. The normalized mean Ct was estimated as ∆Ct by subtracting the mean Ct of GAPDH from that of the muscle genes. The value ∆∆Ct was calculated as the difference between control ∆Ct and values obtained for each sample. The n-fold change in gene expression relative to untreated control was calculated as 2−∆∆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-
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′-AGGGTGGGGGTGGGGCAAA-3′; reverse, 5′-GGCCTGAAAGGCCCTGAA-3′). Myogenin gene distal promoter region (forward, 5′-GCTTACAGTGTCCTCCAAAAATC-3′; reverse, 5′-TCTGTCCCTGCTATCCCTGCT-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 Ct ± S.E. were calculated from individual Ct values obtained from triplicate determinations per stage. The normalized mean Ct was estimated as ΔCt by subtracting the mean Ct 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 (GenDEPOT) 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 TFDGKREDDYPDL (amino acids 70–82 of mouse SETD3) as immunogens.

**In Vitro Transcription and Translation Reactions**—For in vitro transcription and translation, 35S-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.

**Immunocytocytochemistry**—C2C12 cells were seeded in 4-well chamber slides and transiently co-transfected with pcEGFP-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 (Biomedia), 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 (ENSGO0000183576) 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. 1A). 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. 1A).

**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. 1B), 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.

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**TABLE 1**

Oligonucleotides used for RT-PCR

| Target mRNA | Organism     | Primer sequence 1 | Primer sequence 2 |
|-------------|--------------|-------------------|-------------------|
| SETD3       | Mus musculus | F: 5′-GGCTCGAAGCCCTGAA-3′ | R: 5′-AGGAGAAGAAGAAGGAGG-3′ |
| Myogenin    | Mus musculus | F: 5′-AGAGGACGCCCTGAA-3′ | R: 5′-ACCCGATCGGAGAAGAAGGAGG-3′ |
| MCK         | Mus musculus | F: 5′-AGAGGACGCCCTGAA-3′ | R: 5′-ACCCGATCGGAGAAGAAGGAGG-3′ |
| Myf6        | Mus musculus | F: 5′-AGAGGACGCCCTGAA-3′ | R: 5′-ACCCGATCGGAGAAGAAGGAGG-3′ |
| GAPDH       | Mus musculus | F: 5′-GGCTCGAAGCCCTGAA-3′ | R: 5′-AGGAGAAGAAGAAGGAGG-3′ |

*F, forward; R, reverse.*
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. 2A). 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. 2B). 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. 2C). 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 vitro 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. 2D). Deletion of the SET domain (SETD3-C) but not Rubis-subs-bind domain (SETD3-N) failed to dimethylate H3K4 and H3K36 (Fig. 2E). 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. 3C). 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. 1B). 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. 4A). 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.
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. 4B). 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. 4C). 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. 4D). 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 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.
SETD3 into C2C12 cells. As the concentration of SETD3 increased, the luciferase activity increased up to a level of 2.5-fold (Fig. 5A). 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-FLAG antibodies confirmed the interaction between SETD3 and MyoD (Fig. 5B). 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. 5C). We further analyzed the association between endogenous MyoD and SETD3 upon induction of muscle differentiation. 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.
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. 5F). The depletion of MyoD by $\text{si-MyoD}$ in C2C12 cells also resulted in failed recruitment of SETD3 and MyoD to the myogenin gene promoter under DM conditions (Fig. 5G). 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
SETD3 overexpression induces muscle cell differentiation that HMTase activity is required for myofiber maturation (6, 20). In particular, the results of our muscle development (6, 25). In particular, the results of our previous studies that 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 and K36 methylation status of H3K4 and K36.

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.

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