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## The Protein Arginine Methyltransferase Prmt5 Is Required for Myogenesis because It Facilitates ATP-Dependent Chromatin Remodeling<sup>∇</sup>

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**Skeletal muscle differentiation requires the coordinated activity of transcription factors, histone modifying enzymes, and ATP-dependent chromatin remodeling enzymes. The type II protein arginine methyltransferase Prmt5 symmetrically dimethylates histones H3 and H4 and numerous nonchromatin proteins, and prior work has implicated Prmt5 in transcriptional repression. Here we demonstrate that MyoD-induced muscle differentiation requires Prmt5. One of the first genes activated during differentiation encodes the myogenic regulator myogenin. Prmt5 and dimethylated H3R8 (histone 3 arginine 8) are localized at the myogenin promoter in differentiating cells. Modification of H3R8 required Prmt5, and reduction of Prmt5 resulted in the abrogation of promoter binding by the Brg1 ATPase-associated with the SWI/SNF chromatin remodeling enzymes and all subsequent events associated with gene activation, including increases in chromatin accessibility and stable binding by MyoD. Prmt5 and dimethylated H3R8 were also associated with the myogenin promoter in activated satellite cells isolated from muscle tissue, further demonstrating the physiological relevance of these observations. The data indicate that Prmt5 facilitates myogenesis because it is required for Brg1-dependent chromatin remodeling and gene activation at a locus essential for differentiation. We therefore conclude that a histone modifying enzyme is necessary to permit an ATP-dependent chromatin remodeling enzyme to function.**

Gene expression is a highly regulated process that frequently requires coordinated function between transcription factors and chromatin remodeling enzymes. These enzymes are divided into two classes: ATP-dependent remodelers that hydrolyze ATP and alter nucleosome structure and histone modifiers that covalently modify specific histone residues posttranslationally. The activation of skeletal muscle differentiation is regulated by members of the basic helix-loop-helix family of tissue-specific transcription factors, including MyoD, Myf5, Mrf4, and myogenin, as well as by members of the Mef2 family of transcriptional regulators, which act cooperatively with basic helix-loop-helix proteins (8, 34, 43). Numerous chromatin remodeling enzymes have been shown to both positively and negatively affect myogenic gene expression. These include histone acetyl transferases; types I, II; and III histone deacetylases; histone lysine methyltransferases; and members of the SWI/SNF family of ATP-dependent remodeling enzymes (17, 47, 50). The relationships between the different classes of chromatin remodeling enzymes during myogenesis have been largely unexplored.

The protein arginine methyltransferases (PRMTs) are an additional class of enzymes that can be linked to histone modification and gene regulation during skeletal muscle differentiation. This family has 9 members (Prmt1 to Prmt9), six of

which have been shown to generate asymmetric (type I) or symmetric (type II) dimethylarginine and to affect a range of cellular processes through arginine methylation of substrate proteins (6, 50). Of particular note, the Prmt4 enzyme, also called Carm1, has been linked to skeletal muscle differentiation and to control of estrogen-mediated gene activation via methylation of histones H3 and H4 (5, 9, 12, 53). Prmt4 functions at estrogen-induced promoters as part of a multiprotein complex that also contains the Brg1 ATPase of SWI/SNF chromatin remodeling complexes (53). Another family member, Prmt5, has also been isolated as part of an enzymatic complex containing Brg1, though in this case the complex is associated with transcriptional repression of genes involved in growth control and tumor suppression (41, 42). Other studies indicated that Prmt5 acts as a repressor of cyclin E (21, 44). Prmt5 was also shown to associate with and methylate the elongation factor Spt5, which decreased this protein's affinity for RNA polymerase II and impeded transcriptional elongation (30). Thus, Prmt5 negatively affects gene expression via symmetric arginine methylation of both histones and components of the transcriptional machinery. Prompted by association between Prmt5 and Brg1 as well as by the fact that Prmt4 is involved in the transcriptional regulation of myogenic genes, we sought to determine whether Prmt5 contributes to skeletal muscle differentiation.

Previous work described NIH 3T3-based cell lines that constitutively express a Prmt5 antisense vector and thereby cause a significant reduction in Prmt5 mRNA and protein levels (41). Ectopic expression of MyoD in fibroblast cells induces the

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myogenic differentiation program (13); this system has been extensively utilized to examine the mechanisms of skeletal muscle gene regulation for nearly 20 years (50). Using this system, we determined that cells containing reduced levels of Prmt5 failed to activate myogenic gene expression upon introduction of MyoD. Detailed examination of the events leading to activation of the myogenin locus, the production of which is necessary for the activation of myogenic late genes that encode structural and functional skeletal muscle proteins, determined that Prmt5 dependent dimethylation of histone 3 arginine 8 (H3R8) was required for the interaction of the SWI/SNF ATPase Brg1, for chromatin remodeling of the locus, and for all subsequent events leading to gene activation. We also present evidence that Prmt5 interacts with the myogenin promoter in activated satellite cells isolated from adult skeletal muscle tissue, which further supports the conclusion that Prmt5 functions in myogenic gene activation and is necessary for the induction of skeletal muscle differentiation. Thus, we have determined that a histone methyltransferase is necessary for the function of an ATP-dependent chromatin remodeling enzyme during tissue differentiation.

#### MATERIALS AND METHODS

**Cell culture.** Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 2 mM L-glutamine. Prmt5 antisense lines were maintained in the presence of 2.5  $\mu$ g/ml puromycin, since the antisense vector encodes a puromycin resistance gene (41). Cells were differentiated as previously described (15, 18) except that the pBABE-MyoD retroviral construct was modified to contain a blasticidin resistance gene instead of one for puromycin. Briefly, cycling cells were split so that they would be 75% confluent 24 h later. At that time, retroviral infection with the MyoD encoding retrovirus was performed for 30 h. Subsequently, differentiation medium (Dulbecco's modified Eagle's medium containing 2% horse serum, 2 mM L-glutamine, 5  $\mu$ g/ml blasticidin, 2  $\mu$ g/ml puromycin, and 10  $\mu$ g/ml insulin) was added to the cells, and samples were collected at the indicated times. Control samples from both NIH 3T3 cells and antisense lines were mock infected and subjected to the differentiation protocol and are designated "mock differentiated."

B22 cells expressing a Flag-tagged, dominant negative version of the SWI/SNF ATPase subunit BRG1 in the absence of tetracycline were described previously (14). Cycling cells were grown in the presence or absence of tetracycline for 3 days, were split to be 50 to 60% confluent 24 h later, and then were subjected to the differentiation protocol described above.

**mRNA analysis.** RNA was isolated from mock- or MyoD-differentiated samples using Trizol (Invitrogen) and reverse transcribed as previously described (16). The cDNA was amplified using a QIAGEN HotStarTaq Master Mix kit (QIAGEN) containing 0.1  $\mu$ g of specific primers and SYBR green. Reverse transcription-PCR (RT-PCR) and real-time PCR were performed using procedures and primers described previously (10, 39, 51). The dystrophin primer set was 5'-AAG TTT GGA AAG CAA CAC ATA-3' and 5'-GTT CAG GGC ATG AAC TCT TG-3'. Prmt5 primers were 5'-GAT GGC GGC GAT GGC A-3' and 5'-CTG TGT GTG TAG TCG G-3'. All data sets are the average  $\pm$  standard deviation of three or more independent experiments.

**Fluorescence-activated cell sorting (FACS).** Cells were differentiated or mock differentiated as stated above and fixed as previously described (29). Propidium iodide incorporation was measured by flow cytometry to determine the percentage of cells in each phase of the cell cycle.

**Protein extracts, Western analysis, and antibodies.** Whole-cell extracts were generated as previously described (18, 20). For Western analysis, 100  $\mu$ g of each extract was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to nitrocellulose. Coimmunoprecipitation procedures were previously described (16, 39). Antibodies used for chromatin immunoprecipitation (ChIP) included polyclonal rabbit antisera against Prmt5 and dimethylated H3R8 (41, 42), Brg1 (14), Flag epitope, and MyoD (16). Western analysis also made use of commercial antibodies against Prmt5/JBP1/Skb1Hs (catalog no. 611538; BD Biosciences) and MyoD (catalog no. 554130 and 554099; BD Biosciences).

**ChIP.** ChIPs were performed by modifying the Upstate protocol as previously described (46). Cultured cells or nuclei isolated from tissues (see below) were cross-linked in 1% formaldehyde and lysed in buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1. Samples were incubated on ice, and the DNA was sheared by sonication to obtain an average length of 500 bp. A total of 100  $\mu$ g of sonicated DNA was diluted 10-fold in immunoprecipitation buffer (0.01% SDS, 1.1% Triton-X100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml pepstatin A) and precleared with a 50% slurry of protein A beads (Amersham) at 4°C for at least 1 h. Cleared lysates were incubated with antibodies described above at 4°C for 4 h or overnight. Protein A beads were added to precipitate immune complexes from the cell lysates and incubated for 1 h at 4°C. Beads were collected by centrifugation and then washed as previously described (46), and immune complexes were eluted from the beads using 1% SDS. Cross-links were reversed, and DNA was purified by a Qiaquick PCR purification kit (QIAGEN). Analysis of immunoprecipitated DNA was performed by PCR amplification (16) or by quantitative PCR (QPCR) using a Quantitect SYBR Green PCR kit. Primers were described previously (16, 39). Chromatin reimmunoprecipitation (re-ChIP) experiments were performed as previously described (36, 39). QPCR data are the average  $\pm$  standard deviation of three independent experiments.

**REAA.** Restriction enzyme accessibility assay (REAA) and detection by a modified version of ligation-mediated PCR were described previously (16, 39). Data are the average  $\pm$  standard deviation of three independent experiments.

**Isolation of satellite cells and myofibers from mouse skeletal muscle.** Skeletal muscle was dissected from the upper hind limbs of 4- to 6-week-old C57/BL6 mice and minced to approximately 1 mm<sup>3</sup>. Tissues were then digested using 87.5 U/ml collagenase type II (Invitrogen) in phosphate-buffered saline supplemented with 1 mM CaCl<sub>2</sub> and incubated at 37°C for 1 h with agitation (3, 11). Satellite cells were separated from mature myofibers by filtration with a cell strainer (70 $\mu$ m; Becton Dickinson catalog no. 352350) (40). Flowthrough material was enriched for satellite cells, while material that did not pass through the filter was enriched for myofibers. Aliquots were taken for isolation of RNA as described above. Satellite and myofiber preparations were kept on ice throughout the process. The separated fractions were separately pelleted by centrifugation and resuspended in 7 volumes of lysis buffer (10 mM HEPES-KOH (pH 7.3), 10 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 3  $\mu$ g/ml cytochalasin B, 10  $\mu$ g/ml leupeptin). Samples were homogenized and dounced using pestle A to release nuclei, and then samples were incubated on ice for 30 min. Nuclei release and integrity were checked under a light microscope with Hoechst 33258 dye, and the samples were then centrifuged to remove cell debris. Nuclei were resuspended in 2.5 volumes of 10 STM buffer (5 mM MgCl<sub>2</sub>, 10 mM triethanolamine pH 7.5, 5 mM MgCl<sub>2</sub>, 10% sucrose, 10  $\mu$ g/ml leupeptin) and subsequently 2 volumes of 2 M sucrose-10 mM Tris-HCl-5 mM MgCl<sub>2</sub>. In an ultracentrifuge tube (Beckman catalog no. 344057), 750  $\mu$ l of the 2 M sucrose-10 mM Tris HCl-5 mM MgCl<sub>2</sub> mixture was aliquoted and overlaid with the nuclei mixture described above. Samples were ultracentrifuged at 116,140  $\times$  g for 1 h. The supernatant was aspirated and discarded, and the pellet was resuspended in 500  $\mu$ l of lysis buffer containing 0.1% NP-40. Following cross-linking with 2% formaldehyde at room temperature, nuclei were centrifuged at 13,500  $\times$  g, pellets were frozen in liquid nitrogen, and samples were stored at -80°C or immediately thawed, resuspended in lysis buffer described above, and sonicated.

#### RESULTS

**Transcription of skeletal muscle genes is significantly impaired in cells with reduced levels of Prmt5.** Cooperation between myogenic transcription factors and chromatin remodeling enzymes is needed to properly regulate the transcription of muscle-specific genes. To address the function of the Prmt5 arginine methyltransferase in skeletal muscle differentiation, we utilized two independently derived NIH 3T3 cell lines that express a Prmt5 antisense vector. These cell lines were previously shown to have significantly decreased levels of this protein and its corresponding mRNA (41).

In vitro skeletal muscle differentiation was initiated by infection of both the control and antisense lines (c15 and c12 cells) with a retrovirus encoding MyoD (15, 38) for 30 h. After

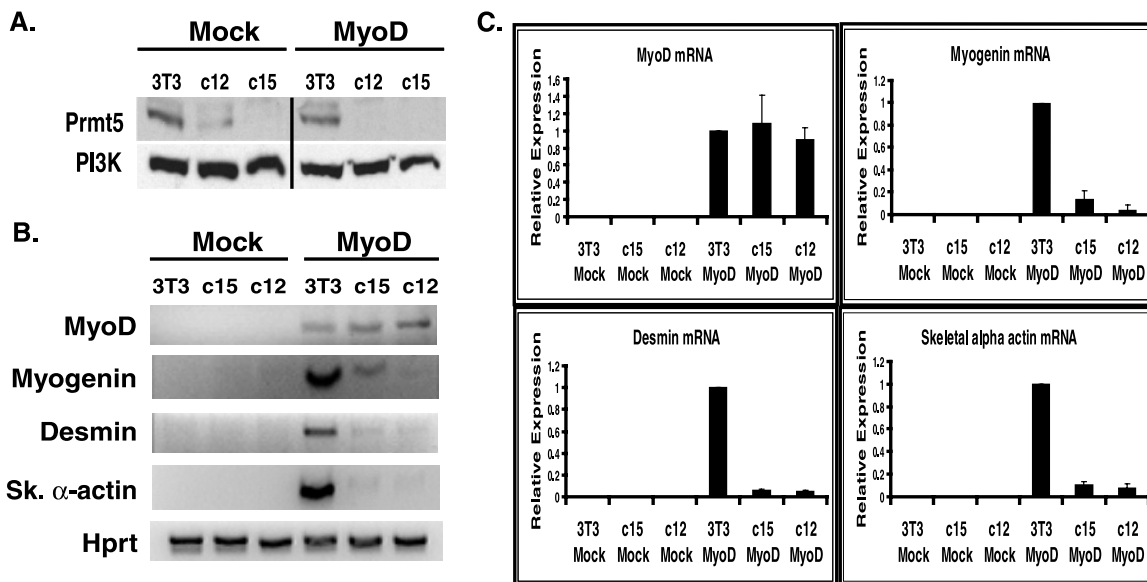


FIG. 1. Prmt5 is required for skeletal muscle differentiation. NIH 3T3 cells and two independently derived NIH 3T3 lines expressing an antisense vector against Prmt5 (c15 and c12 cells) were mock-differentiated or differentiated by ectopic expression of MyoD. (A) Western blots show that protein levels of Prmt5 were significantly reduced in differentiated and mock-differentiated antisense lines compared to levels in the parental NIH 3T3 cells. Cells were differentiated as described in Materials and Methods for 36 h prior to sample collection. (B) Expression of myogenic target genes myogenin, desmin, and skeletal alpha-actin (Sk.  $\alpha$ -actin) and ectopic expression of MyoD were monitored by RT-PCR in mock- and MyoD-differentiated cells collected 36 h postdifferentiation. (C) Quantification of muscle-specific gene expression by quantitative real-time PCR confirms that early and late differentiation markers are significantly decreased in cells containing reduced levels of Prmt5. PI3K, phosphatidylinositol 3-kinase.

infection, low-serum differentiation medium was added to the cells, and the cells were allowed to undergo differentiation for 36 h. Control samples that were mock infected were also placed in differentiation medium and are referred to as mock differentiated. Western analysis was performed to demonstrate that the antisense vector present in these two clones reduced the amount of Prmt5 in both mock- and MyoD-differentiated c15 and c12 cells (Fig. 1A).

RT-PCR was performed to determine if the reduction of Prmt5 had an effect on the transcription of myogenic target genes. As shown in Fig. 1B, expression of MyoD mRNA was comparable in each cell line, indicating that each cell line was infected by the retrovirus and expressed equivalent levels of MyoD. As expected, both the early muscle marker myogenin and the late genes desmin and skeletal alpha actin were induced upon differentiation with MyoD in the control cells. In contrast, in MyoD-differentiated Prmt5 antisense lines, the expression of all three marker genes was significantly reduced. Quantitative real-time PCR was used to more precisely measure the differences in transcript levels (Fig. 1C). Impaired gene activation of early and late muscle-specific transcripts suggests a role for Prmt5 in the activation of myogenic transcription during the differentiation process.

In order to initiate the process of skeletal muscle differentiation, MyoD promotes cell cycle arrest, which involves the induction of cyclin-dependent kinase inhibitors such as p21 and cell cycle regulators like retinoblastoma protein (32, 54). As a consequence, the activity of cyclins and cyclin-dependent kinases is downregulated, and cell cycle arrest is achieved. To eliminate the possibility that defects seen in myogenic gene expression upon reduction of Prmt5 levels stem from aberrant

cell cycle arrest, we performed FACS analysis following propidium iodide incorporation to determine whether the cells were arresting properly (Table 1). The percentage of cells in S phase did not differ between the control and antisense cell lines under any of the conditions evaluated. Cycling cell populations contained approximately 30% of cells in S phase. In mock- or MyoD-differentiated cells, the percentage of cells in S phase was 12 to 14% (Table 1), which is consistent with the level of cell cycle withdrawal that can be achieved with immortalized fibroblasts under these conditions (18, 45). These data indicate that the control and both Prmt5 antisense lines withdrew from the cell cycle upon differentiation. RT-PCR analysis indicated that p21, cyclin D3, p16, and retinoblastoma protein mRNAs were upregulated equivalently in each of the cell lines upon differentiation with MyoD (data not shown), further corroborating these findings. Thus, the reduction in myogenic gene expression observed upon reduction of Prmt5 levels was not due to a failure of the cells to undergo cell cycle arrest. These

TABLE 1. Results of FACS analysis

Cell line	Percentage of indicated cell type in S phase <sup>a</sup>		
	Cycling	Mock differentiated	MyoD differentiated
NIH 3T3	29.7 $\pm$ 1.6	13.0 $\pm$ 3.0	13.6 $\pm$ 2.1
c15	27.4 <sup>b</sup>	10.8 $\pm$ 4.5	11.8 $\pm$ 2.2
c12	28.3 <sup>b</sup>	12.4 $\pm$ 4.2	11.8 $\pm$ 0.4

<sup>a</sup> Results are expressed as the average  $\pm$  standard deviation of three experiments.

<sup>b</sup> Average of two experiments.



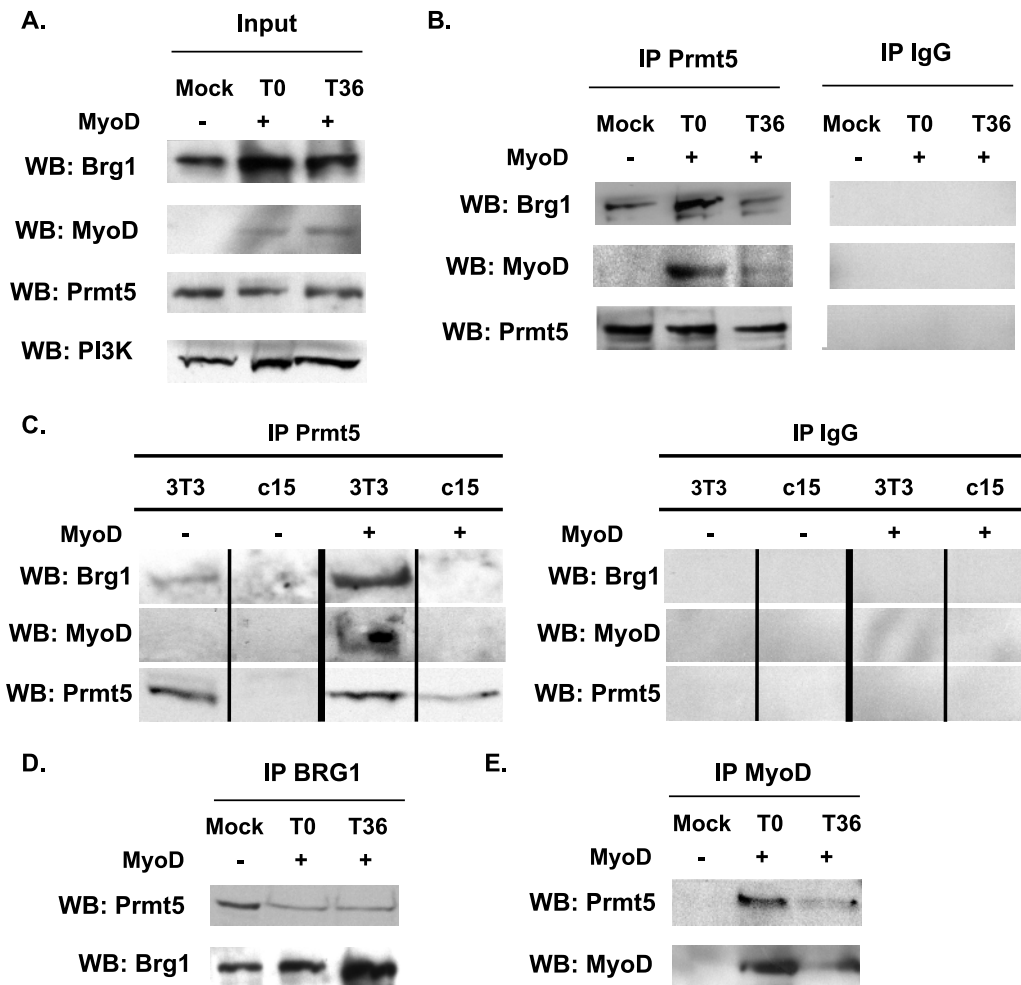


FIG. 2. Prmt5 coimmunoprecipitates with Brg1 and MyoD. Mock-differentiated cells, nondifferentiated (time zero [T0]) MyoD-infected cells, or MyoD-infected cells that were exposed to differentiation conditions for 36 h were harvested and used to perform coimmunoprecipitation experiments with the indicated antibodies. (A) Indicated proteins were detected by Western blotting using 10% of the input sample. (B) Whole-cell extracts from mock and differentiated samples were coimmunoprecipitated with Prmt5 antibody (catalog no. 611538; BD Biosciences) or purified immunoglobulin G (IgG). Immunoprecipitated material was run on SDS-PAGE gels, transferred to a membrane, and probed for the presence of Brg1, MyoD, and Prmt5. (C) As a control, the experiment in panel B was repeated using the c15 Prmt5 antisense line mock differentiated or MyoD differentiated for 36 h. Each Western blot panel contains data from the same blot; intervening samples were removed for clarity. (D) Coimmunoprecipitations were performed using Brg1 antisera and probed for Prmt5 and Brg1. (E) Coimmunoprecipitations were performed using MyoD antisera and probed for the presence of Prmt5 and MyoD. WB, Western blot; PI3K, phosphatidylinositol 3-kinase.

data indicate that Prmt5 is not needed for cell cycle arrest under the conditions utilized for these experiments. Furthermore, Prmt5 is not required for all MyoD-induced gene expression, since the cell cycle regulators upregulated during MyoD-mediated differentiation were upregulated normally when Prmt5 levels were reduced.

The requirement for Prmt5 during MyoD-induced differentiation suggests that Prmt5 may physically associate with MyoD and other regulators of differentiation. Coimmunoprecipitation experiments revealed that endogenous Prmt5 was associated with MyoD in differentiating cells (Fig. 2). As expected, no interactions were observed in mock-differentiated cells or in Prmt5 antisense cells. Additional experiments showed that endogenous Prmt5 was associated with endogenous Brg1 (Fig. 2). This association was not dependent upon differentiation, consistent with previous reports demonstrating that a subset of

Brg1 containing SWI/SNF chromatin remodeling enzymes is associated with Prmt5 in tumor-derived cell lines (41, 42).

**Prmt5 binding and H3R8 dimethylation at the myogenin promoter are required for binding of Brg1 and MyoD.** We wished to determine if Prmt5 was directly influencing the expression of the myogenin gene promoter by directly interacting with its regulatory regions. We examined the myogenin promoter because transcriptional activation at this locus is necessary for the subsequent expression of late marker genes, such as desmin and skeletal alpha-actin, and for terminal differentiation. ChIP for Prmt5 was performed in mock- and MyoD-differentiated NIH 3T3 and Prmt5 antisense lines (Fig. 3A and B). In the NIH 3T3 cells, binding of Prmt5 to the myogenin promoter required MyoD-induced differentiation. H3R8 is a known substrate for Prmt5 (41, 42). Dimethylation of H3R8 at the myogenin promoter was enriched upon differentiation. As

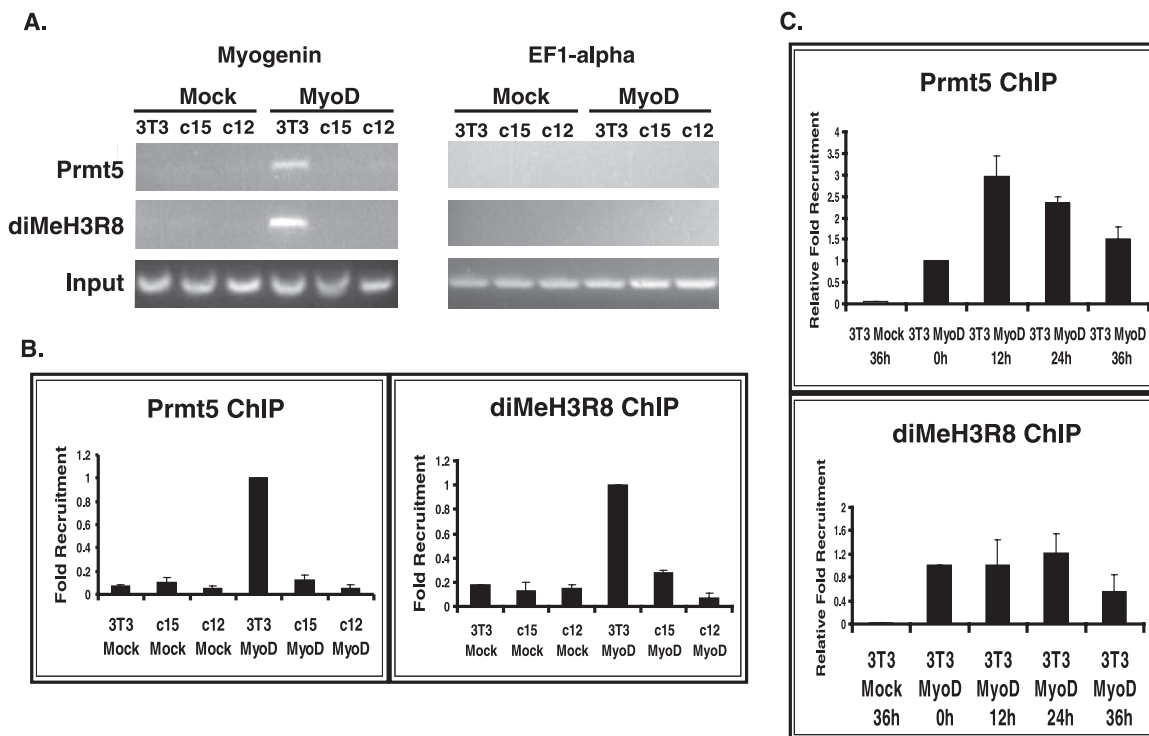


FIG. 3. Prmt5 binds to the myogenin promoter and dimethylates H3R8. ChIPs were performed using antibodies against Prmt5 and dimethylated (diMe) H3R8 in mock- and MyoD-differentiated NIH 3T3 and Prmt5 antisense cell lines. (A) ChIPs demonstrate that binding of Prmt5 and dimethylated H3R8 at the myogenin promoter in differentiated cells is significantly reduced in the antisense lines. Amplification of the coding region of elongation factor EF1-alpha was performed as a negative control. (B) Quantification of Prmt5 binding and H3R8 dimethylation at the myogenin promoter was performed by QPCR. (C) Time course of Prmt5 and dimethylated H3R8 association with the myogenin promoter. QPCR analysis of binding in differentiated cells at the indicated times is shown. Values are expressed relative to the values obtained at time zero, which was set at 1.

expected, Prmt5 binding was significantly reduced in the antisense lines and was comparable to binding seen in mock-differentiated cells. Similarly, in the antisense lines, dimethylation of H3R8 was reduced to the level observed in mock-differentiated samples, indicating that dimethylation of H3R8 at the myogenin promoter required Prmt5.

To further investigate the interaction of Prmt5 with the myogenin promoter, ChIPs were performed over a time course of differentiation. Both Prmt5 and dimethylated H3R8 were detected at the time when differentiation medium was first added (time zero), and the interaction of these proteins was observed throughout the time course (Fig. 3C). These interactions precede the activation of myogenin expression (16), suggesting that the Prmt5 methyltransferase contributes to the initial promoter reorganization that promotes myogenin expression. Curiously, though the levels of dimethylated H3R8 at the myogenin promoter were relatively constant during the differentiation protocol, a reproducible increase in amount of Prmt5 present at the promoter was observed between 0 and 12 h postdifferentiation (Fig. 3C). The significance of this observation, if any, is not known.

Prior work showed that induction of myogenin transcription required the binding and activity of Brg1, an ATPase that is the catalytic subunit of some of the SWI/SNF chromatin remodeling enzymes (15). To ascertain whether a reduction in Prmt5 affected Brg1 binding to the myogenin promoter, additional

ChIPs were performed. These experiments showed that in cells with reduced levels of Prmt5, recruitment of Brg1 at the myogenin promoter was diminished to the level of binding seen in mock-differentiated cells (Fig. 4A and B). Control Western blots showed that the lack of Brg1 binding to the myogenin locus was not due to changes in Brg1 levels in the Prmt5 antisense cells (Fig. 4C). Thus, Prmt5 binding and dimethylation of H3R8 are prerequisites for Brg1 binding. The lack of Brg1 at the myogenin promoter implies a lack of chromatin remodeling at this locus. An REAA allowed us to detect accessibility changes in the chromatin at the myogenin locus in response to induction of differentiation by MyoD in the presence of normal and reduced levels of Prmt5. When mock-differentiated, none of the cell lines displayed significant enzyme accessibility at the myogenin locus (Fig. 4D). Upon differentiation there was an expected increase in accessibility in the NIH 3T3 cells, but little or no accessibility was observed in the Prmt5 antisense lines. These findings reiterate the requirement for Brg1 to alter myogenin promoter structure in a manner that permits restriction enzyme accessibility and indicate that the presence of Prmt5 and dimethylated H3R8 is insufficient to cause such structural changes at the myogenin locus.

Previous work has implicated the Pbx/Meis homeodomain factors as playing an important role during the induction of myogenin transcription by providing a mechanism to initially target MyoD to the locus (7, 28). Further studies showed that

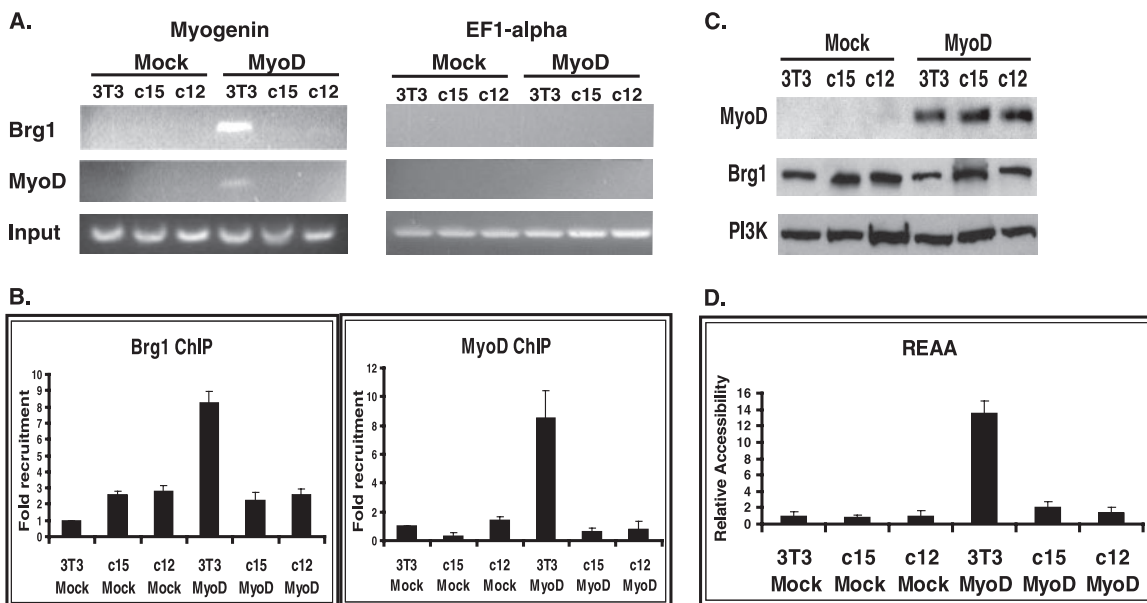


FIG. 4. Brg1 and MyoD binding at the myogenin promoter require Prmt5. (A) ChIPs were performed using antibodies against Brg1 and MyoD in mock- and MyoD-differentiated cell lines harvested 36 h postdifferentiation, and the myogenin promoter and elongation factor EF1-alpha coding region (input control) were PCR amplified. Data in panel A and Fig. 3A were from the same experiment; thus, the input control bands are the same for both panels. (B) Brg1 and MyoD binding were quantified by QPCR. (C) Western blot showing that Brg1 and MyoD protein levels were unaffected by the reduction in Prmt5 protein levels. (D) An REAA was performed to evaluate the accessibility of a Pvu II site at -320 relative to the myogenin mRNA start site. Chromatin accessibility was dependent upon Prmt5 expression.

Brg1-mediated chromatin remodeling at the myogenin promoter subsequently permits stable binding of MyoD to its consensus binding sites upstream and downstream of the Pbx/Meis site (16). Since Brg1 did not bind to or remodel chromatin at the myogenin promoter in Prmt5 antisense cell lines, we would predict that stable binding of MyoD would also not be observed in these cells. As expected, an additional consequence of decreased Brg1 interactions in the antisense lines was that binding of MyoD was reduced to background levels (Fig. 4A and B). In summary, the reduction in Prmt5 levels resulted in a failure to activate myogenin expression because neither Prmt5 nor Brg1 interacted with the promoter. Thus, subsequent events such as histone methylation, ATP-dependent chromatin remodeling, and MyoD binding did not occur.

To further probe the molecular events occurring at the myogenin locus, we assessed whether binding of Prmt5 and dimethylation of H3R8 required functional Brg1. We previously described and characterized tetracycline-suppressible cell lines that in the absence of tetracycline (Tet) express a Flag-tagged, ATPase-deficient, dominant negative Brg1 protein (14, 15) and showed that expression of dominant negative Brg1 blocks activation of myogenic early and late genes because chromatin remodeling at each inducible locus is blocked (15, 16, 39). Upon differentiation with MyoD, Prmt5 was able to bind to the myogenin promoter in cells expressing functional Brg1 (with Tet) as well as in cells expressing the dominant negative version of Brg1 (without Tet) (Fig. 5A). Dimethylation of H3R8 was also enriched at the promoter, regardless of the functional status of Brg1 (Fig. 5A). Control Western blots demonstrated that Prmt5 protein levels were unaffected by the expression of dominant negative Brg1, and, as previously documented, expression of dominant negative Brg1 did not alter the overall

levels of Brg1 in the cells (Fig. 5B) (16). Additional mRNA analyses indicated that MyoD was equivalently expressed in cells expressing or lacking dominant negative Brg1 and that the expression of dominant negative Brg1 inhibited subsequent myogenic gene expression (Fig. 5C and data not shown). We conclude that chromatin remodeling by Brg1 is not required to facilitate the binding of Prmt5 at the myogenin promoter. Thus, Prmt5 binding is required for the binding of Brg1, but Brg1 function is not needed for the binding of Prmt5.

**Prmt5 and dimethylated H3R8 are present at the myogenin locus of satellite cells in vivo.** To determine whether Prmt5 binds to the myogenin promoter in vivo, we isolated hind limb muscle from 4- to 5-week-old BL6 mice and separated satellite cells and myofibers (see Materials and Methods). Satellite cells are quiescent muscle progenitor cells located in the basal lamina of mature muscle fibers (33, 48). Upon activation, they express myogenic regulatory factors and initiate the differentiation program by proliferating and fusing with existing myofibers or fusing to form new myofibers (reviewed in references 19 and 25). Investigation of the molecular roles of transcriptional regulators and chromatin remodeling enzymes during myogenic gene activation in these cells has been limited by the difficulties associated with isolation and analysis of these cell populations.

The separation protocol utilized results in a satellite cell pool that contains both quiescent and activated cells. To evaluate whether the purification scheme adequately separated satellite cells from myofibers, quantitative PCR was performed to examine the expression of satellite cell and myofiber marker genes. Pax3 is a transcription factor that is a member of the paired box/homeodomain family and is expressed in satellite cells (10). Pax3 is highly expressed in satellite cells compared to

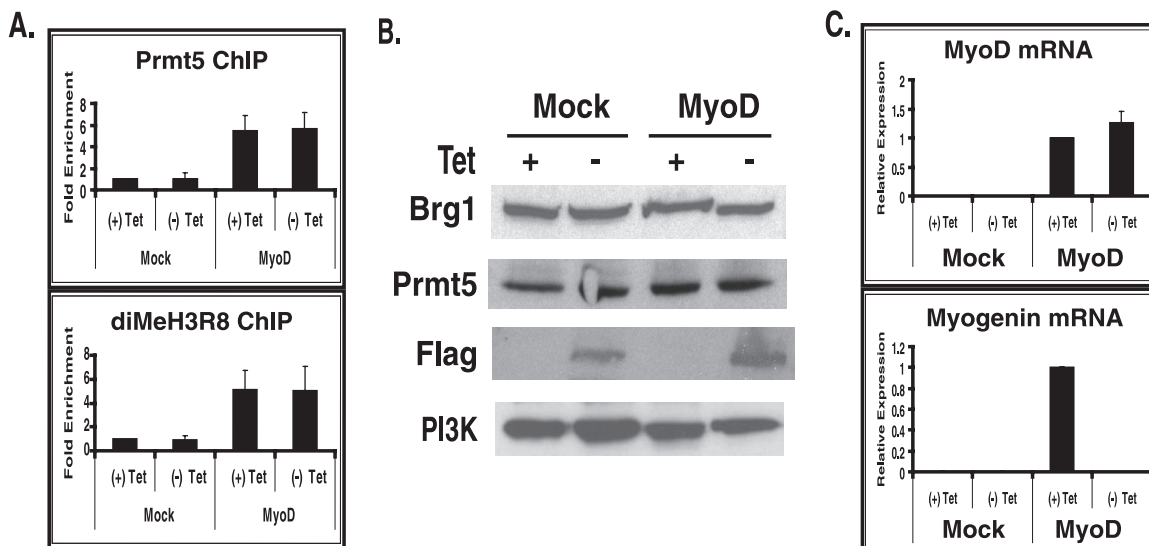


FIG. 5. Prmt5 is able to bind to and dimethylate H3R8 at the myogenin promoter in the absence of functional Brg1. ChIPs were performed 36 h postdifferentiation in a cell line containing a Tet-suppressible vector expressing a Flag-tagged, ATPase-deficient, dominant negative Brg1. (A) QPCR shows binding of Prmt5 and dimethylated H3R8 at the myogenin promoter in the presence (+ Tet) and absence (–Tet) of functional Brg1. (B) Protein levels of Brg1, Prmt5, and the Flag-tagged dominant negative mutant Brg1 were evaluated by Western blotting. Expression of Flag in samples without Tet indicates expression of dominant negative Brg1. (C) QPCR evaluation of MyoD mRNA, to demonstrate that differentiated cells expressed MyoD, and myogenin, to demonstrate that MyoD-dependent gene expression was inhibited by dominant negative Brg1. PI3K, phosphatidylinositol 3-kinase.

mature myofibers or the negative control liver tissue, indicating that this fraction is enriched for satellite cells while the myofiber preparation contains few if any satellite cells (Fig. 6A). Since the satellite cell fraction contains both quiescent and activated satellite cells, both satellite cell and myofiber fractions displayed expression of MyoD and myogenin, as expected. Expression of the late skeletal muscle marker dystrophin was seen only in the myofiber fraction, establishing that the satellite cell fraction was not contaminated with myofibers (Fig. 6A).

We then isolated nuclei from these separated tissue samples and performed ChIP experiments to assess factor interactions at the myogenin locus. Prmt5 was highly enriched at the myogenin promoter in satellite cells, but this enrichment was significantly reduced in the myofibers (Fig. 6B). H3R8 dimethylation also was highly enriched at the myogenin promoter in satellite cells while enhanced to a much lesser extent at the myogenin promoter in myofibers, which is consistent with the reduced binding of Prmt5 that was observed. Comparable levels of Prmt5 were expressed in both tissue samples (Fig. 6C).

Since the satellite cell population contains both quiescent and activated cells, we could not definitively conclude that Prmt5 association with the myogenin promoter was related to the activation of gene expression in these cells. To better address this question, we asked whether Prmt5 and MyoD could be colocalized to the myogenin promoter. Since only activated satellite cells express MyoD, the simultaneous presence of MyoD and Prmt5 would indicate that Prmt5 was present at the myogenin locus in cells that were actively expressing myogenin. Re-ChIP experiments were performed in which chromatin from satellite cell nuclei immunoprecipitated with Prmt5 antibodies was subsequently immunoprecipitated with MyoD antibodies. The results show that Prmt5

and MyoD are present together at the myogenin locus in activated satellite cells (Fig. 6D).

Collectively, these results indicate that the Prmt5 arginine methyltransferase is required for MyoD-mediated differentiation. Examination of one of the early myogenic target genes shows that Prmt5 binds to the myogenin promoter and dimethylates H3R8. Prmt5 binding was required for association of the Brg1 chromatin remodeling enzyme with the promoter and for all subsequent events that occur during the activation of myogenin expression. Thus, cells containing reduced levels of Prmt5 failed to activate myogenin and, due to the absence of myogenin, failed to activate myogenic late genes, leading to the observed block in differentiation. The corecruitment of Prmt5 and MyoD to the myogenin locus in primary satellite cells corroborates a role for Prmt5 in myogenic differentiation *in vivo*. The findings presented here provide evidence for an important physiological role for Prmt5 during the induction of skeletal muscle differentiation because it facilitates ATP-dependent chromatin remodeling at myogenic loci, which then leads to gene expression.

## DISCUSSION

**Prmt5 is necessary for the activation of myogenin expression and myogenesis.** The results indicate that the protein methyltransferase Prmt5 is required for MyoD-mediated skeletal muscle differentiation. Prmt5 localizes to the myogenin promoter, a MyoD inducible gene whose expression mediates terminal differentiation (24, 27, 37). In both cell culture and satellite cells isolated from muscle tissue, the presence of Prmt5 at the myogenin promoter is coincident with the presence of dimethylated H3R8, a known substrate for Prmt5 (41). Manipulated cells expressing low levels of Prmt5 were unable



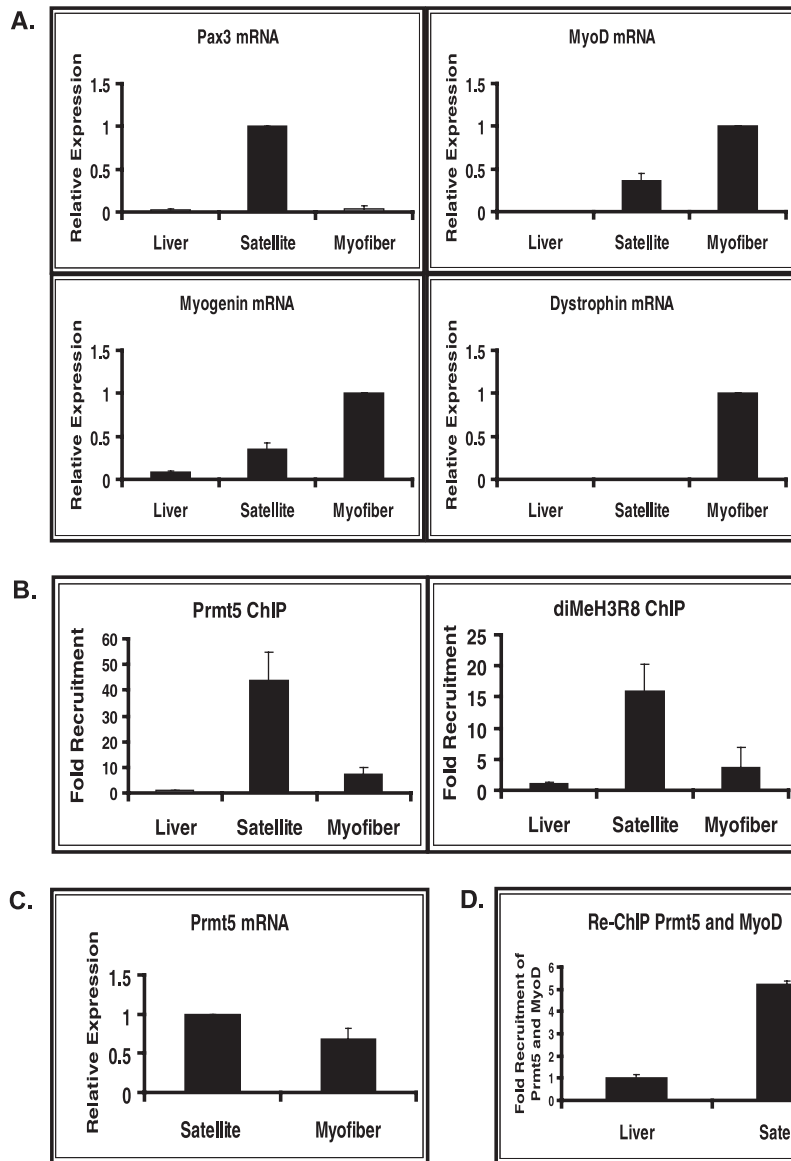


FIG. 6. Prmt5 binds to and dimethylates H3R8 at the myogenin promoter in muscle satellite cells. (A) The separation and purification of satellite cells and myofibers were monitored by QPCR of marker gene mRNAs. (B) ChIP analysis using nuclei obtained from muscle satellite cells and myofibers indicates that Prmt5 binding and dimethylated H3R8 were present at the myogenin promoter in satellite cells and at reduced levels in myofibers. (C) Transcript levels of Prmt5 in both cell types were quantified by QPCR. (D) Re-ChIP analysis quantified by QPCR. Materials immunoprecipitated with Prmt5 antibodies were subsequently immunoprecipitated with MyoD antibodies. QPCR data are the average  $\pm$  standard deviation of three independent experiments.

to activate the expression of myogenin or other muscle-specific genes, and the lack of activation correlated with the lack of Prmt5 and dimethylated H3R8 at the myogenin locus. Moreover, both Prmt5 and dimethylated H3R8 were present at the myogenin locus prior to, at the onset of, and following myogenin expression. Finally, re-ChIP experiments from satellite cell nuclei indicate the presence of both MyoD and Prmt5 at the myogenin promoter in primary tissue. The satellite cell pool contains both quiescent cells as well as activated cells that have initiated differentiation. Since only the activated cells express MyoD, the re-ChIP experiment places Prmt5 on the myogenin promoter in primary cells that are actively expressing myogenin and are in the process of differentiating. The

results support the conclusion that Prmt5 promotes gene activation during skeletal muscle differentiation.

There are two previous reports suggesting that Prmt5 promotes reporter gene activation; all other studies suggest that Prmt5 functions in transcriptional repression. In one study, dose-dependent reduction of Prmt5 by RNA interference methods resulted in corresponding decreases in NFAT- and interleukin-2 (IL-2)-driven promoter activity and in IL-2 secretion, suggesting that Prmt5 promotes IL-2 expression (44). In a different report, Prmt5 was purified in a multiprotein complex with p44, an androgen receptor (AR) interacting protein that enhances AR-dependent transcription. Prmt5 acted synergistically with p44 to mediate AR-dependent reporter

gene expression. However, activation by Prmt5 in this system did not require its methyltransferase function; thus, the mechanism by which Prmt5-activated transcription occurred was not clear (26). In contrast, Prmt5 involvement in gene repression is better understood. Interestingly, multiple mechanisms appear to be involved. Prmt5 can methylate the elongation factor, Spt5, which decreases its affinity for polymerase II (Pol II) and impedes transcription elongation (30). In contrast, Prmt5 directly binds to regulatory sequences controlling expression of cyclin E, the tumor suppressors NM23 and ST7, and the *c-myc* target gene, CAD, and methyltransferase function was required for repression where examined (21, 41, 42). Microarray and confirmatory RT-PCR experiments identified additional Prmt5 target genes also involved in cell growth control (41). Dimethylation of H3R8 at promoter sequences was associated with repression of the genes examined, and purification of Prmt5 as part of a multienzyme complex with histone deacetylase 2 and the SWI/SNF ATPase Brg1 (42) suggests mechanisms by which nucleosomes on gene regulatory sequences can be altered and/or repositioned and by which histones can be dimethylated and deacetylated in a manner that leads to transcriptional repression.

Prmt5 also functions in complexes with RNA Pol II and the Pol II-associated phosphatase FCP1 and with several cytosolic proteins that together with Prmt5 promote snRNP assembly (2, 4, 35). Thus, it is established that Prmt5 can function in multiple capacities in multiple complexes, likely through its protein methylase activity, to regulate disparate cellular processes. Our results suggest that Prmt5 may also be a component of one or more additional complexes that function as coactivators of gene expression.

Interestingly, while Prmt5 was required for myogenin expression as well as for the expression of several skeletal muscle-specific late genes, cell cycle regulators that are upregulated during MyoD-mediated differentiation to promote cell cycle withdrawal were induced normally in the Prmt5 antisense cells lines. This result indicates that Prmt5 is not required for the activation of every MyoD target gene and is reminiscent of previous studies that showed that the SWI/SNF chromatin remodeling enzymes were required for activation of myogenin and many skeletal muscle-specific late genes but not for the activation of cell cycle regulators (18, 45). cDNA expression array analysis ultimately revealed that while almost all of the genes induced by MyoD in this culture system showed at least a modest dependence on the presence of functional SWI/SNF enzymes, only about one-third of these genes were highly dependent on functional SWI/SNF enzymes (16). We predict that the set of genes that showed the greatest requirement for SWI/SNF enzymes will also be highly dependent upon Prmt5; GeneChip analyses will be required to definitively address this issue.

**Prmt5 function facilitates ATP-dependent chromatin remodeling in the cascade of events leading to myogenin expression.** The observation that Prmt5 is required for the binding and function of the Brg1 ATP-dependent chromatin remodeler indicates that the histone methyltransferase is required for ATP-dependent chromatin remodeling to occur. Though there are reports of specifically modified histones serving as recognition sites for the interaction of specific subunits of ATP-dependent chromatin remodeling enzymes with chromatin (1,

22, 23, 31, 52), we believe this is the first demonstration that a specific histone modification enzyme is required for an ATP-dependent chromatin remodeling enzyme to modify chromatin structure and activate gene expression from an endogenous locus.

A number of studies have examined the factors involved in and the order of events that lead to activation of the myogenin locus (7, 16, 49). Prior studies showed the homeodomain factor Pbx constitutively interacting with the myogenin promoter and providing a mechanism to initially target MyoD to the promoter. This led to promoter-specific histone acetylation, followed by binding of the Brg1 ATPase of SWI/SNF chromatin remodeling enzymes. Subsequent steps, including increased chromatin accessibility, stable binding of MyoD and Mef2 to the promoter, and activation of myogenin transcription, were absolutely dependent upon Brg1 function. The kinetics of histone acetylation and Brg1 binding, coupled with protein-protein interactions between endogenous Brg1 and endogenous Pbx at the initiation of the differentiation process, supported the idea that chromatin modifying enzymes were targeted to the promoter via the MyoD/Pbx/Meis complex to permit histone modifications and ATP-dependent chromatin remodeling, thereby enabling stable occupancy by transcription factors required for myogenin transcription. The dependency of Brg1 binding on the presence of Prmt5 places Prmt5 function early in the activation process, coincident with or preceding Brg1 interaction with the promoter. The timing of Prmt5 function and the observed physical associations between Prmt5, MyoD, and Brg1 suggest a similar mechanism of remodeling enzyme targeting to the myogenin promoter.

The observation that Prmt5 is required for the association of Brg1 with the myogenin promoter and its subsequent chromatin remodeling functions suggests that either the physical presence of Prmt5 at the myogenin promoter and/or the dimethylation of H3R8 at the promoter facilitates Brg1 binding. Histone acetylation also precedes Brg1 interaction at the myogenin promoter (16), and both *in vivo* and *in vitro* data indicate that the bromodomain present in Brg1 interacts with acetylated histones and that SWI/SNF complex function can be facilitated by histone acetylation (1, 22, 23). We speculate that Brg1 has a higher affinity for chromatin that contains H3R8 dimethylated by Prmt5 and that H3R8 dimethylation might combine with histone acetylation to create a more permissive substrate for Brg1-dependent chromatin remodeling.

**Functional interplay between different methyltransferases during myogenesis.** A distinct protein methyltransferase, Prmt4/Carm1, was previously shown to contribute to myogenesis (9). Prmt4, along with the Mef2C protein, were localized the muscle creatine kinase promoter in differentiating cell cultures by ChIP, and inhibition of Prmt4 expression blocked the expression of myogenic genes. The fact that Prmt4 was found in a complex with Brg1 that promoted estrogen receptor-stimulated gene expression (53) raises the possibility that both PRMTs can cooperate with SWI/SNF chromatin remodeling enzymes during gene activation events. However, the ATPase activity of Brg1 was required for Prmt4 histone methylation *in vitro* (53), whereas our data indicate that Prmt5 was necessary for Brg1 association with the myogenin promoter and subsequent changes in promoter chromatin accessibility. Thus, the mechanisms by which these two PRMTs function may differ.

Furthermore, Prmt4 and Prmt5 do not share the same histone substrates so far as is known. Whether Prmt5 and Prmt4 can work synergistically to modify histones at the same myogenic loci remains to be determined.

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