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Analysis of Protein Arginine Methyltransferase Function during Myogenic Gene Transcription: A Dissertation

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A Dissertation Presented

By

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July 9th, 2008

Analysis of Protein Arginine Methyltransferase Function during Myogenic Gene Transcription

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By
Caroline S. Dacwag

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Abstract

Skeletal muscle differentiation requires synergy between tissue-specific transcription factors, chromatin remodeling enzymes and the general transcription machinery. Here we demonstrate that two distinct protein arginine methyltransferases are required to complete the differentiation program. Prmt5 is a type II methyltransferase, symmetrically dimethylates histones H3 and H4 and has been shown to play a role in transcriptional repression. An additional member of the Prmt family, Carm1 is a type I methyltransferase, and asymmetrically methylates histone H3 and its substrate proteins. MyoD regulates the activation of the early class of skeletal muscle genes, which includes myogenin. Prmt5 was bound to and dimethylates H3R8 at the myogenin promoter in a differentiation-dependent fashion. When proteins levels of Prmt5 were reduced by antisense, disappearance of H3R8 dimethylation and Prmt5 binding was observed. Furthermore, binding of Brg1 to regulatory sequences of the myogenin promoter was abolished. All subsequent events relying on Brg1 function, such as chromatin remodeling and stable binding by muscle specific transcription factors such as MyoD, were eliminated. Robust association of Prmt5 and dimethylation of H3R8 at myogenin promoter sequences was observed in mouse satellite cells, the precursors of mature myofibers. Prmt5 binding and histone modification were observed to a lesser degree in mature myofibers. Therefore, these results indicate that Prmt5 is required for dimethylating histone at the myogenin locus during skeletal muscle differentiation in order to facilitate the binding of Brg1, the ATPase subunit of the chromatin remodeling complex SWI/SNF.

Further exploration of the role of Prmt5 during the activation of the late class of muscle genes revealed that though Prmt5 is associated with and dimethylates histones at the regulatory elements of late muscle genes in tissue and in culture, it was dispensable for late gene activation. Previous reports had indicated that Carm1 was involved during late gene activation. We observed that Carm1 was bound to and responsible for dimethylating histones at late muscle gene promoters in tissue and in culture. In contrast to Prmt5, a complete knockout of Carm1 resulted in abrogation of late muscle gene activation. Furthermore, loss of Carm1 binding and dimethylated histones resulted in a disappearance of Brg1 binding and chromatin remodeling at late muscle gene loci. Time course chromatin immunoprecipitations revealed that Carm1 binding and histone dimethylation occurred concurrently with the onset of late gene activation. *In vitro* binding assays revealed that an interaction between Carm1, myogenin and Mef2D exists. These results demonstrate that Carm1 is recruited to the regulatory sequences of late muscle genes via its interaction with either myogenin or Mef2D and is responsible for dimethylates histones in order to facilitate the binding of Brg1. Therefore, these results indicate that during skeletal muscle differentiation, distinct roles exist for these Prmts such that Prmt5 is required for activation of early genes while Carm1 is essential for late gene induction.

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Abbreviations

MRF	Muscle Regulatory Factor
MEF2	Myocyte Enhancer Factor 2
HAT	Histone Acetyltransferase
HMT	Histone Methyltransferase
CER	Core Enhancer Region
bHLH	basic helix loop helix
MADS	MCM1, Agamous, Deficiens, Serum Response Factor
DBD	DNA binding domain
NTD	Amino terminus domain
CTD	Carboxy terminus domain
Brg	Brahma related gene
BRM	Brahma
MHC	Myosin Heavy Chain
MCK	Muscle creatine kinase
FACS	Fluorescence activated cell sorting
ChIP	Chromatin immunoprecipitation
PCAF	p300/CBP associated factor
TSA	Trichostatin A
CaMK	Calcium calmodulin kinase
SET	Su(var), Enhancer of Zeste, Trithorax
CARM1	Coactivator of arginine methyltransferase 1

ER	Estrogen Receptor
MEF	Mouse embryo fibroblast
TAD	Transcription activation domain
AdOX	Adenosine dialdehyde
diMeH3R8	dimethylated histone H3 arginine 8
diMeH3R17	dimethylated histone H3 arginine 17
PRMT	Protein arginine methyltransferase
REAA	Restriction Enzyme Accessibility Assay
WT	Wild type
KO	Knockout

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Preface

Parts of this dissertation have been published in the following works:

Dacwag, CS., Bedford, MT., Sif, S., and AN Imbalzano. Distinct protein arginine methyltransferases promote ATP-dependent chromatin remodeling function at different stages of skeletal muscle differentiation. 2008. (*Submitted to MCB, minor revisions)*

Dacwag, CS., Ohkawa, Y., Pal, S., Sif, S., and Imbalzano, AN. The protein arginine methyltransferase Prmt5 is required for myogenesis because it facilitates ATP-dependent chromatin remodeling. 2007. Mol. Cell Biol. 27(1):384-94.

Ounzain, S., **Dacwag, CS**., Samani, NJ., Imbalzano, AN., and NW Chong. Comparative *in silico* analysis identifies bona fide MyoD binding sites within the Myocyte Stress 1 gene promoter. 2008. BMC Molecular Biology. May 19;9:50.

Ohkawa, Y., Yoshimura, S., Higashi, C., Marfella, CG., **Dacwag, CS**., Tachibana, T., and AN Imbalzano. Myogenin and the SWI/SNF ATPase Brg1 maintain myogenic gene expression at different stages of skeletal myogenesis. 2007. Jounal of Biological Chemistry. 282(9):6564-6570.

De la Serna, IL., Ohkawa, Y., Berkes, CA., Bergstrom, DA., **Dacwag, CS**., Tapscott, SJ., and AN Imbalzano. MyoD Targets Chromatin Remodeling Complexes to the Myogenin Locus Prior to Forming a Stable DNA-Bound Complex. 2005. Molecular and Cellular Biology. (25)10:3997-4009.

CHAPTER I

INTRODUCTION

Cellular differentiation events require the exquisite temporal regulation of specific subsets of genes. This is accomplished through the seamless interplay between transcription factors, coactivators, chromatin remodelers and transcriptional machinery. The expression of specific muscle regulatory factors (MRFs) initiates specification of cells to the myogenic lineage while expression of other MRFs permit the activation of structural genes required for terminal differentiation. This process requires the combined efforts of the MyoD family of transcription factors, tissue specific co-factors such as Myocyte Enhancer Factor 2 (MEF2), ATP-dependent chromatin remodelers and histone modifying enzymes such as histone acetyltransferases (HATs) and histone methyltransferases (HMTs).

Skeletal muscle development

During early embryonic development, gastrulation generates the formation of the mesodermal layer, which lies between the ectoderm and endodermal layer. Mesoderm forms the blood, vasculature, bones, cartilage, connective tissue and muscles of the body trunk (27, 40). The mesoderm is divided into the axial mesoderm (notochord), intermediate mesoderm, paraxial mesoderm, and the lateral plate mesoderm. In the developing embryo, skeletal muscle progenitors arise from the paraxial mesoderm. The paraxial mesoderm is comprised of bilateral strips of mesodermal tissue that run parallel to the notochord and the neural tube and are delimited by the lateral plate and

intermediate mesoderm (27, 40). Following gastrulation, somite maturation is initiated when the paraxial mesoderm begins to segment into pairs in a rostral to caudal fashion, or head to toe (175, 176, 210, 212). These newly formed somites then assemble to form the dorsal epithelial dermomyotome and ventral mesenchymal sclerotome. Cells of the ventral somites undergo an epithelial to mesenchymal transition, which comprise the sclerotome and eventually form the vertebrae and ribs. The dermomyotome is further partitioned into the epaxial and hypaxial dermomyotome, which are the sources of lateral trunk & deep back, and intercostal, & abdominal musculature, respectively (175, 176, 210, 212). The ventral sclerotome goes on to form the cartilage and bone of the vertebral column and ribs. Cells from the dorsal medial lip and ventral lateral lip migrate underneath the dermomyotome to form the epaxial and hypaxial myotome (175, 176, 210, 212). Myogenic precursors that originate from the hypaxial dermomyotome express homeodomain proteins Pax3 and Pax7 as well as low levels of the muscle regulatory factor Myf5 (83, 96, 105, 109). However, signaling from the lateral plate mesoderm maintains these cells in a proliferative but undifferentiated state (5). Cells from the ventral lateral lip also undergo an epithelial to mesenchymal transition, delaminate and ultimately migrate to sites of limb bud, ventral body wall, diaphragm, tongue and other areas of muscle development (40, 161).

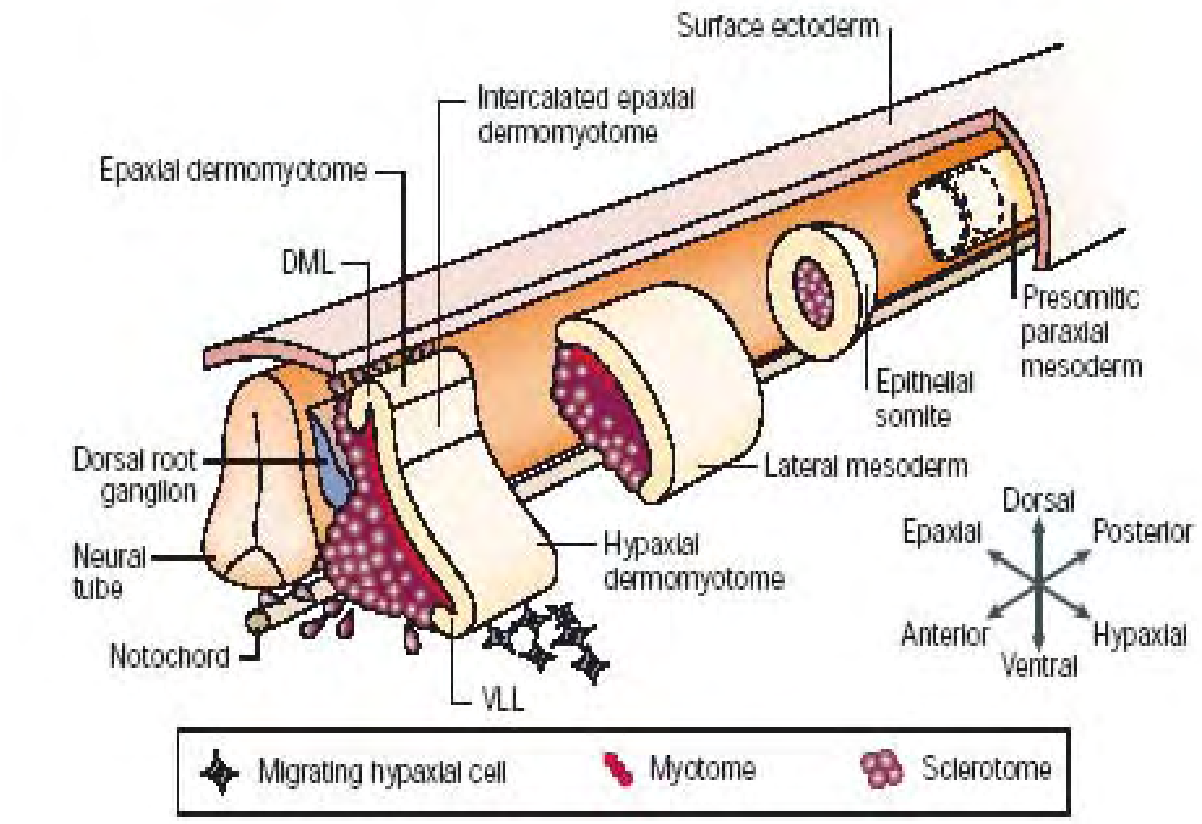

Figure 1-1.

(Buckingham, M et al. The formation of skeletal muscle: from somite to limb. J. Anat. 202;59-68. 2003)

Figure 1-1.

Mature somites originate from the paraxial mesoderm, a bilateral strip of tissue that is localized adjacent and parallel to the notochord and neural tube. It is delimited by the intermediate mesoderm and lateral plate. Upon maturation, the paraxial mesoderm segments into pairs that are further partitioned into the dorsal epithelial dermomyotome and ventral mesenchymal sclerotome. The dermomyotome is divided into a hypaxial and epaxial dermomyotome. Cells delaminating from the dermomyotome migrate underneath to form the epaxial and hypaxial myotome. Cells originating from the hypaxial dermomyotome delaminate to sites of limb bud development or other areas destined to become skeletal muscle such as tongue, diaphragm or the ventral body wall.

Delamination and migration of skeletal muscle precursors are dependent on the tyrosine kinase receptor c-met and its ligand, spatter factor (HGF), which is produced by non-somitic mesodermal cells that mark the route these delaminating cells must travel (58). The paired-box homeodomain-containing transcription factor Pax3 transcriptionally regulates c-met (68). Pax3 expressing cells could be visualized using a lacZ reporter introduced into a Pax3 allele. Pax3 was integral for this process since in Pax3 mutant mice, cells failed to delaminate from the hypaxial dermomyotome and as a result, never migrated to sites of skeletal muscle development (77, 213, 214) (Figure 1-2).

Another homeodomain-containing protein, Lbx1, has been implicated in migration (197). In Lbx1 mutant mice, skeletal muscle progenitors were able to delaminate from the hypaxial dermomyotome, but did not migrate to sites of skeletal muscle development such as limb buds, and therefore these cells adopt different fates (197). It was postulated that Lbx1 functions as a transcription factor, though its targets have not been identified in muscle progenitor cells (99). Msx1, which was present in migrating muscle precursor cells at the level of the forelimb, maintains muscle precursor/progenitor cells (mpcs) in a proliferative state (99). Additionally Msx1 has been shown to contribute to repression of muscle-specific genes as it is able to interact with endogenous H1b but not with Histone H3 and Pol II in C2C12 myoblasts (124). Msx1 also associates with the core enhancer region (CER) of the MyoD promoter. The N-terminus of Msx1 (105-138) is required for the interaction with H1b but the homeodomain is required for DNA binding. Msx1 increases the amount of H1b associated with the CER of MyoD, which renders the chromatin at this locus in an

inaccessible and therefore repressed state (124). In fact, overexpression of Msx1 in terminally differentiated c2c12 myotubes resulted in reduction of myogenic transcripts and reversion of cells to a proliferative state (156) indicating that a mechanism of repression must be in place to coordinate the proper temporal activation of muscle-specific genes since muscle precursors migrating from the hypaxial dermomyotome do not yet express muscle determination factors until they arrive at the limb bud or other sites of skeletal muscle development.

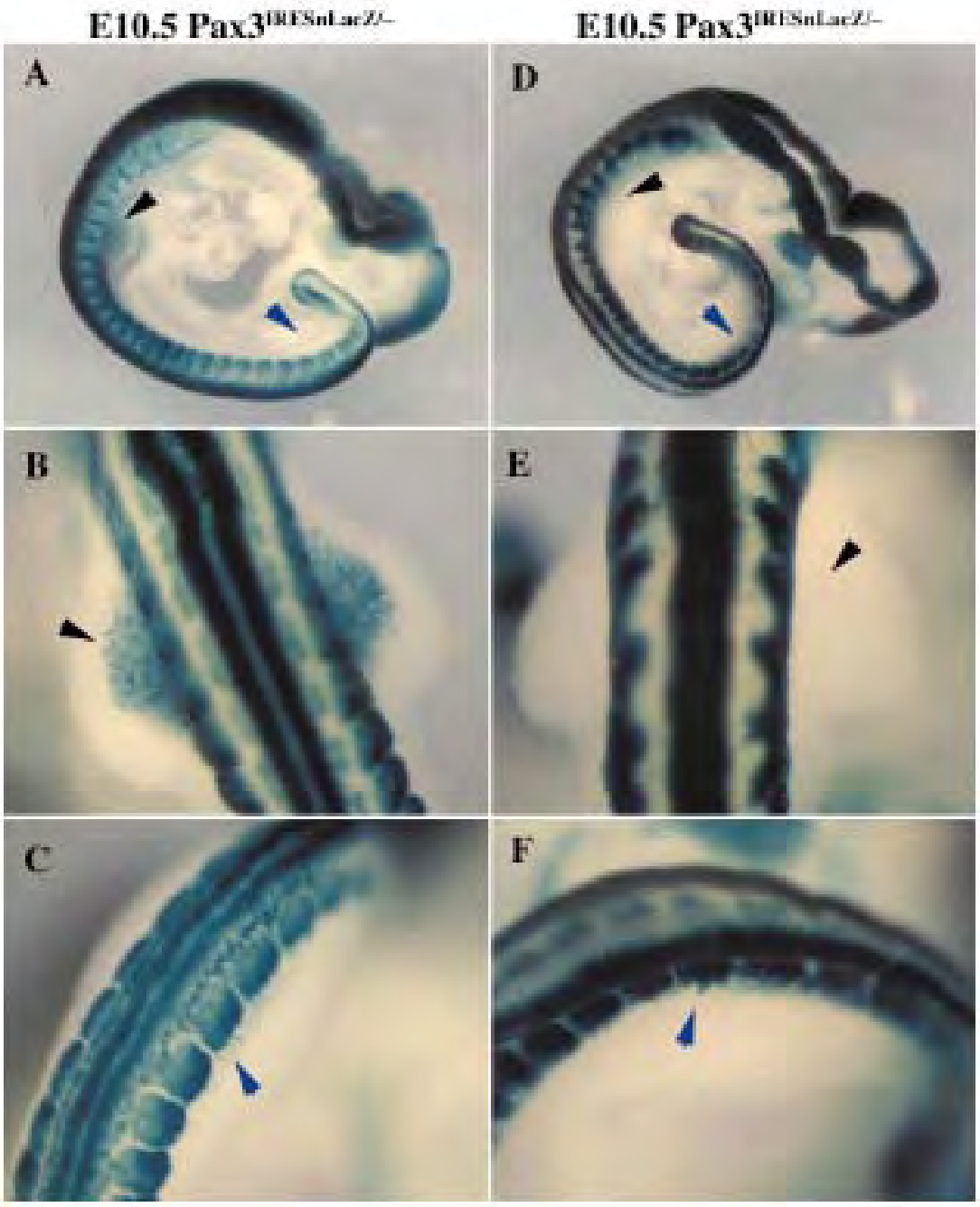

Figure 1-2.

(Buckingham, M et al. The formation of skeletal muscle: from somite to limb. J. Anat. 202;59-68. 2003)

Figure 1-2.

Mouse embryos at embryonic day (E) E10.5 with one (A-C) or two (D-F) alleles of Pax3 targeted with an nlacZ reporter sequence. Black arrows denote the location of forelimb bud while blue arrows indicate hindlimb bud. ß-galactosidase (Pax3)-positive cells can be observed in the forelimb while ß-galactosidase (Pax3)-positive cells can be seen delaminating from somites and migrating. In homozygous null animals, spinal bifidia and exencephaly are apparent in addition to reduced dermomyotome formation. No labeled Pax3-positive cells were observed in the forelimb or hindlimb, nor is there any indication of migrating cells.

Adult Skeletal Muscle

Pax3 is expressed in pre-somitic mesoderm and early epithelial somites (83). Pax7, a paralogue of Pax3, is not expressed in the pre-somitic mesoderm but is induced during somite formation (105). Pax7 is dispensable for embryonic myogenesis (202), but limb and diaphragm muscles are not formed due to defective lateral migration and reduced proliferation of the dermomyotome in Pax3 mutant mice (23, 47, 219). Ectopic expression of Pax3 in embryonic tissues activates MyoD, Myf5 and Myogenin expression (17, 135).

Pax7 expressing cells derived from the central dermomyotome give rise to most if not all satellite cells (202). Satellite cells arise during the late stages of embryogenesis in chick and mouse. This subpopulation of cells provides most of the myonuclei to adult muscles during postnatal muscle growth. In adult muscle, satellite cells exist in a quiescent state and are located between the basal lamina and the sarcolemma of myofibers. Pax3, Pax7, M-Cadherin and c-Met are identifying markers of satellite cells (93). It has been proposed that these cells are of somitic origin, but recent studies have shown that satellite cells may derive from cells associated with the embryonic vasculature, specifically the dorsal aorta (201). Somitic angioblasts derived from the paraxial mesoderm colonize the dorsal aorta. Other groups have found considerable heterogeneity in satellite cell populations, which could reflect a more varied developmental origin than previously demonstrated (13, 244).

Quiescent satellite cells do not express MRFs, though in response to muscle damage or heavy exercise, they become activated, commit to the myogenic lineage, and

express Myf5 or MyoD, during which time the cells undergo a proliferative phase (22, 84). Coexpression of Myf5 and MyoD in satellite cell derived progenitors defines myogenic precursors that proliferate in response to growth factors and cytokines (93, 201). Activation of downstream MRFs such as myogenin or MRF4 is a hallmark of terminal differentiation and fusion of myogenic precursor cells to new or existing fibers.

The capacity of satellite cells to renew is essential to perpetuate muscle regeneration or repair over the life span of the organism. Without this ability to self renew, the number of satellite cells would decline after repetitive injury and in the course of normal tissue turnover. Two possible mechanisms have been proposed to describe satellite cell renewal (reviewed in (97, 121, 122, 148, 204). During asymmetric division, two daughter cells are formed, of which one remains quiescent and the other undergoes myogenic activation and ultimately terminal differentiation. In contrast, during symmetric division, both daughter cells are proliferative and activated. The majority of daughter cells down-regulate Pax7 and differentiate into myofibers, while a sub-population retain Pax7 expression and returns to a quiescent state, earmarked for replenishing the satellite cell pool (reviewed in (97, 121, 122, 148, 204).

During the regulation of adult myogenesis, an essential role exists for MyoD (42, 193, 238). In mice that lack MyoD (MyoD-/-), there are increased numbers of satellite cells and deficient regenerative process in skeletal muscle (42, 193, 238). Similar phenotypic signatures were found in MyoD and Dystrophin null (MyoD-/-,Dmd-/-) mice indicating that MyoD deficient satellite cells have increased potential for renewal rather than differentiation (144). Alternatively, others have shown that MyoD-deficient mice

have defects in differentiation which is accompanied by increases in Myf5 expression, consistent with generated mouse knockout models (42, 193, 238). In the absence of Myf5, Lacz-expressing epaxial myogenic progenitors were reduced, indicating that Myf5 may regulate myoblast proliferation (211). Adult myoblasts that are deficient for MyoD but express high levels of Myf5 proliferate rapidly but differentiate poorly, whereas Myf5-/- myoblasts from newborn mice have reduced proliferative potential (42, 147, 193, 238). Transcription profile analysis using MyoD-/-, Myf5-/- myoblasts expressing oestradiol inducible MyoD demonstrate that MyoD regulates several gene clusters, which orchestrate differentiation events.

Approximately 20 years ago, subtractive hybridization led to the identification of a factor that was able to direct conversion of 10T1/2 fibroblast to the myogenic lineage (82). This transcript was aptly termed MyoD and was capable of orchestrating the myogenic conversion of many cell types including fibroblasts, chondrocytes, and neurons. Identification of MyoD, which belongs to a class of DNA binding proteins that contain a basic helix-loop-helix (bHLH) domain, was closely followed by the identification of other member of this bHLH family; Myf5, Myogenin, and MRF4. These transcription factors bind to a consensus sequence (CANNTG) called an E-box that is present in the regulatory regions of muscle-specific genes. MRFs interact with Myocyte Enhancer Factor 2 (MEF2) proteins, which bind A/T rich sequences in regulatory regions of muscle-specific genes to synergistically activate downstream muscle gene expression. MEF2 was originally identified as a muscle-specific DNA binding protein that was induced when myoblasts differentiated into myotubes (82). The 4 genes of the MEF2

family have 85% homology within the 56 amino acid MADS (MCM1, Agamous, Deficiens, SRF) domain, which has been shown to regulate serum-inducible and muscle gene expression (205).

MEF2 binding sites $CTA(A/T)_4$ TG have been identified in the promoter sequences of muscle-specific genes and were shown to be important for skeletal muscle and cardiac muscle gene expression (65, 160). The MEF2 binding site within the myogenin promoter has been shown to be essential for transcription activation in cultured mouse cells and converted mouse fibroblasts (38, 64, 243). Transfection of MEF2 family members into fibroblasts failed to induce myogenic conversion unless coexpressed with myogenic bHLH proteins. This transcriptional cooperativity required interactions between the DNA-binding domain (DBD) of MEF2 and bHLH of MRFs.

In mammalian cells, the MAPK p38 was initially identified with a member of the MEF2 family, MEF2C (87). Induction of the host immune system defense cascade can be triggered by lipopolysaccaride, the endotoxin secreted by Gram (-) bacteria, and is mediated by MAPKs. Phosphorylation of this protein potentiates its trancriptional activity. It was further established that p38 substrates include another member of the MEF2 family; MEF2A but not MEF2B or MEF2D (249). Phosphorylation of threonine residues within the transcriptional activation domain of MEF2A results in enhanced MEF2-dependent gene expression (249).

As many MAPKs phosphorylate similar motifs, the question of specificity can be resolved with an additional determinant. Members of the MAPK family interact with their target substrates via a docking domain for efficient phosphorylation and maximal

activation (242). MEF2A and MEF2C were preferentially phosphorylated by p38• and p38ß and the phosphorylation and resulting transactivation were dependent on a kinase docking domain (242).

Additional members of the MAPK are essential during muscle differentiation. Expression of a dominant negative version of PI3K or growth of myoblasts in the PI3K inhibitors, LY294002, inhibits the transcriptional activity of MEF2 but its DNA binding activity is intact (215). Phosphorylation of MEF2 proteins during muscle differentiation is prevented if PI3K is non-functional. Interestingly, the activity of p38 is not affected by PI3K in muscle cells and can substitute for PI3K during the induction of MEF2 and muscle gene activation, indicating that these kinases function in distinct but parallel pathways to transactivate myogenic targets (215).

Regulatory sequences within the myogenin proximal promoter include the E-box, MEF2 and MEF3 sites. MyoD, MEF2s and Six proteins are bound to these sites and function in parallel but nonredundant pathways to control myogenin expression. Inhibition of any of these pathways results in reduced activation of myogenin (235). The association of MEF2 with various cis-elements was examined in primary human skeletal muscle and isolated rat muscle following various stimuli (2). Exposure to insulin, hydrogen peroxide, osmotic stress or AICAR led to robust increases in MEF2 DNA binding. Preincubation of human skeletal muscle cells with inhibitors of p38 MAPK, MEK1, PKC, PI3K or AMPK. Cells failed to differentiate when treated with MAPK inhibitors and were partially blocked in response to inhibition of PKC, PI3K and AMPK (2).

During differentiation, activation of p38 MAPK facilitates the binding of MyoD and MEF2 at a subset of late gene promoters (166). Mef2D binding is able to recruit the active phosphorylated and elongating form of RNA Pol II. Precocious activation of p38 and coexpression of Mef2D are able to shift the activate the expression of late myogenic genes to earlier stages of differentiation (166). Extracellular factors initiate signal cascades within the cell. Activity of MAPK p38 is induced during terminal differentiation of rat L8 cells (245). The addition of the p38 inhibitors SB 203580 to myoblasts prevented them from accomplishing fusion into multinucleated myotubes and inhibited expression of MyoD and MEF2 family members as well as terminal differentiation markers such as myosin light chain 2 (MLC2) (245).

During developmental myogenesis, MEF2A and p38 expression is concurrent with Myf5 protein expression in the somite at E9.5. Inhibition of p38 signaling using SB 203580 resulted in the failure of MEF2 activation as well as blockage of myogenic differentiation in somite culture and embryos (51). Malignant cells such as rhabdomyosarcomas (RMS), a tumor that arises from muscle precursors, are characterized by a block in the differentiation program and deregulated proliferation despite MyoD expression. Ectopic overexpression of p38 by activated MKK6, restores MyoD function and augments MEF2 activity in RMS, resulting growth arrest and terminal differentiation (179). GRIP1, a cofactor of MEF2 is expressed in proliferating myoblasts, but upon differentiation its localization becomes punctuate and is observed in nuclear bodies (36). MEF2 and GRIP are coexpressed in the nucleus of differentiated myotubes. In RMS cells derived from human tumors, GRIP1 expression is weak and

MEF2-dependent activation of transgenes is defective in ARMS cells. Aberrant localization of GRIP1 and its cofactor MEF2 contribute to RMS phenotype (36). Collectively, these findings indicate that an integral exists for MEF2 proteins during skeletal myogenesis and their enzymatic function is required for potentiate the activity of tissue specific transcription factors such as MyoD but also to facilitate the recruitment of chromatin remodeling enzymes and general transcription machinery.

Transgenic and Knockout mice

Genetic disruption of the MyoD locus was used to generate MyoD null mice (191). These mice were viable and had no gross abnormalities in the musculature. Interestingly Myf5 mRNA was elevated in the postnatal skeletal muscle of these mice, suggesting that Myf5 is regulated by MyoD and that Myf5 possibly compensates for MyoD (191). Null mutations of the Myf5 locus resulted in mice that were unable to breathe and died immediately after birth due to the absence of a major portion of distal ribs (26). Though the mice were not viable, no gross morphological defects were observed in skeletal muscle. Of note, the appearance of myotomal cells in somite was delayed by several days and complete ossification of the sternum occurred (26). As no muscle-specific phenotype was noted in either MyoD or Myf5 null mice, these factors were postulated to behave redundantly and to compensate for each other. To address this assertion, MyoD/Myf5 double knockout mice were generated. These mice were born, but were immobile and died shortly after birth. These mice were characterized by a complete absence of skeletal muscle, when analyzed using histology (192). Gene

expression analysis indicated no detectable skeletal muscle specific mRNAs and examination by immunocytochemistry showed absence of desmin-expressing myoblast-like cells. Studies conducted using the double knockout (DKO) MyoD/Myf5 null mice indicate that MyoD or Myf5 are essential for the determination of skeletal myoblasts and are also required for propagation and specification of myoblasts during development (192).

Myogenin null mice were generated by disrupting the myogenin locus (92, 152). These mice were able to survive fetal development but died shortly after due to a severe reduction in mature skeletal muscle fiber, though the population of proliferating myoblasts was unperturbed. These studies indicated that myogenin was dispensable for specification to the myogenic lineage but was necessary for terminal differentiation of myofibers (92, 152). To examine the relationship between MyoD and/or Myf5 with other MRFs, DKO Myog/Myf5 null mice were generated (183). These mice were not viable and died within first few minutes of birth due to absence of functional skeletal muscle and ribs, respectively. Since MyoD/Myf5 null mice were nonviable this suggests that two viable copies of Myf5 are required for survival in the absence of MyoD. When examined by by hematoxylin and eosin histology, the phenotype of the Myog/Myf5 DKO was indistinguishable from Myog null mice; severe reduction in myofibers in a background of mononucleate myoblasts. Immunostaining of total MHC was reduced and highly disorganized as was also seen in myogenin null cells, though the additional disruption of the Myf5 gene did not exacerbate the phenotype, indicating that MyoD or Myf5 do not share overlapping functions with myogenin. Gene expression analysis via

RT PCR showed no upregulation of MyoD, indicating that MyoD did not compensate for the lack of myogenin. Additionally, a failure to activate MRF4 and MCK was observed, implying that these genes are activated by myogenin. MyoD/Myog DKO mice were generated to further probe the relationship between these MRFs. When stained with hematoxylin and eosin, the phenotype of these mice was indistinguishable from myogenin null mice. Similarities included a severe reduction in myofibers, accompanied by an unperturbed population of mononucleate myoblasts. As seen in Myog/Myf5 DKO mice, MHC staining was reduced and highly disorganized. Interestingly, RT PCR showed upregulation of Myf5 twofold in response to the null mutation of MyoD. Myog/MyoD double mutant also failed to activate MRF4 and MCK (183).

The Mrf4 locus was disrupted to generate Mrf4 null mice (25). Interestingly perturbation of the Mrf4 locus also resulted in pronounced down-regulation of Myf5, rendering this mouse model essentially Mrf4/Myf5 knockout. Mrf4 mutants resembled the phenotype observed in Myf5 null mice, with aberrant and delayed early myotome formation with lack of distal rib structure and a reduction in size of axial muscles in the back. Existing myofibers appeared fairly normal, implying that Mrf4 has no major role in maturation of myotubes (25). In contrast, others have revealed that a Myf5/MyoD DKO mouse bears no muscle phenotype when Mrf4 is intact, asserting that Mrf4 too, is able to modulate specification of cell fate to the myogenic lineage (107). Mrf4/MyoD double null embryos did not survive beyond birth due to an inability to breathe. These mice presented with lordokyphosis, absence of skeletal muscle and an accumulation of fat at the neck. Gross examination indicated a similar phenotype to the myogenin null

mouse. Residual myofibers were found surrounded by mononucleated cells. Intriguingly, the residual myofibers had centrally located nuclei, indicating they were newly fused. Gene expression analysis indicated that expression of MCK, MHC and alpha-skeletal actin was severely affected in double Mrf4/MyoD null mice. These studies indicate that Myogenin and MRF4 are involved in the differentiation of skeletal muscle but are not functionally redundant and do not compensate for each other.

Chromatin Organization

A paradox exists in eukaryotic cells since over a meter of DNA must be condensed into the limited volume of the nucleus and yet still be accessible for processes such as transcription, replication and DNA repair. The simplest unit of chromatin is the nucleosome, which is comprised of 146 bp of DNA and 8 core histones; a tetramer of $(H3\text{-}H4)_2$ and 2 dimers of H2A-H2B (44, 66, 114, 115, 170, 188). The central 121 bp of DNA bind $(H3\text{-}H4)_2$ while the peripherally localized H2A-H2B dimers bind DNA at the entrance and exit of the nucleosome, bringing the total length of DNA wound around the nucleosome to ~147 bp (67, 94, 129, 154). The disc-shaped nucleosome is approximately 11nm in diameter x 5.7nm in height and has 1.7 turns of DNA associated with it (73, 187). The histones interact with the phosphodiester backbone of DNA and these points of contact occur every 10 bp (132). As such the minor groove of the DNA is turned inside, facing the nucleosome. DNA bases do not interact with histones, therefore DNA interactions are not sequence dependent, though it is noteworthy that some DNA sequences favor the positioning and formation of nucleosomes (132).

Each histone has two domains; the histone fold motif which is necessary for histone-histone and histone-DNA interactions, and the histone tail motifs in the N and C-termini, which contain residues for post-translational modification that affect the organization of chromatin into a transcriptionally permissive or non-permissive state (89, 95). Limited proteolysis was used to create tailless core histones. Nucleosome arrays without N-terminal domains (NTDs) were unable to form secondary or tertiary chromatin structures (3, 75, 80, 200, 221, 230). Therefore, the 14-38 amino acids from the NTD extend outside the disc-shaped complex and are required for formation of condensed higher order chromatin structures. The NTD of core histones is required for linker DNA and internucleosomal interactions (3, 75, 80, 200, 221, 230).

Linker histones are another integral component of chromatin structure. Linker histones are not structurally related to core histones, but do exist in practically stoichiometrical association with nucleosomes, indicating they are involved in the assembly and formation of higher order chromatin structures and dynamics (9, 228, 229). The linker histones is composed of a short unstructured NTD, a central globular winged helix domain and a long C-terminal domain (CTD) consisting of approximately 100 amino acid residues (9, 228, 229). The association of linker histones with core histones and linker DNA protects an additional 20 bp of DNA from nuclease digestion, stemming from a proposed “stem-loop” structure which forms upon binding of H1 linker histone. Linker histones are also required for stabilization of the 30nm fiber and oligomeric tertiary chromatin structures (16, 33).

The chromatin fiber is defined as an assembly of linker histone and non-histone proteins associated with nucleosomal arrays forming compacted chromatin structures in vivo. Repeating nucleosomal units exist as “beads on a string” in low salt conditions. This 10nm fiber exists in equilibrium with highly compacted chromatin structures and can be condensed into the 30nm fiber in a solution containing the ionic conditions 1-2mM divalent cation (8, 241). Self-association of these condensed fiber structures is accomplished through the NTDs of the core histones. In fact, the NTD of H4 is critical for full fiber folding (61, 200, 221). In eukaryotic cells during mitosis, further compaction of the 30nm chromatin solenoid into a 60-300nm fiber is observed, though this process is far from elucidated. Linker histones are thought to aid in the condensation of chromatin structure either by continuing the coiling or assembling into a zig-zag formation (230). Formation of higher order chromatin structures may occur through further compaction of these 30nm fibers or through side-by-side arrangement of the fibers (45, 72, 131, 231, 232).

Packaging of promoter elements into nucleosomes *in vitro* inhibits the initiation of transcription by bacterial and eukaryotic RNA polymerases (112, 130). Further, the genetic abrogation of histone synthesis in yeast results in loss of nucleosome formation and consequently, precocious activation of previously inactive genes (88). Thus, the chromatin structure at specific gene loci are non-permissive and must be manipulated in a fashion to render DNA sequences accessible to the transcriptional machinery. Alteration of chromatin structure can be achieved through the modification of N-terminal histone tails by acetylation, deacetylation, methylation, phosphorylation, ubiquitination, or

sumoylation. ATP-dependent chromatin remodelers are able to affect nucleosomal structure by "sliding" or changing the path of DNA around the histone octamer. The interplay between ATP-dependent remodelers and histone methyltransferases during the temporal regulation of skeletal muscle target genes will be emphasized in this introduction.

ATP- dependent chromatin remodelers

ATP-dependent remodeling enzyme are divided into four major subfamilies, characterized by the identity of the central catalytic subunit Brg1 (or hBrm), ISWI, Mi-2 and Ino80, which belong to SWI/SNF, ISWI, NuRD and INO80 complexes, respectively (reviewed in (63, 206). These enzymes use energy derived from ATP hydrolysis to actively alter nucleosomal structure, which is an essential step for transcriptional regulation of many eukaryotic genes (28, 103, 106, 108, 169, 181, 185). Specific temporal regulation of gene activation must occur during skeletal muscle differentiation. The chromatin structure of inactive genes precludes access of transcription factors, coactivators and the transcriptional machinery to the DNA. In order for muscle specific gene expression to proceed, MyoD must form heterodimers with ubiquitous E proteins via helix-loop-helix (HLH) domains (120, 151). The basic regions of MyoD act as a sequence specific DNA binding domain that recognizes a consensus sequence CANNTG termed the E-box. MyoD-E protein heterodimers recognize E-boxes commonly found in the regulatory regions of skeletal muscle genes in order to regulate transcription (81).

In addition to interctions between MyoD and cofactors with requisite cis-elements, post-translation modifications of MyoD are essential for determining its functional status. MyoD contains two putative PKC phosphorylation sites (Thr 115 and Ser 200), which are analogous to Thr85 within the highly conserved basic domain of myogenin (128). Phosphorylation of these residues results in reduced transactivation of myogenin. Thr 115-Ala was mutated to determine if this site would also attenuate MyoD function. CH310T1/2 fibroblasts expressing wt or mutant MyoD were able to induce terminal differentiation and fuse into multinucleated myotubes. Mutant MyoD was able to bind a consensus E-box sequence when subjected to EMSA (128). However, when cells were differentiated and maintained in high mitogen conditions, only mutant MyoD was able to induce cell cycle arrest, late gene activation and E-box oligonucleotide binding. Cells maintained in high mitogen conditions contained phosphorylated MyoD and significant levels of phosphorylated threonine. In RMS cells, MyoD was phosphorylated and its function attenuated (128).

Using nuclease accessibility studies, it was determined that the gene loci regulated by MyoD exist in a heterochromatic state prior to MyoD expression in the cell (81). MyoD expression was accompanied by induction of chromatin remodeling at these loci. The functional status of MyoD is an essential event during the induction of skeletal muscle transcripts and therefore, modulation of MyoD activity is essential for the initiation of gene expression.

To determine the requirement for chromatin remodeling enzymes in cellular differentiation events, fibroblasts inducibly expressing dominant negative versions of

human Brahma-related gene 1 (BRG1), and Brahma (Brm), the ATPase subunits of distinct SWI/SNF enzymes (30, 153, 167, 206, 209, 227), were used to examine MyoD-induced skeletal muscle differentiation. Induction of myogenin and myosin heavy chain (MHC) transcripts and protein was abrogated in cells expressing dominant negative enzymes (53). Extracts from mock or MyoD-differentiated cells were incubated with a oligonucleotide probe encompassing the MyoD binding site at the muscle creatine kinase (MCK) enhancer, and the bound complex was specifically super-shifted by MyoD antibody. These results demonstrated that a MyoD-associated complex was able to bind the MCK enhancer and this interaction was unaffected by dominant negative BRG1. Compromised muscle-specific gene activation correlated with ablation of nuclease accessibility at the myogenin promoter indicating that ATP-dependent SWI/SNF-mediated chromatin remodeling is required for activation of muscle-specific targets(53).

Though the transcriptional activation of muscle regulatory factors and structural genes was inhibited by dominant negative SWI/SNF, activation of cell cycle regulators was unperturbed. Fluorescence activated cell sorting (FACS) indicated that cells arrested normally in the presence of dominant negative proteins (56). Further, transcript and protein synthesis of cell cycle genes were unaffected in cells expressing mutant SWI/SNF, indicating that ATP-dependent chromatin remodelers are required for muscle-specific genes but not cell cycle targets (56). Since ATP-dependent chromatin remodeling is required for MyoD-mediated muscle differentiation, the interplay between SWI/SNF and the other muscle regulatory factors (MRFs), Myf5, myogenin and MRF4 was examined (190). Induction of muscle-specific genes by Myf5 and MRF4 was inhibited by

dominant-negative BRG1, but myogenin was unable to activate any myogenic transcripts. In contrast, all four MRFs were able to induce cell cycle regulatory transcripts and proteins even in the presence of mutant SWI/SNF proteins, indicating that MRFs have a similar requirement for the activity of SWI/SNF during the activation of myogenic targets but not cell cycle genes (190).

Microarray analysis determined that approximately 30% of MyoD-regulated genes required the activity of SWI/SNF (19, 54). To finely dissect the interplay between MyoD and SWI/SNF, chromatin immunoprecipitations (ChIPs) were performed on the myogenin promoter, revealing that hyperacetylated histone H4 was enriched in a MyoD-dependent manner prior to the binding of Brg1. As chromatin remodeling enzymes require the function of a transcription factor to recognize and bind regulatory sequences, a two-step mechanism of MyoD-mediated gene activation was revealed. Though stably bound MyoD does not appear at the myogenin promoter until ~8 hours post-differentiation, at the time of myogenin activation, MyoD interacts with the constitutively bound Pbx/Meis homeodomain protein complex, which permits targeting of chromatin remodelers such as HATs and SWI/SNF (19, 54). SWI/SNF family members contain a bromodomain motif that allows recognition and binding to acetylated lysine residues within histone N-terminal tails in vitro. Indeed, co-immunoprecipitation studies demonstrate that Pbx1 and BRG1 are able to associate prior to the appearance of stable MyoD binding and well before the onset of myogenin gene activation (19, 54). These findings revealed a two-step mechanism of gene activation, in which MyoD associates

with the constitutively bound heterodimer Pbx/Meis at the myogenin promoter in order to target HAT and SWI/SNF activity to histones. (Figure 1-3)

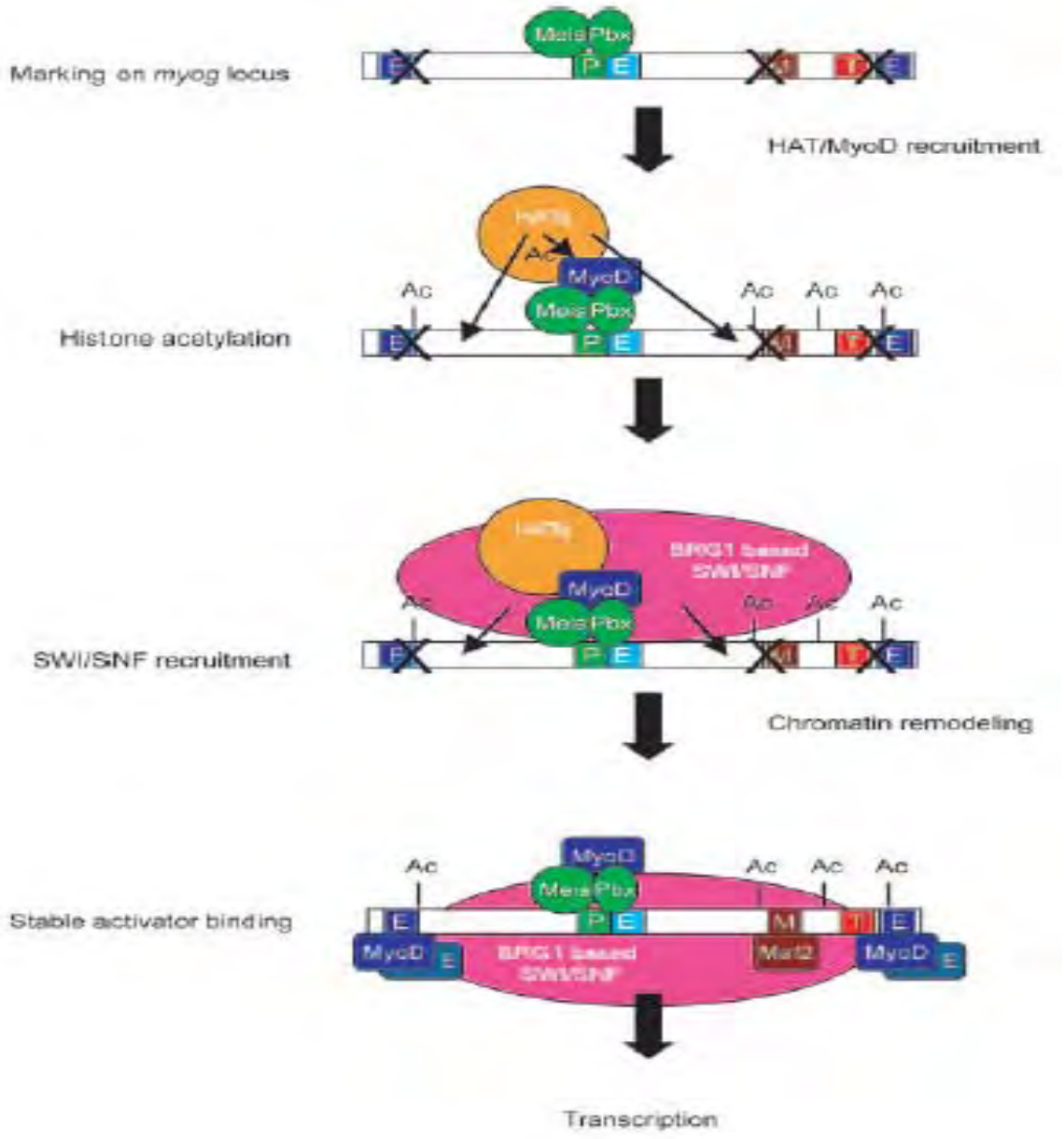

Figure 1-3.

(de la Serna, IL et al. 2005. MCB)

Figure 1-3.

The heterodimer containing homeodomain proteins Pbx1 and Meis is constitutively bound to the myogenin promoter. MyoD associates with this heterodimer in order to target HATs and mediate acetylation of histones at the myogenin promoter. Hyperacetylated histones allow binding of Brg1 to target sequences in the myogenin promoter and subsequent chromatin remodeling allowing binding of sequence-specific binding of transcription factors such as MyoD and Mef2, which initiate transcription activation.

Additional regulatory mechanisms are in place to modulate the activity of early muscle specific gene transcription. The p38 pathway, which is activated by extracellular signaling upon differentiation, causes targeting of p38 kinase to the chromatin of muscle-specific regulatory sequences and facilitates the binding of SWI/SNF to myogenic loci (207). Inhibition of p38 binding resulted in a loss of SWI/SNF complex binding at these elements but did not affect other factor binding. Forced activation of p38 in myoblasts elicited precocious recruitment of SWI/SNF to the myogenin promoter (207). Inflammation induced MKK6-p38 and insulin growth factor 1 (IGF1) signaling induced PI3K/AKT function in concert to target transcriptional components to the chromatin of muscle specific genes (203). p38 kinases recruit SWI/SNF while PI3K/AKT promote the association of MyoD with p300 and pCAF by direct phosphorylation of p300. Perturbation of either pathway leads to disassembly of chromatin bound complexes. PI3K,AKT permitted recruitment of MEF2-SWI/SNF complexes that were defective for chromatin remodeling activity in the absence of MyoD and HAT activity (203). In the context of malignant cells, the combination of MyoD and myogenin is not sufficient to induce terminal differentiation (126). Treatment of cells with tetradecanoylphorbol-13-acetate (TPA), resulted in recruitment of PCAF and BRG1 to the myogenin promoter. Recruitment of chromatin modifying enzymes was dependent on p38 MAPK activity (126). These findings demonstrate that transcriptional regulation of early myogenic gene expression is governed by a complex array of factors that can translate cellular signaling events into chromatin changes at muscle specific loci.

As the functional relationship between SWI/SNF and MRFs has been well described at the promoter regions of early myogenic markers, analysis of molecular events at late muscle-specific loci remained. In embryonic tissue and transdifferentiated cell culture, MyoD is bound concomitantly with HDAC2 at the promoter regions of late myogenic targets upon the onset of differentiation but prior to late gene activation (157). Upon induction of muscle-specific transcripts, a transition in which MyoD is replaced with myogenin, occurs concurrently with the loss of HDAC2, association of the Mef2d coactivator, and binding of the BRG1 remodeling enzyme at these regulatory regions. Expression of dominant-negative BRG1 results in abrogation of late myogenic gene activation and diminished nuclease accessibility at these loci, indicating that SWI/SNF activity is required for the transcription activation of these genes. Indeed, co-immunoprecipitation and ChIPs demonstrate that BRG1, Mef2d1b, and myogenin associate with each other and are present at the promoter regions of these loci concomitantly. From these studies it is apparent that myogenin and Mef2d1b are sufficient to induce activation of late myogenic target genes in the absence of MyoD in a BRG1-dependent manner. Therefore, myogenin cooperates with Mef2d to facilitate recruitment of SWI/SNF to the regulatory sequences of late myogenic loci to activate gene transcription (157).

Histone Acetyltransferases

20 years ago studies done in chicken myoblasts indicated that histone acetyltransferase activity increased robustly for 24 hours and declined after several days

in culture (127). Since the levels of HAT transcript and protein were temporally regulated during differentiation, it is likely that HAT activity is required for the transcription of myogenic genes (127). FLAG-tagged MyoD was able to coimmunoprecipitate with p300/CBP-associated factor (PCAF). Acetylation of lysine residues in the N-terminus of MyoD was mediated by PCAF, which enabled MyoD to bind its target DNA with greater affinity (196). Mutation of these residues from lysine to arginine abrogated the activity of MyoD to transactivate reporter DNA in c2c12 myoblasts, though dimerization with E12 and binding of DNA were still intact. When these arginine mutants were exogenously expressed in 10T1/2 cells, myogenic differentiation failed to occur as immunohistochemical analysis revealed few MyoD and MCK double positive cells (196).

Other groups indicated that MyoD is modified by additional HATs such as CBP/p300 (171). Anti-acetyl lysine antibody was able to coimmunoprecipitate MyoD in c2c12 myoblasts and reciprocal pulldowns using MyoD antibody also precipitate acetyl-lysine. Recombinant MyoD could be acetylated by p300 in vitro though lysine to arginine mutations in the N-terminus precluded p300-mediated acetylation (171). To further examine the interaction between these two coactivators, coimmunoprecipitations were performed and demonstrated that acetylated MyoD interacts with the bromodomain of p300/CBP . Deletion of the bromodomain rendered p300 unable to associate with MyoD. K-R mutants, or the non-acetylatable forms of MyoD were unable to associate with p300/CBP, though the acetylated form of MyoD exhibits enhanced affinity for CBP. Additionally, acetylated MyoD which interacts with p300/CBP, was also proficient at

transactivating an MCK-Luciferase promoter. Association of p300/CBP with myogenic promoters corresponded to specific increases in acetylated histone H4, implying that the activity of histone acetyltransferases was needed for potentiation of transcription factor activity but also for histone modification (172-174).

Cooperation between MRFs and E proteins is required for optimal activation of muscle-specific genes. To determine if coactivators such as p300/CBP were also required for full-fledged induction, transactivation studies were performed using FLAG-tagged MyoD, and FLAG-tagged E47 in concert with PCAF, and p300/CBP (59). Maximal activation of a luciferase reporter containing 4 tandem E-boxes required the synergistic activity of both p300/CBP and PCAF. Addition of p300/CBP or PCAF alone did not achieve robust transactivation. Temporal analysis indicated that p300/CBP is required to acetylate histones while PCAF was needed for MyoD acetylation, and this order of modifications was required for maximal transactivation (59).

The MEF2 family of coactivators interacts with MyoD to potently activate myogenic gene targets (133). To describe the interplay between HATs and this coactivator, coimmunoprecipitations were performed, demonstrating that acetylated MEF2C was able to associate with p300/CBP. Transfection of MEF2C and p300 into 293T cells indicated that p300/CBP specifically acetylates MEF2C as si-RNA-mediated knockdown of p300 resulted in compromised MEF2C acetylation (133). Truncation mutants without the MADS and MEF2 domain were unable to bind p300/CBP and were consequently, hypoacetylated. Mutations in acetylated lysines of MEF2C resulted in diminished transactivation of a luciferase reporter containing 3 tandem MEF2 sites.

Failure to transactivate was likely due to inability of unacetylated MEF2C to bind DNA, as EMSA analysis indicates that lysine to arginine mutants that cannot be acetylated, have reduced ability to bind DNA. Transfection of these mutants concurrently with myogenin in 10T1/2 cells results in failed myogenic conversion and down-regulation of MHC, and myogenin at early time points of differentiation (133).

Histone Deacetylases

Histone deacetylases are divided into three classes based on their homology to yeast homologues Rpd3, Hda1, and Sir2. Class I and II HDACs are expressed in proliferating undifferentiated myoblasts where they are thought to repress gene activation (143). The muscle regulatory factor MyoD associates with HDAC1, a class I HDAC, and prevents precocious activation of myogenic genes (11, 134). MyoD is released from HDAC1, which then associates with hypophosphorylated Rb upon differentiation, allowing the activation of skeletal muscle targets (18, 119, 177). Rb and HDAC1 stimulate myogenic differentiation by complexing with the transcription factor E2F and repressing downstream gene targets that promote cell proliferation. Forced ectopic expression of HDAC1 appears to block activation of late myogenic genes, therefore the reciprocal interactions between MyoD, HDAC1, and Rb serve to regulate expression of transcripts from late but not early stages during differentiation (18, 119, 177).

The activity of HDACs must be carefully manipulated to coincide with temporal gene regulation. Post-translational modification, specifically phosphorylation, of HDAC1 at residues in the extreme C-terminus is mediated by the serine/threonine kinase

Casein kinase II (CKII) (29, 78, 79, 168). Alanine substitution of these residues reduces the deacetylase activity of HDAC1 even though these residues are not part of the catalytic domain (49). The phosphorylation of residues at this site inhibits an interaction between repressors and/or cofactors like Rb, p38, and Sin3A, that stimulate HDAC1 activity and ultimately affect specific gene induction. Intriguingly, HDAC1 is also sumoylated on lysine residues in the C-terminus. SUMO is a small ubiquitin-like moiety which is attached by an E3 ubiquitin ligase as a monomer, but does not target the protein for degradation. Sumoylation increases protein stability, and/or alters the sub-cellular localization of a protein. It has been observed that sumoylated proteins have altered enzymatic properties and protein interactions with cofactors are disrupted. In the case of HDAC1, sumoylation enhances its ability to promote gene repression. C-terminus lysine to arginine mutants cannot be efficiently sumoylated and have reduced repressive capacity (49).

Inhibitors of HDAC activity include trichostatin A (TSA), valproic acid, and sodium butyrate (21, 74, 104, 208). These compounds were capable of stimulating or inhibiting muscle differentiation depending on the time and duration they were added to differentiation media. HDAC inhibitors added at the onset of differentiation, concurrently with serum withdrawal in culture, resulted in the failure of myoblasts to differentiate (101). Differentiation media supplemented with inhibitors prior to differentiation, or prior to serum withdrawal exhibited enhanced muscle differentiation (101). MEF2 interactions with class II HDACs also regulated skeletal muscle gene activation (140, 142). Lysine 9 on histone H3 N-terminal tails located near a MEF2

binding site were hypermethylated in a proliferating undifferentiated myoblast (247). However, upon differentiation, expression of myogenin was upregulated and H3 lysine 9 became hyperacetylated. HDACs formed a complex with heterochromatin protein 1 (HP1), which targeted the activity of lysine methyltransferases to the site, generating methylated hypo-acetylated chromatin (247). HDAC and HP1 interactions could be disrupted by calcium calmodulin kinases (CaMKs) without phosphorylation of 14-3-3 binding sites. Myogenic signals overcome the MEF2-HDAC interactions by stimulating nuclear export. CaMK I-IV could phosphorylate HDACs 4, 5, 7, and 9 at two conserved serine residues. These phosphor-serine residues could be bound by 14-3-3, which led to a disruption between MEF2 and HDAC and exposure of a nuclear export sequence in HDACs 4, 5, 7, and 9 (62, 86, 139, 141, 224). CaMK-independent regulation of class II HDAC phosphorylation also has been identified (246, 247). This putative kinase, mediating CaMK-independent regulation was capable of phosphorylating 14-3-3 binding site residues on HDACs 4, 5, 7, and 9 but is resistant to CaMK inhibitors, and pharmacologic antagonists of PKC, PKA, PKG, & various MAPKs. Interestingly, this unidentified kinase was sensitive to broad-spectrum antagonists. The identity of this kinase is incredibly important as it regulates transcriptional control of skeletal muscle, cardiac muscle, and neuronal gene expression (246, 247).

Histone Methyltransferases

Lysine Methylation

A conserved feature found in a few chromatin-associated proteins was termed the Su(var), Enhancer of zeste, Trithorax (SET) domain (102, 220) (184, 189, 225). The homology of the SET domain to the plant protein Rubisco, which was characterized as a methyltransferase, led to the identification of several proteins identified as histone lysine methyltransferases (184, 189, 225). The methyltransferase Ezh2 is present in proliferating myoblasts and in embryos at E9.5. Ezh2 overexpression inhibits muscle gene activation while a SET-deficient mutant allows gene transcription (32). Analysis by ChIP indicates that Ezh2 is present at the MHC II and MCK, but not the myogenin promoters in myoblasts. Coimmunoprecipitation demonstrates that Ezh2 associates with HDAC1 and the transcriptional repressor YY1 and these proteins are present at the MCK and MHC II promoters exclusively in myoblasts but not in myotubes, while SRF was present at MCK and MHC II promoters in myotubes. Ezh2 association with the regulatory regions of MCK and MHC II resulted in increased histone H3 K27 tri-methylation. Si-RNA-mediated knockdown of YY1 resulted in loss of Ezh2 binding, and also H3 K27 tri-methylation at the MCK and MHC II promoters, indicating that Ezh2 binds YY1 to access the DNA. The proposed model postulates that DNA-bound YY1 interact with Ezh2 and HDAC1 prior to gene activation, but are replaced with SRF, which causes hypomethylation at H3K27 and loading of MyoD and other chromatin remodelers and transcription factors (32).

Arginine Methylation

Protein arginine methyltransferases are evolutionarily conserved from yeast to humans and are classified based on their inclusion in class I, II, or III methyltransferases

(reviewed in (163) and references therein). Type I PRMTs mono- or asymmetrically dimethylate their substrates and type II PRMTs mono- or symmetrically dimethylate their substrates, while type III PRMTs simply mono-methylate their substrates. To date, eleven members of the PRMT family have been identified. With the exception of PRMTs 2, 10, and 11 all others have been experimentally shown to catalyze arginine methylation. PRMTs 1, 4, 5, 7, 8 and 9 are capable of methylating arginines on histone substrates (reviewed in (163) and references therein).

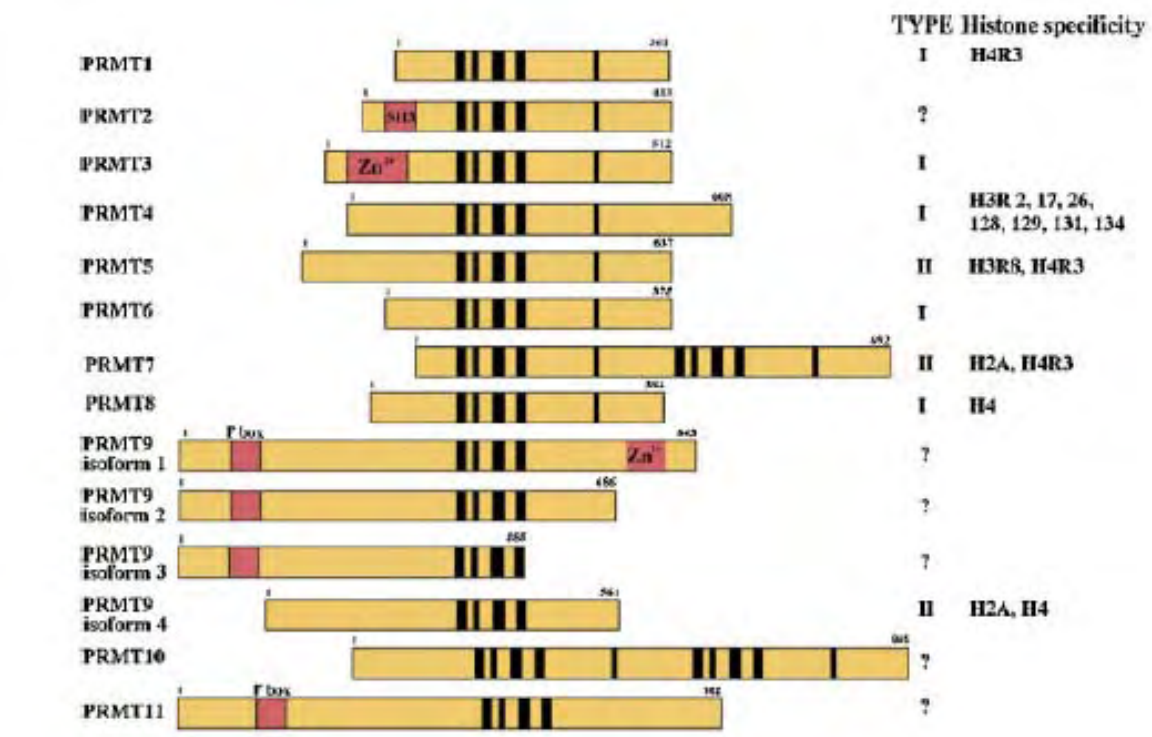

Figure 1-4.

(Pal, S et al. 2007. J Cell Phys)

Figure 1-4.

The PRMT family is comprised of 11 members to date. The conserved catalytic domains and specific motifs of the PRMT are shown. Prmt7 and Prmt10 are unique as they contain two copies of the catalytic domain. Histone substrates of each member are listed though the histone substrates of PRMT2, PRMT9 isoforms 1-3, PRMT10 and 11 are currently unknown.

Prmt4, which is also known as coactivator arginine methyltransferase 1 (Carm1) was identified in a yeast two hybrid screen designed to identify proteins that interact with the AD2 region of p160 coactivators (199). Carm1 has also been shown to interact with and methylate transcriptional coactivators such as p300 and GRIP1. Arginine residues in the N- and C-terminus of histone H3 are methylated by Carm1, specifically Arg 2, 17 and 26 in the N-terminus and Arg 128, 129, 131, and 134 in the C-terminus. Cell lines expressing FLAG-tagged Carm1 were used to purify a multi-subunit complex termed NUMAC, which was found to contain subunits of the Brg1-based SWI/SNF complex. These findings suggest interplay exists between Carm1 and Brg1 (237). Carm1-mediated methylation of H3 is associated with enhanced GRIP-1 and Estrogen-Receptor • (ER•) transcriptional activation (12, 34). Analysis of mice with a genetic disruption of Carm1 reveals that Carm1 is an essential gene since knockout mice undergo normal development but are perinatal lethal (240). At embryonic day E12.5, homozygous null embryos appear normal though at E18.5-19.5, 60% of embryos are smaller when compared to wild type embryos. Mouse embryo fibroblasts (MEFs) isolated from homozygous null animals at E12.5 are deficient for methylation of Carm1 substrates such as Poly-A binding protein (PABP1), p300, and H3R17 (240).

Glutathione-agarose-immobilized GST and GST-Carm1 incubated with 35-S Methionine radiolabel led GRIP-1, MEF2C, MyoD or Myogenin revealed that an interaction exists between Carm1 and Mef2C (35). Though the MADS box of MEF2C was not required for interaction with Carm1, it was required for association with GRIP1. The C-terminal domain of MEF2C is essential for its interaction with Carm1. GST-

MEF2C chimeras containing functional domains of MEF2C were created to determine the specificity of this interaction. Deletion of MADS box or MEF2 domain did not compromise the association between MEF2C and Carm1. However, perturbation of the C-terminal transactivation domain (TAD) ablated any interaction between MEF2C and Carm1 (35).

To further characterize the relationship between these coactivators and their contribution to transcriptional regulation, mammalian two-hybrid was performed. Robust transactivation of a luciferase reporter was achieved when MEF2C, GRIP1, and Carm1 interacted, indicating these cofactors cooperate synergistically during transcription activation of muscle-specific genes (35). A luciferase reporter containing 3 tandem MEF2 sites was transfected into 10T1/2 fibroblasts. Robust transactivation of the reporter was accomplished in 10T1/2 fibroblasts when MEF2C, GRIP1, and Carm1 were expressed concurrently, indicating that Carm1 coactivates MEF2C-dependent transcription. To determine the interaction of these cofactors *in vivo,* ChIPs were performed demonstrating that Carm1 and Mef2 were present at the muscle creatine kinase promoter in differentiated myotubes but not proliferating myoblasts. These findings are consistent with the findings that Carm1 protein and transcripts are expressed in both proliferating myoblasts and differentiated myotubes though its activity was required for the activation of myogenic marker genes in differentiated cells (35). Immunohistochemical analysis indicated that Carm1 is localized to both the cytoplasm and nucleus in proliferating myoblasts but becomes nuclear during differentiation. Treatment of c2c12 myoblasts with the methyltransferase inhibitor adenosine dialdehyde

(AdOX) inhibited differentiation. Analysis of gene expression indicates that activation of early and late myogenic targets was compromised when cells were treated with AdOX. Antisense-mediated knockdown of Carm1 in c2c12 myoblasts results in compromised gene activation of myogenin, corroborating the assertion that Carm1 is essential for myogenic gene activation (35).

Prmt5 was first identified in a yeast two hybrid screen to identify proteins interacting with JAK2. Biochemical characterization revealed that Prmt5 is a type II arginine methyltransferase that mono- and symmetrically di-methylates Arg 8 and Arg 3 in the N-terminal tails of histone H3 and H4, respectively (6, 24, 123, 164, 165, 216). Other cellular proteins that are substrates of Prmt5 include, MBD2, SmD1, and SmD3 (6, 24, 123, 164, 165, 216). Prmt5 is associated with multiple complexes where it directs various processes such as RNA processing, transcriptional regulation, germ line and skeletal muscle differentiation (6, 69, 123, 164, 165). Prmt5 has been purified in association with chromatin remodeling complexes hSWI/SNF and NuRD. These interactions have also been shown to be important for transcriptional repression of cell cycle regulator and tumor suppressor genes (6, 69, 123, 164, 165), indicating that Prmt5 is involved in the transcriptional regulation of a diverse array of genes.

Thesis Aims

Skeletal muscle differentiation is a complex process and has been demonstrated by many groups, to require the combined action of transcription factors, HDACs, HATs and HMTs. However, the role of protein arginine methyltransferases and their relationship with muscle regulatory factors and other chromatin remodeling enzymes is poorly understood. Exquisite temporal regulation of gene transactivation is a hallmark of development and differentiation events, though the mechanisms which govern this process are not well understood. The objective of this thesis was to analyse the mechanism of protein arginine methyltransferase function during myogenic gene transcription.

In Chapter I, we began by utilizing a cell line containing antisense-mediated knockdown against Prmt5 in order to determine whether muscle-specific gene expression was perturbed.

- When Prmt5 protein levels were reduced, activation of myogenic transcripts was diminished.
- Further analysis by ChIP revealed that Prmt5 directly targets the promoter sequences of myogenin, a gene required for terminal differentiation of skeletal muscle.
- Enrichment of di-methylated histone H3 arginine 8 (diMeH3R8) at this locus was mediated by Prmt5.

A time course of ChIPs demonstrated that Prmt5 association with the myogenin promoter occurred at early time points, and did not require the activity of ATP-dependent chromatin remodeling in order to target the DNA.

- Co-IPs indicated that Prmt5 interacts with MyoD, in order to access the regulatory regions of the myogenin locus.

Though many groups have catalogued the dependence of one type of histone modification on another, few reports exist detailing the requirement for histone modifications prior to nucleosome remodeling. In this thesis we uncover that a requirement exists for Prmt5 and its enzymatic activity during myogenesis, specifically to facilitate the recruitment of the ATP-dependent chromatin remodeling enzymes SWI/SNF. In order to determine the dependence of Brg1 binding on Prmt5 binding and activity, we performed ChIPs in cells where Prmt5 recruitment to gene sequences of myogenin was lost.

- Failure of Prmt5 to bind the regulatory regions of myogenin resulted in loss of Brg1 interaction with the promoter and consequently, ablation of nucleosome remodeling, which precluded sequence specific transcription factors such as MyoD from associating with the promoter and intiating transcription.
- ChIPs performed using muscle satellite cells and mature myotubes revealed that Prmt5 associated with and regulated H3R8 dimethylation at myogenin promoter sequences in activated but not quiescent satellite cells *in vivo.*

In order to further examine the role of Prmt5 during myogenic differentiation, we wished to explore the possibility that Prmt5 was associated with and required for the activation of late stage muscle genes.

- ChIPs performed using mouse skeletal muscle tissue samples revealed that Prmt5 was also bound to late myogenic promoters such as desmin and muscle creatine kinase (MCK).
- Time course ChIPs indicated that Prmt5 was bound to late muscle promoter sequences at early times prior to the onset of late gene activation, or differentiation.

To further examine the role of Prmt5 during the transcription activation of late muscle-specific target genes we induced late genes using a combination of exogenously expressed myogenin and Mef2d1b. Cells were also induced using ectopically expressed MyoD. In cells containing intact levels of Prmt5, binding was enriched in late muscle gene loci in cells differentiated using MyoD, but interestingly, Prmt5 was absent on late gene promoters when cells were differentiated with myogenin and Mef2d1b. Using cells in which Prmt5 was knocked down, we examined late myogenic gene expression and observed that when late genes were induced using myogenin and Mef2d1b, no defect in activation was seen. However, in cells where differentiation was accomplished using MyoD alone, late gene activation was significantly reduced. This observation was in agreement with our initial findings, that Prmt5 and dimethylation of H3R8 were necessary for activation of myogenin, a gene required for the induction of late muscle-

specific transcripts. Thus, Prmt5 did not directly influence transcription of late myogenic targets, but was necessary for myogenin activation.

To further our analysis of PRMT involvement during myogenic gene transcription we performed ChIPs in muscle tissue and discovered that

- Carm1 was present at the regulatory sequences of late muscle-specific genes *in vivo.*
- ChIP time course analysis revealed that Carm1 binding occurred concurrently with the onset of late myogenic gene activation.

Using Carm1 -/- null and +/+ wild type MEFs, we observed that Carm1 was associated with the promoter regions of late gene loci in MyoD- or myogenin/Mef2d1b-differentiated wild type samples.

- Loss of Carm1 binding in null MEFs resulted in a loss of Brg1 binding, which consequently led to abrogation of nucleosome remodeling at these loci.

Regardless of MyoD-mediated or myogenin/Mef2d1b-mediated myogenic conversion, null MEFs failed to robustly activate late myogenic targets but were still able to activate myogenin. Thus, Carm1 is directly required for the activation of late muscle-specific transcripts as it facilitates the targeting of SWI/SNF.

CHAPTER II

Prmt5 facilitates ATP-dependent chromatin remodeling and is required during myogenesis

Introduction

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 post-translationally. The activation of skeletal muscle differentiation is regulated by members of the basic helix-loop-helix (bHLH) 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 bHLH proteins (20, 140, 178). 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 (55, 195, 217). 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 (Prmt 1-9), 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 (15, 217). 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 (12, 35, 48, 237). Prmt4 functions at estrogen induced promoters as part of a multi-protein complex that also contains the Brg1 ATPase of SWI/SNF chromatin remodeling complexes (237). 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 (164, 165). Other studies indicated that Prmt5 acts as a repressor of cyclin E (69, 186). 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 (117). 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 NIH3T3 based cell lines that constitutively express a Prmt5 antisense vector and thereby cause a significant reduction in Prmt5 mRNA and protein levels (164). Ectopic expression of MyoD in fibroblast cells induces the myogenic differentiation program (50); this system has been extensively utilized to examine the mechanisms of skeletal muscle gene regulation for nearly twenty years (217).

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.

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 NIH3T3 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 (164).

In vitro skeletal muscle differentiation was initiated by infection of both the control and antisense lines (c15, c12) with a retrovirus encoding MyoD (53, 155) for 30 hours. After infection, low-serum differentiation media was added to the cells and the cells were allowed to undergo differentiation for 36 hours. Control samples that were mock infected were also placed in differentiation media, and are referred to as mock-differentiated. Westerns were 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.

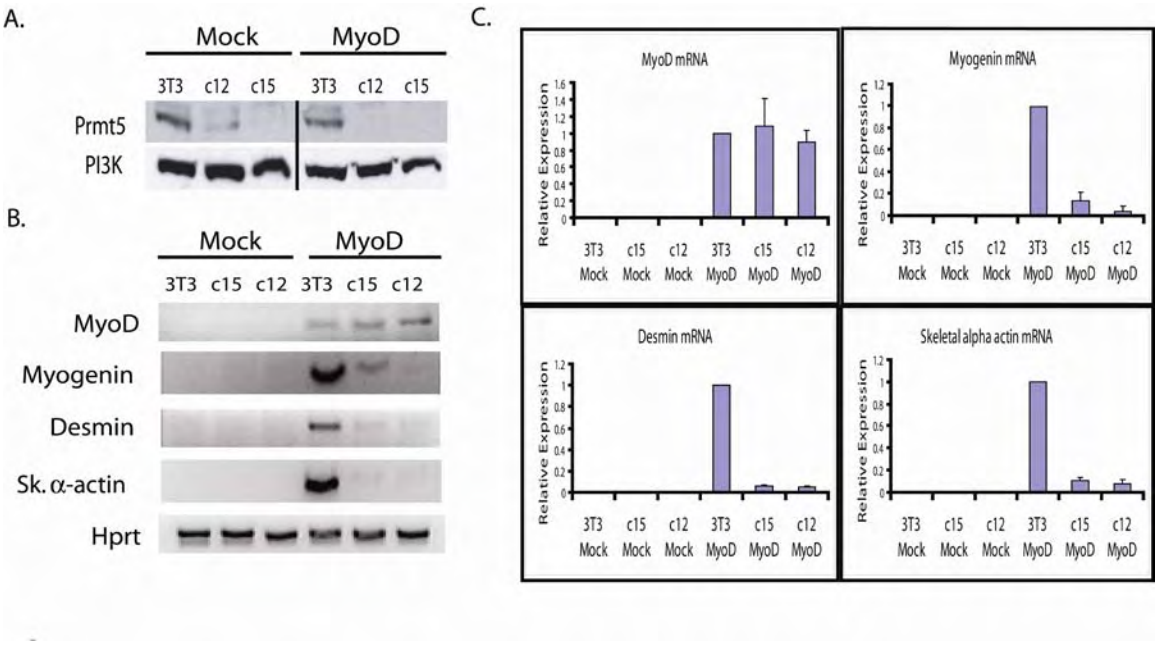
A.
Mock
MyoD
3T3 c12 c15 3T3 c12 c15
Prmt5
PI3K
B.
Mock
MyoD
3T3 c15 c12 3T3 c15 c12
MyoD
Myogenin
Desmin
Sk. α-actin
Hprt
C.
MyoD mRNA
Myogenin mRNA
Desmin mRNA
Skeletal alpha actin mRNA
Relative Expression
3T3 Mock
c15 Mock
c12 Mock
3T3 MyoD
c15 MyoD
c12 MyoD

Figure 2-1

Figure 2-1: Prmt5 is required for skeletal muscle differentiation. NIH3T3 cells and two independently derived NIH3T3 lines expressing an antisense vector against Prmt5 (c15, c12) 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 NIH3T3 cells. Cells were differentiated as described in the methods section for 36 hours prior to sample collection. (B) Expression of myogenic target genes myogenin, desmin, skeletal α-actin and ectopic expression of MyoD were monitored by RT-PCR in mock- and MyoD-differentiation cells collected 36 hours post-differentiation. (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.

In order to initiate the process of skeletal muscle differentiation, MyoD promotes cell cycle arrest, which involves the induction of cdk inhibitors such as p21 and cell cycle regulators like Rb (136, 248). As a consequence, the activity of cyclins and cyclin dependent kinases is down-regulated 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 fluorescence activated cell sorting (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 12-14% (Table 1), which is consistent with the level of cell cycle withdrawal that can be achieved with immortalized fibroblasts under these conditions (56, 190). These data indicate that the control and both Prmt5 antisense lines withdrew from the cell cycle upon differentiation. RT-PCR analysis indicated that p21 and Rb 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. Further, the data indicate that Prmt5 is not needed for cell cycle arrest under the conditions utilized for these experiments.

Table 1.

	Percentage of Cells in S-Phase		
	cycling	Mock	MyoD
3T3	29.7 +/- 1.6	13.0 +/- 3.0	13.6 +/- 2.1
c15	27.4*	10.8 +/- 4.5	11.8 +/- 2.2
c12	28.3*	12.4 +/- 4.2	11.8 +/- 0.4

* average of two experiments, n=3 experiments

The requirement for Prmt5 during MyoD induced differentiation suggests that Prmt5 may physically associate with MyoD and other regulators of differentiation. Co-immunoprecipitation experiments revealed that endogenous Prmt5 was associated with MyoD in differentiating cells (Fig. 2). As expected, no interactions were observed in mock-differentiated 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 are associated with Prmt5 in tumor derived cell lines (164, 165).

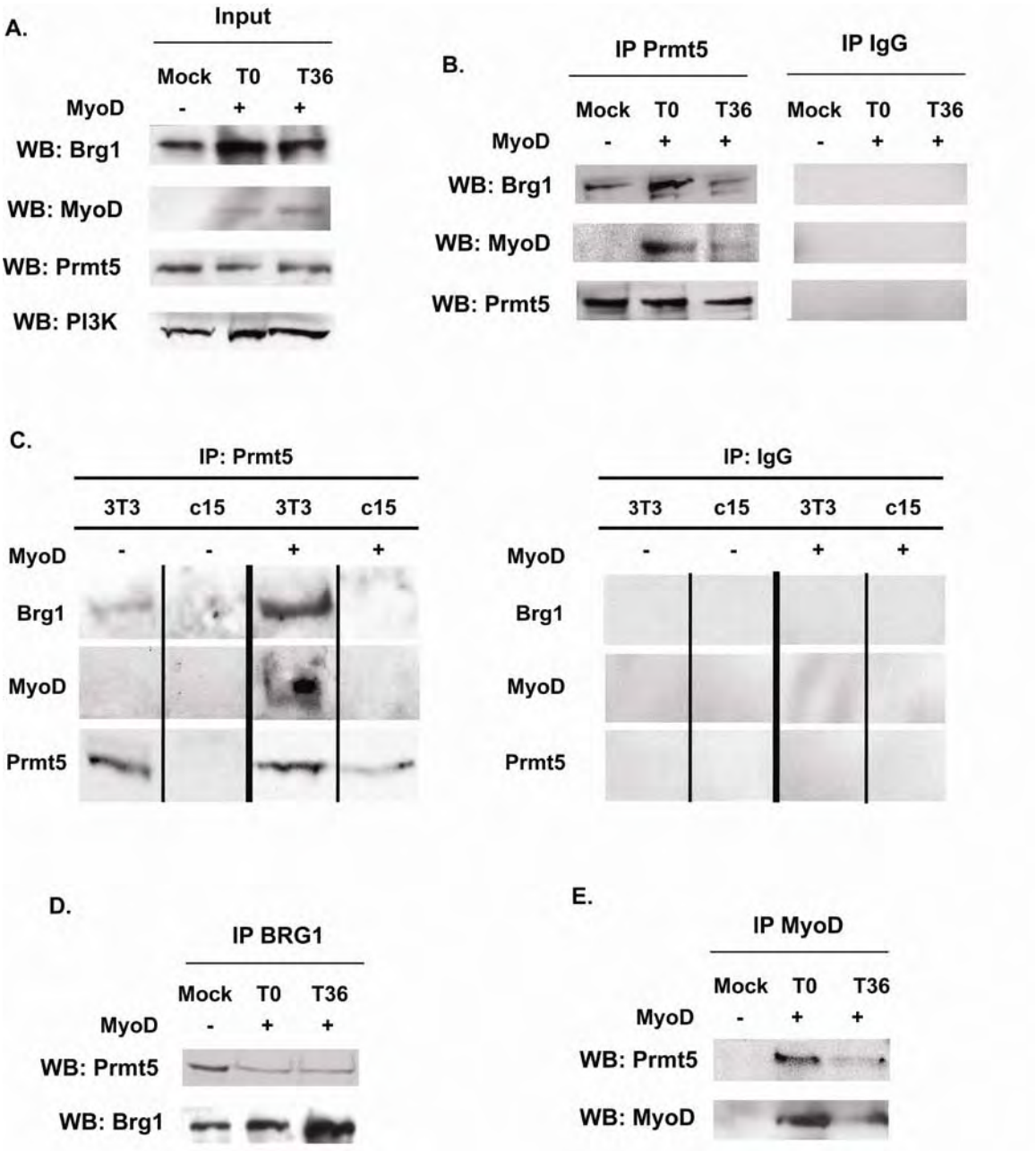
A.
Input
Mock T0 T36
MyoD - + +
WB: Brg1
WB: MyoD
WB: Prmt5
WB: PI3K
B.
IP Prmt5
IP IgG
Mock T0 T36
Mock T0 T36
MyoD - + +
WB: Brg1
WB: MyoD
WB: Prmt5
C.
IP: Prmt5
IP: IgG
3T3 c15 3T3 c15
3T3 c15 3T3 c15
MyoD - - + +
Brg1
MyoD
Prmt5
D.
IP BRG1
Mock T0 T36
MyoD - + +
WB: Prmt5
WB: Brg1
E.
IP MyoD
Mock T0 T36
MyoD - + +
WB: Prmt5
WB: MyoD

Figure 2-2.

Figure 2-2: Prmt5 co-immunoprecipitates with Brg1 and MyoD. Mock differentiated or cells differentiated for 0h, at which point the MyoD infected cells were exposed to differentiation conditions, or for 36h were harvested and used to perform co-immunoprecipitation experiments with the indicated antibodies.

(A) In 10% of input samples, indicated proteins were detected by western blot. (B) Whole cell extracts from mock and differentiated samples were co-immunoprecipitated with Prmt5 antibody (#611538 – BD Biosciences) or purified IgG. Immunoprecipitated material was run on SDS-PAGE, transferred to a membrane and probed for the presence of Brg1, MyoD and Prmt5. (C) Co-immunoprecipitations were performed using Brg1 antisera and probed for Prmt5 and Brg1. (D) Co-immunoprecipitations were performed using MyoD antisera and probed for the presence of Prmt5 and MyoD.

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 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. Chromatin immunoprecipitation (ChIP) for Prmt5 was performed in mock and MyoD-differentiated NIH3T3 and Prmt5 antisense lines (Figs. 3A-B). In the NIH3T3 cells, binding of Prmt5 to the myogenin promoter required MyoD-induced differentiation. Arginine 8 of histone H3 (H3R8) is a known substrate for Prmt5 (164, 165). Dimethylation of H3R8 at the myogenin promoter was enriched upon differentiation. As 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 media was first added ($t = 0$) and the interaction of these proteins was observed throughout the time course (Fig. 3C). These interactions precede the activation of myogenin expression (54), 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 hours post-differentiation (Fig. 3C). The significance of this observation, if any, is not known.

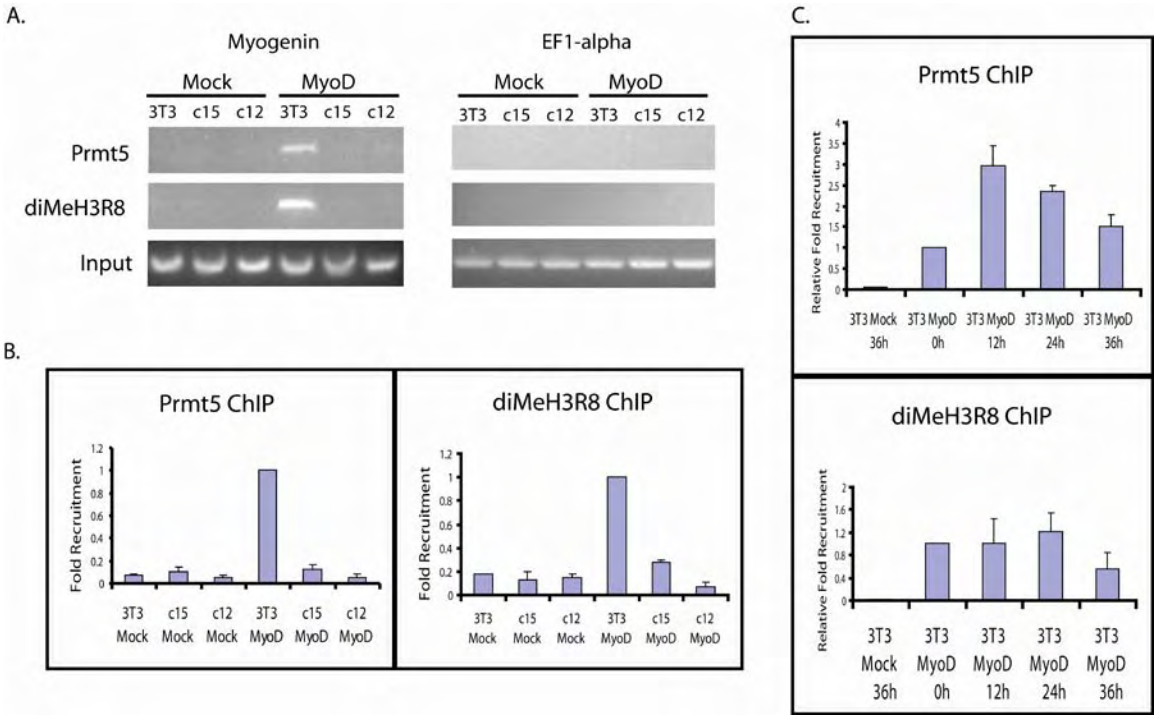
A.
Myogenin
EF1-alpha
Mock
MyoD
3T3 c15 c12 3T3 c15 c12
Prmt5
diMeH3R8
Input
B.
Prmt5 ChIP
diMeH3R8 ChIP
Fold Recruitment
3T3 Mock
c15 Mock
c12 Mock
3T3 MyoD
c15 MyoD
c12 MyoD
C.
Prmt5 ChIP
Relative Fold Recruitment
3T3 Mock 36h
3T3 MyoD 0h
3T3 MyoD 12h
3T3 MyoD 24h
3T3 MyoD 36h
diMeH3R8 ChIP

Figure 2-3.

Figure 2-3: Prmt5 binds to the myogenin promoter and dimethylates H3R8. Chromatin immunoprecipitations (ChIPs) were performed using antibodies against Prmt5 and dimethylated H3R8 in mock and MyoD differentiated NIH3T3 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 Q-PCR. (C) Time course of Prmt5 and dimethylated H3R8 association with the myogenin promoter. Q-PCR analysis of binding in differentiated cells at the indicated times is shown. Values are expressed relative to the values obtained at time 0, which was set at 1.

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 SWI/SNF chromatin remodeling enzymes (53). 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 (Figs. 4A-B). Control westerns 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. A restriction enzyme accessibility assay (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 NIH3T3 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.

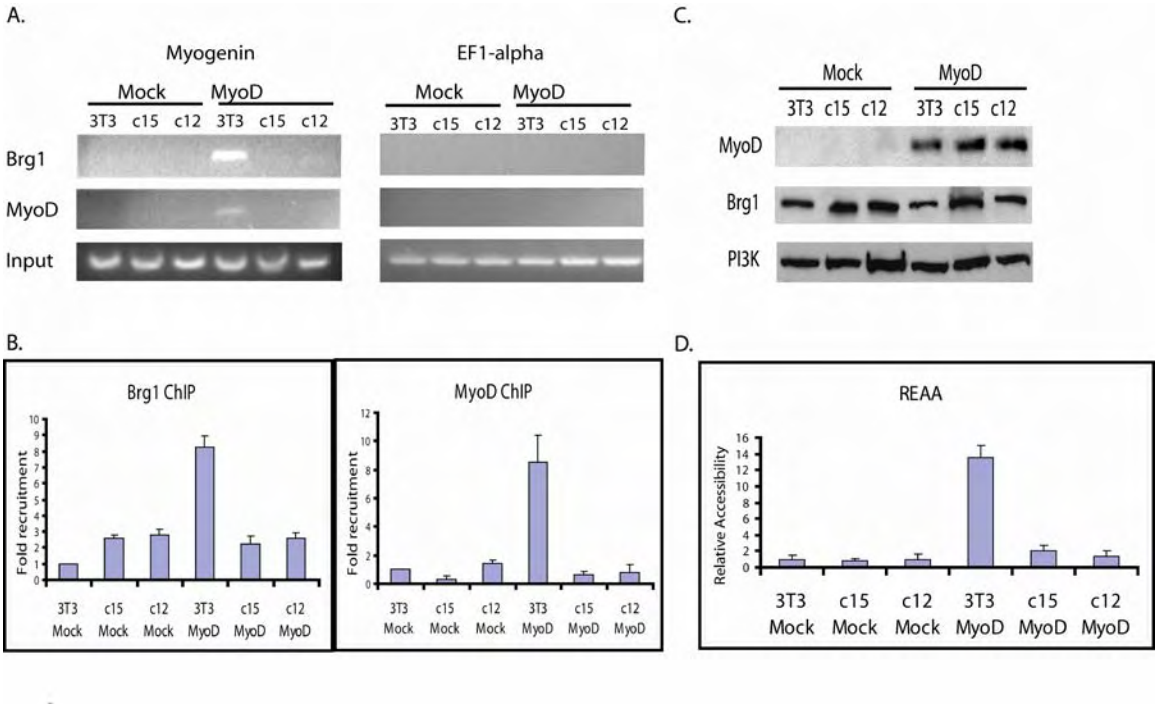
A.
Myogenin
EF1-alpha
Mock
MyoD
3T3 c15 c12 3T3 c15 c12
Brg1
MyoD
Input
C.
Mock
MyoD
3T3 c15 c12 3T3 c15 c12
MyoD
Brg1
PI3K
B.
Brg1 ChIP
MyoD ChIP
Fold recruitment
3T3 Mock
c15 Mock
c12 Mock
3T3 MyoD
c15 MyoD
c12 MyoD
D.
REAA
Relative Accessibility

Figure 2-4.

Figure 2-4: 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 post-differentiation, and the myogenin promoter and EF1α coding region (input control) were PCR amplified. Data from Figs 3A and 4A were from the same experiment, thus the input control bands are the same for both panels. (B) Brg1 and MyoD binding were quantified by Q-PCR. (C) Western blot showing that Brg1 and MyoD protein levels were unaffected by the reduction in Prmt5 protein levels. (D) A restriction enzyme accessibility assay (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.

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 (19, 113). Further studies showed that 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 (54). 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 (Figs. 4A-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 (52, 53) 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 (53, 54, 157). Upon differentiation with MyoD, Prmt5 was able to bind to the myogenin promoter in cells expressing functional Brg1 (+ Tet) as well as in cells expressing the dominant negative version of Brg1 (- Tet; Fig. 5A).

Dimethylation of H3R8 was also enriched at the promoter, regardless of the functional status of Brg1 (Fig. 5A). Control westerns 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; (54)). 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.

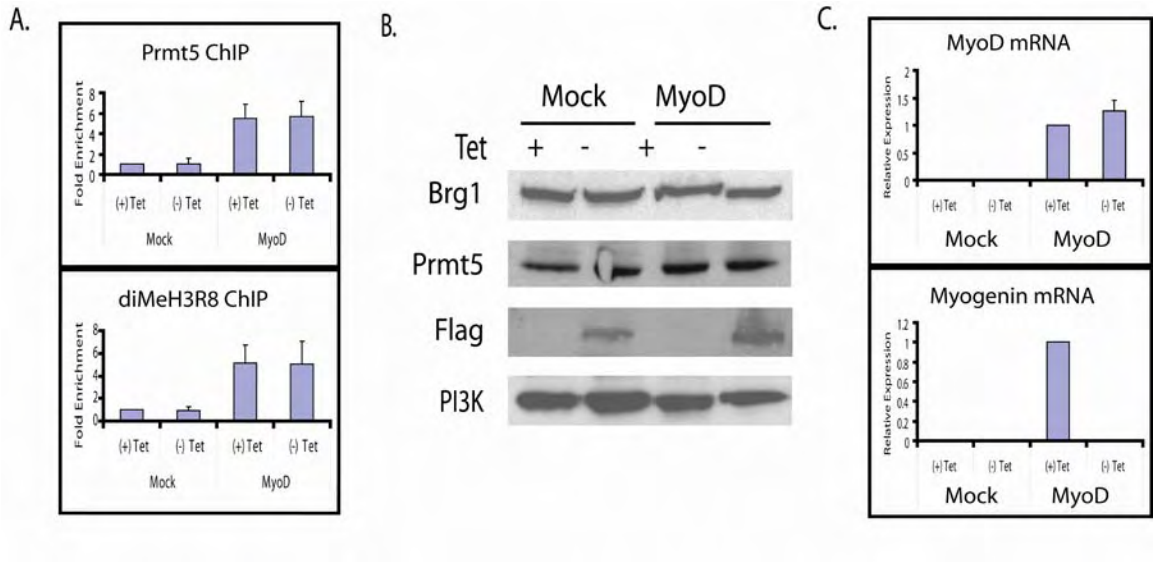
A.
Prmt5 ChIP
Fold Enrichment
(+)Tet
(-) Tet
(+)Tet
(-) Tet
Mock
MyoD
diMeH3R8 ChIP
Fold Enrichment
(+)Tet
(-) Tet
(+)Tet
(-) Tet
Mock
MyoD
B.
Mock
MyoD
Tet
+
-
+
-
Brg1
Prmt5
Flag
PI3K
C.
MyoD mRNA
Relative Expression
(+)Tet
(-) Tet
(+)Tet
(-) Tet
Mock
MyoD
Myogenin mRNA
Relative Expression
(+)Tet
(-) Tet
(+)Tet
(-) Tet
Mock
MyoD

Figure. 2-5.

Figure 2-5: Prmt5 is able to bind to and dimethylate H3R8 at the myogenin promoter in the absence of functional Brg1. ChIPs were performed 36 h post-differentiation in a cell line containing a Tet-suppressible vector expressing a FLAG-tagged, ATPase deficient, dominant negative Brg1. (A) Q-PCR 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 blot. Expression of FLAG in (-) Tet samples indicates expression of dominant negative Brg1. (C) Q-PCR 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.

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 4-5 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 (138, 198). 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 (57, 97)). 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 that is expressed in satellite cells (41). Pax3 is highly expressed in satellite cells compared to 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 co-localized 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 preformed 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 co-recruitment 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.

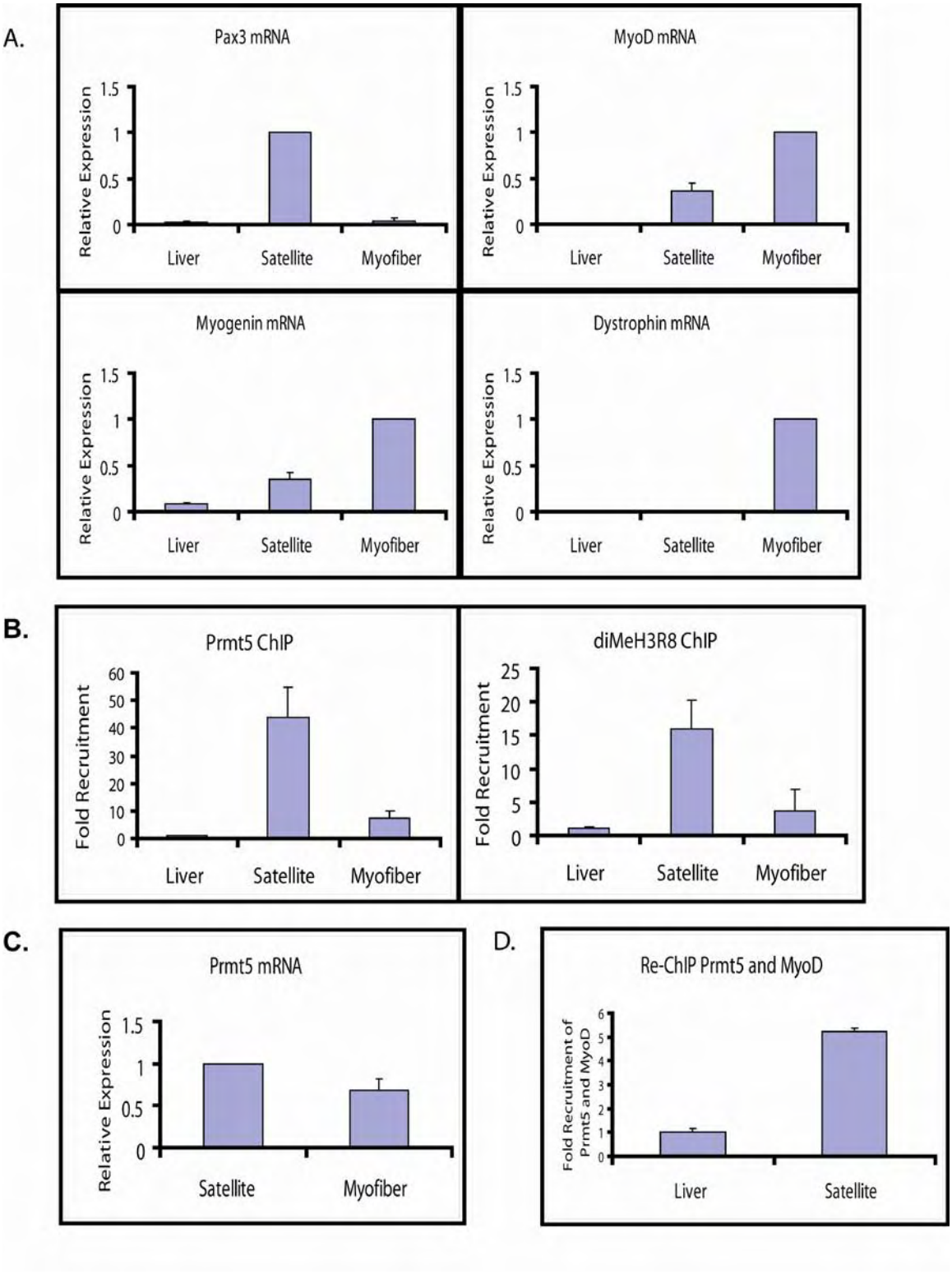
A.
Pax3 mRNA
MyoD mRNA
Myogenin mRNA
Dystrophin mRNA
Relative Expression
1.5
1
0.5
0
Liver
Satellite
Myofiber
B.
Prmt5 ChIP
diMeH3R8 ChIP
Fold Recruitment
60
50
40
30
20
10
0
25
20
15
10
5
0
C.
Prmt5 mRNA
Satellite
Myofiber
D.
Re-ChIP Prmt5 and MyoD
Fold Recruitment of Prmt5 and MyoD
6
5
4
3
2
1
0

Figure 2-6.

Figure 2-6: 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 Q-PCR 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 Q-PCR. (D) Re-ChIP analysis quantified by Q-PCR. Material immunoprecipitated with Prmt5 antibodies were subsequently immunoprecipitated with MyoD antibodies. Q-PCR data are the average +/- standard deviation of 3 independent experiments.

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 (92, 111, 152) . 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 (164). Manipulated cells expressing low levels of Prmt5 were unable to activate the expression of myogenin or other myogenic 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 Prmt5 promotes reporter gene activation; all other studies suggest that Prmt5 functions in transcriptional repression. In one study, dose dependent reduction of Prmt5 by RNAi methods resulted in

corresponding decreases in NFAT and IL-2 driven promoter activity and in IL-2 secretion, suggesting that Prmt5 promotes IL-2 expression (186). In a different report, Prmt5 was purified in a multi-protein 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 (98). 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 pol II and impedes transcription elongation (117). 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 (69, 164, 165). Microarray and confirmatory RT-PCR experiments identified additional Prmt5 target genes also involved in cell growth control (164). Dimethylation of H3R8 at promoter sequences was associated with repression of the genes examined, and purification of Prmt5 as part of a multi-enzyme complex with HDAC2 and the SWI/SNF ATPase Brg1 (165) suggests mechanisms by which nucleosomes on gene regulatory sequences can be altered and/or repositioned and histones can be dimethylated and deacetylated in a manner that leads to transcriptional repression.

Prmt5 also functions in complexes with RNA polymerase II and the pol II-associated phosphatase FCP1, and with several cytosolic proteins that together with

Prmt5 promote snRNP assembly (4, 10, 145). 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.

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, 90, 91, 125, 234), 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 (19, 54, 207). 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 facilitate Brg1 binding. Histone acetylation also precedes Brg1 interaction at the myogenin promoter (54), 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, 90, 91). 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 (35). 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 (237), 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 (237), 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.

Materials and Methods

Cell Culture

Cells were maintained in DMEM supplemented with 10% calf serum and 2mM L-glutamine. Prmt5 antisense lines were maintained in the presence of 2.5ug/mL puromycin, since the antisense vector encodes a puromycin resistance gene (164). Cells were differentiated as described (53, 56) 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 hours later. At that time, retroviral infection with the MyoD encoding retrovirus was performed for 30 hours. Subsequently, differentiation media (DMEM + 2% horse serum, 2mM L-glutamine, 5ug/mL blasticidin, and 2ug/mL puromycin, and 10ug/mL insulin) was added to the cells and samples were collected at the indicated times. Control samples from both 3T3s 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 (52). Cycling cells were grown in the presence or absence of tetracycline for 3 days, were split to be 50-60% confluent 24 hours later, 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 (54). The cDNA was

amplified using the Qiagen HotStarTaq Master Mix kit (Qiagen) containing 0.1ug of specific primers and SYBR green. RT-PCR and real time PCR were performed using procedures and primers previously described (222) (41, 157). 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 +/- standard deviation of 3 or more independent experiments.

FACS

Cells were differentiated or mock-differentiated as stated above and fixed as previously described (116). 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 described (56, 60). For Western analysis, 100 ug of each extract were used for SDS-PAGE followed by transfer to nitrocellulose. Co-IP procedures were previously described (54, 157). Antibodies used for ChIP included polyclonal rabbit antisera against Prmt5 and dimethylated H3R8 (164, 165), Brg1 (52), FLAG epitope, and MyoD (54). Western analysis also made use of commercial antibodies against Prmt5/JBP1/Skb1Hs (#611538 - BD Biosciences) and MyoD (#554130 and #554099 - BD Biosciences).

Chromatin immunoprecipitation (ChIP)

ChIPs were performed by modifying the Upstate protocol as described (194). Cultured cells or nuclei isolated from tissues (see below) were cross-linked in 1%

formaldehyde and lysed in buffer containing 1% SDS, 10mM EDTA, 50mM 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. 100ug of sonicated DNA was diluted 10-fold in IP 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 PMSF, 1 ug/ml aprotinin, 1 ug/ml pepstatin A) and precleared with a 50% slurry of protein A beads (Amersham) at 4°C for at least 1 hour. Cleared lysates were incubated with antibodies described above at 4°C for 4 hours or overnight. Protein A beads were added to precipitate immune complexes from the cell lysates and incubated for 1 hour at 4°C. Beads were collected by centrifugation then washed as described (194), and immune complexes were eluted from the beads using 1% SDS. Crosslinks were reversed and DNA was purified by Qiaquick PCR purification kit (Qiagen). Analysis of immunoprecipitated DNA was performed by PCR amplification (54) or by Q-PCR using a Quantitect SYBR Green PCR kit. Primers were described (54, 157). Re-ChIP experiments were performed as described (146, 157). Q-PCR data are the average +/- standard deviation of 3 independent experiments.

Restriction Enzyme Accessibility Assay (REAA)

REAA and detection by a modified version of ligation-mediated PCR were described previously (54, 157). Data are the average +/- standard deviation of 3 independent experiments.

Isolation of satellite cells and myofibers from mouse skeletal muscle

Skeletal muscle was dissected from the upper hindlimbs of 4-5 week old BL6 mice and minced to approximately $1mm^3$. Tissues were then digested using 87.5 U/ml

collagenase type II (Invitrogen) in PBS supplemented with 1mM $CaCl_2$ and incubated at 37°C for 1 hour with agitation (7, 43). Satellite cells were separated from mature myofibers by filtration with a cell strainer 70um (Becton Dickinson # 352350) (159). Flow through 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 (10mM HEPES-KOH (pH7.3), 10mM KCl, 5mM $MgCl_2$, 0.5mM DTT, 0.2mM PMSF, 3ug/mL cytochalasin B, 10ug/mL leupeptin). Samples were homogenized and dounced using pestle A to release nuclei, then samples were incubated on ice for 30 minutes. Nuclei release and integrity was 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 (5mM MgCl2, 10mM triethanolamine pH 7.5**,** 5mM $MgCl_2$**,** 10% Sucrose**,** 10ug/mL leupeptin) and subsequently 2 volumes of 2.0M Sucrose/10mM Tris-HCl/5mM $MgCl_2$. In an ultracentrifuge tube (Beckman #344057), 750uL of the 1.8M Sucrose/10mM Tris HCl/5mM $MgCl_2$ was aliquoted and overlayed with the nuclei mixture described above. Samples were ultracentrifuged at 50,000 x *g* for 1 hour. The supernatant was aspirated and discarded, and the pellet was resuspended in 500uL of lysis buffer + 0.1% NP-40. Following crosslinking with 2% formaldehyde at room temperature, nuclei were centrifugated at 13,500 x *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.

CHAPTER III

Distinct protein arginine methyltransferases promote ATP-dependent chromatin remodeling function at different stages of skeletal muscle differentiation

INTRODUCTION

Skeletal muscle differentiation involves cooperation between myogenic basic helix-loop-helix transcription factors (MyoD, Myf5, myogenin, Mrf4), ubiquitous E proteins, Myocyte-enhancer factor 2 proteins (MEF2s), histone modifying enzymes and ATP-dependent chromatin remodeling enzymes. The involvement and requirement for individual chromatin modifying and remodeling enzymes during skeletal muscle differentiation has been intensely investigated in recent years. However, the interdependence of different enzymes affecting chromatin structure during myogenesis has not received as much attention. In addition, regulation of myogenic gene expression is further complicated by the temporal regulation that exists and separates myogenic genes into different classes based on when they are activated relative to the onset of differentiation. Whether chromatin altering enzymes specifically and differentially contribute to aspects of temporal regulation is largely unexplored.

We and others have previously demonstrated that SWI/SNF chromatin remodeling enzymes containing the Brg1 ATPase are directly required for the induction of myogenesis because they remodel chromatin structure at the regulatory regions of both

early and late myogenic genes (53, 54, 157, 207). Numerous histone modifying enzymes have also been implicated in the regulation of myogenic genes, including acetyl transferases, deacetylases, lysine methyl transferases, and arginine methyl transferases (reviewed in (55, 195, 217)). Of particular interest to us are the arginine methyltransferases. Type I arginine methyltransferases generate asymmetric dimethyl arginines on substrate proteins, while type II arginine methyltransferases catalyze the formation of symmetric dimethyl arginines (reviewed in (14, 15, 233)). Both Prmt5, a type II arginine methyltransferase, and Carm1/Prmt4, a type I methyltransferase, have been shown to act as a co-regulator for numerous gene activation and repression events (reviewed in (15, 163, 233) and both have been independently purified in large protein complexes containing Brg1 (164, 165, 237). The connections between Prmt5 and Brg1 led us to investigate possible cooperativity between these different types of chromatin altering enzymes in cell differentiation systems shown to be Brg1 dependent.

Our previous work demonstrated that the class II arginine methyltransferase, Prmt5 was required for myogenesis (46). Prmt5 associated with the myogenin promoter and locally di-methylated H3R8. Knockdown of Prmt5 protein levels resulted in a reduction of di-methylation of H3R8 at the myogenin promoter and importantly, a nearly total loss of Brg1 binding, which prevented chromatin remodeling of the promoter. All subsequent transcription factor binding events and the initiation of myogenin expression were inhibited. Thus the arginine methyltransferase was required for the function of the ATP-dependent chromatin remodeling enzyme.

To further probe the relationships between different classes of chromatin altering enzymes and to explore potential differences between the regulation of myogenin, a myogenic early gene, and genes expressed later in the differentiation process, we investigated the requirement for Prmt5 in the expression of myogenic late genes and also examined the involvement of Carm1/Prmt4, which had previously been linked to myogenesis via regulation of myogenin expression (35). Our data demonstrate that both Prmt5 and Carm1/Prmt4 are associated with regulatory elements of representative late myogenic genes *in vivo* and in culture. We also found a concomitant enrichment in di-methylation of H3R8 and H3R17, substrates for Prmt5 and Carm1/Prmt4, respectively, at these loci. Despite the presence of Prmt5 at late gene promoters, it is dispensable for transcriptional activation of late myogenic genes. In contrast, Carm1/Prmt4 was absolutely required for activation of late myogenic targets. In the absence of Carm1/Prmt4, Brg1 association with these promoter elements was lost, as were changes in nuclease accessibility that are concurrent with gene activation. These data support the assertion that a preferential requirement exists for Carm1/Prmt4 during the later stages of myogenic differentiation while Prmt5 governs the activation of early myogenic targets. The results indicate a differential requirement for two distinct protein arginine methyltransferases during the different stages of myogenic differentiation to facilitate loading and function of an ATP-dependent chromatin remodeling enzyme. This relationship between the different classes of enzymes may represent a paradigm for cooperation between PRMTs and ATP-dependent remodeling enzymes.

RESULTS

Binding of Prmt5 and Carm1 to regulatory regions of late myogenic genes

Our previous studies showed that the protein arginine methyltransferase Prmt5 is required to facilitate the activation of myogenin, an essential gene that is induced during the early stages of skeletal muscle differentiation (46). In that study, we also showed that genes expressed later during differentiation were not activated when levels of Prmt5 were reduced. However, it was unclear if the lack of late gene expression was a direct consequence of Prmt5 reduction or was an indirect consequence resulting from the failure to induce myogenin, which is required for late gene activity and terminal differentiation (92, 152).

If specific arginine methyltransferases are functioning at myogenic late genes, the enzymes and histones modified by these enzymes should be localized at regulatory sequences controlling late gene expression. To address physiological relevance, we first performed ChIPs using primary skeletal muscle tissue. We isolated hind limb muscle from 4-6 week old BL6 mice and prepared mature myofibers for ChIP analysis, as we described previously (46, 158). Liver was isolated as a negative control. ChIP results were quantified by real-time PCR. The data demonstrated that Prmt5 was bound to the regulatory regions of the muscle creatine kinase (MCK) locus in mature myofibers while no significant association was observed in the control liver tissue (Fig. 1A). This induction of Prmt5 binding was accompanied by enrichment in the amount of di-methylated H3R8 at the MCK locus in myofibers, thereby correlating the presence of

both Prmt5 and a histone modification known to result from Prmt5 activity (46, 69, 163, 164).

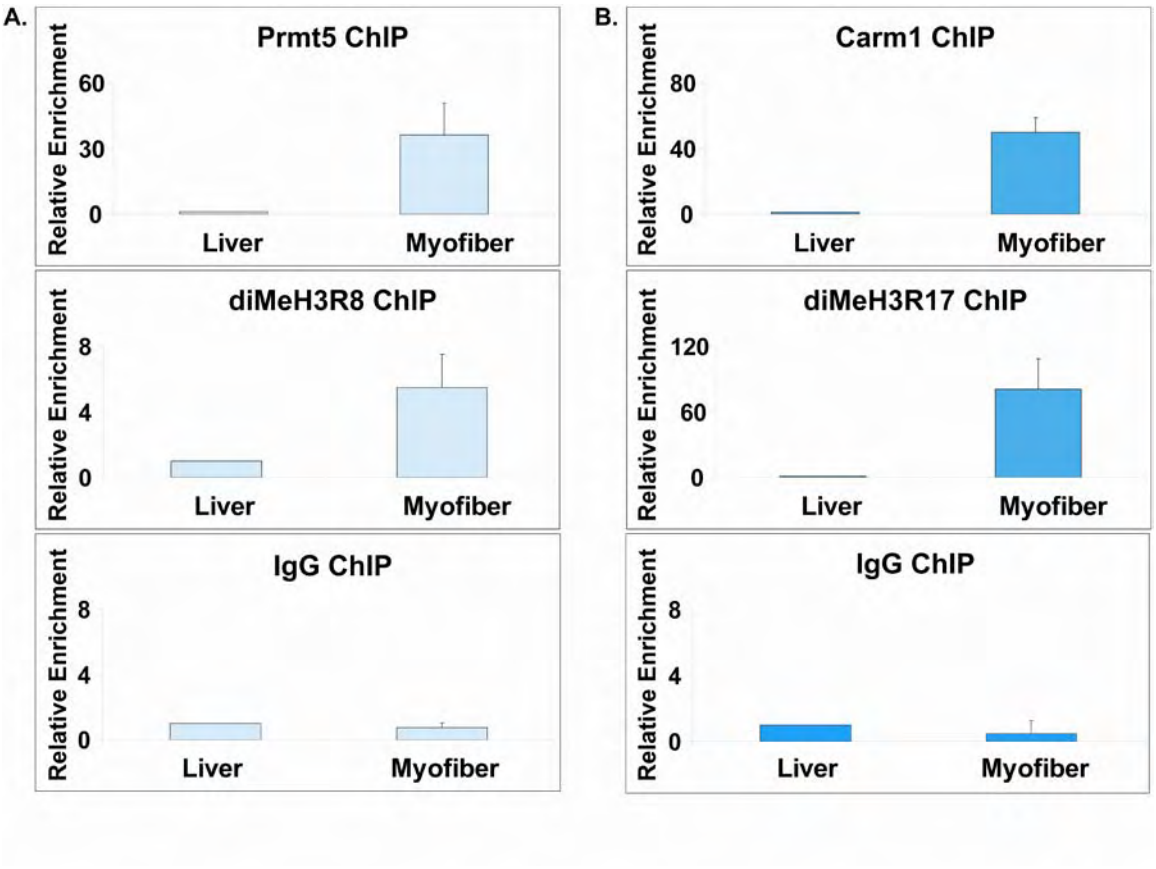
A.
Prmt5 ChIP
Relative Enrichment
60
30
0
Liver
Myofiber
diMeH3R8 ChIP
Relative Enrichment
8
4
0
Liver
Myofiber
IgG ChIP
Relative Enrichment
8
4
0
Liver
Myofiber
B.
Carm1 ChIP
Relative Enrichment
80
40
0
Liver
Myofiber
diMeH3R17 ChIP
Relative Enrichment
120
60
0
Liver
Myofiber
IgG ChIP
Relative Enrichment
8
4
0
Liver
Myofiber

Figure 3-1.

Figure 3-1. Prmt5 and Carm1 both bind to the MCK regulatory sequences *in vivo*. Skeletal muscle was dissected from the hindlimb of 4-6 week old BL6 mice. Nuclei were isolated from muscle tissue and liver. ChIPs were performed using antibodies recognizing Prmt5, dimethylated (diMe) H3R8, Carm1, and dimethylated (diMe) H3R17. Specific binding of Prmt5 and Carm1/Prmt4 was seen at gene regulatory regions of muscle creatine kinase (MCK) in myofibers but not in liver. Corresponding increases in dimethylated H3R8 and H3R17 were also observed specifically in myofibers. Values for binding in liver samples were set at 1. ChIPs with purified IgG generated background signals equivalent to the signals obtained with specific antibodies in liver samples. Data represent the average +/- standard deviation of 4 independent experiments.

Previously, the Carm1/Prmt4 enzyme was shown to localize to the MCK locus in differentiating C2C12 myoblasts, though the functional significance of this event was not addressed (35). Similarly, a considerable increase in the amount of Carm1/Prmt4 binding was seen at the MCK locus in myofibers, which correlated with an induction of dimethylated H3R17, a known epigenetic mark resulting from Carm1/Prmt4 activity (199). These results are the first demonstration that distinct protein arginine methyltransferases and histone modifications caused by these enzymes are present at regulatory regions of a late myogenic gene in terminally differentiated, primary muscle tissue.

We next turned to a well-established, manipulatable cell culture system for myogenic differentiation (50) to determine whether the association of arginine methyltransferases and specifically modified histones at late myogenic gene promoters was recapitulated. Confluent, MyoD-expressing fibroblast cells were exposed to a low serum differentiation cocktail for varying times, and samples were evaluated by ChIP. Both Prmt5 and di-methylated H3R8 were already bound to regulatory regions of both the MCK gene and the desmin gene, which is expressed as a late gene in this and similar tissue culture systems (18, 157) at time 0, which is when the differentiation media was added to the cultures (Fig. 2A). The presence of Prmt5 and dimethylated H3R8 were entirely dependent upon MyoD expression, since these proteins were absent from the loci in mock-differentiated cells (Fig. 2A). In contrast, Carm1/Prmt4 and dimethylated H3R17 did not show significant binding at late gene loci until 8 hours post-differentiation (Fig. 2B), which in this culture system corresponds to the time when desmin and MCK

gene expression is induced (157). As observed with Prmt5 and dimethylated H3R8, binding of Carm1 and dimethylated H3R17 did not occur in mock-differentiated cells and required MyoD expression. Once bound, both arginine methyltransferases and modified histones remained present throughout the differentiation time course (Figs. 2A-B). Thus Prmt5 and dimethylated H3R8 binding preceded early and late gene expression while Carm1/Prmt4 and dimethylated H3R17 binding correlated with the onset of late gene expression.

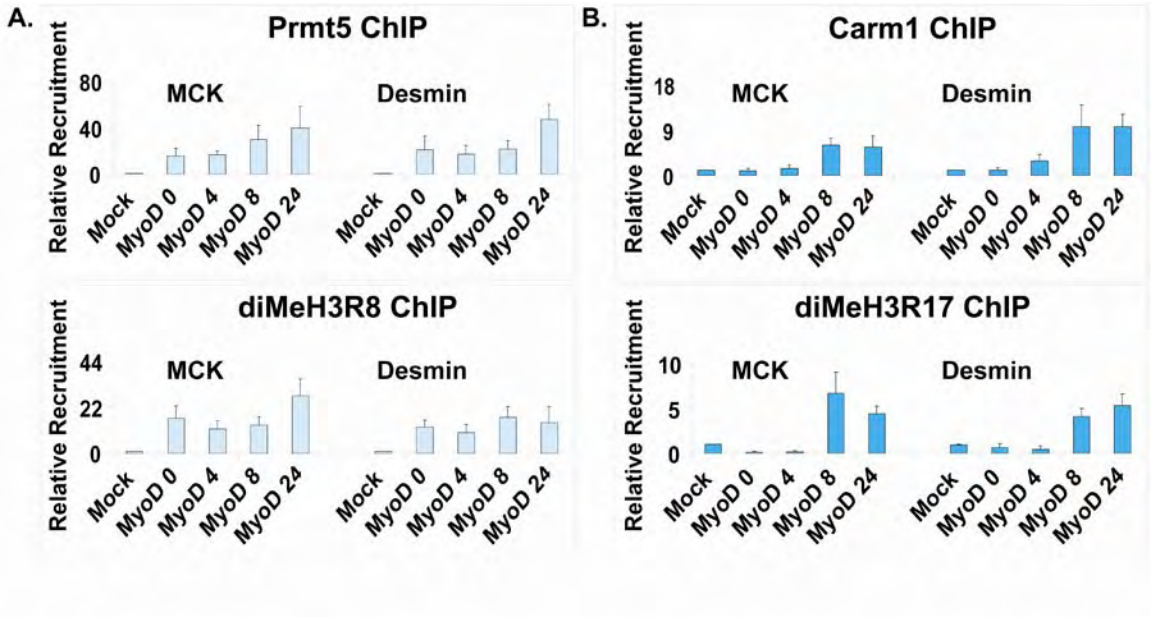
A.
Prmt5 ChIP
Relative Recruitment
80
40
0
MCK
Desmin
Mock
MyoD 0
MyoD 4
MyoD 8
MyoD 24
diMeH3R8 ChIP
44
22
B.
Carm1 ChIP
18
9
diMeH3R17 ChIP
10
5

Figure 3-2.

Figure 3-2. Prmt5 and Carm1 bind to and methylate histones at the regulatory regions of late myogenic target genes in cell culture. A time course of MyoD-differentiated NIH 3T3 cells were harvested for ChIP analysis, using antibodies recognizing Prmt5, dimethylated (diMe) H3R8, Carm1, and dimethylated (diMe) H3R17 to analyze the temporal binding of these PRMTs and the deposition of their corresponding histone modifications at late skeletal muscle target genes. (A) Kinetics of binding of Prmt5 and diMeH3R8 at the regulatory sequences controlling MCK and desmin in mock and MyoD –differentiated cells. (B) Kinetics of binding of Carm1 and diMeH3R17 at the regulatory sequences controlling MCK and desmin in mock and MyoD –differentiated cells. All values are expressed relative to the values obtained for binding in mock-differentiated NIH3T3 cells, which were set at 1. Data represent the average +/- standard deviation of 4 independent experiments.

Prmt5 associates with promoter elements of late myogenic targets but is not required for gene activation

To address the requirement for Prmt5 in the induction of myogenic late genes, we utilized cell lines (c12 and c15) that stably express an antisense construct to Prmt5 (164). Fig. 3A demonstrates that Prmt5 levels are significantly reduced under both mock and MyoD-induced differentiation conditions, in agreement with previous results (46). We previously demonstrated that ectopic expression of myogenin and the muscle specific isoform of Mef2d (Mef2D1b) (137) in combination was sufficient to activate myogenic late genes and to drive myogenesis to completion in culture without inducing endogenous MyoD expression (157). Thus, we can utilize ectopic expression of myogenin and Mef2D1b to address whether Prmt5 and Carm1/Prmt4 are directly required for late gene expression because this method (a) bypasses the requirement for MyoD to synthesize myogenin and (b) provides the muscle specific Mef2D1b isoform to cooperate with myogenin in the activation of late gene loci. We would expect that if either arginine methyltransferase were indirectly required for late gene expression because of a requirement to synthesize early genes, lack of the arginine methyltransferase should not impact late gene expression when myogenin and Mef2D1b were ectopically expressed. In contrast, if there were a direct requirement for either arginine methyltransferase during late gene induction, simply providing myogenin and Mef2D1b should not be sufficient to induce late gene expression. Before experimentally addressing these issues, we confirmed that the cell lines were still deficient for Prmt5 when differentiation was induced by myogenin and Mef2D1b (Fig. 3A).

We first examined whether Prmt5 and dimethylated H3R8 were present on late gene regulatory sequences in wildtype and Prmt5 antisense cell lines differentiated under the different conditions described. Induced binding of Prmt5 at the MCK and desmin promoters was observed in MyoD-differentiated NIH3T3 cells (Fig. 3B). As expected, Prmt5 antisense-expressing cell lines showed reduced levels of Prmt5 binding (Fig. 3B). Similarly, dimethylation of histone H3R8 was observed at the MCK and desmin promoters in the wildtype, MyoD differentiated cells and was significantly reduced in the MyoD differentiated, Prmt5 antisense-expressing lines (Fig. 3C). Prmt5 and dimethylated H3R8 association with late myogenic gene sequences was specific to MyoD differentiated cells as no significant binding was seen in mock-differentiated samples or, interestingly, in myogenin/Mef2D1b differentiated cells (Fig. 3B-C). These results support the assertion that Prmt5 is able to bind late gene promoter regions only in the presence of MyoD, which binds to these loci prior to gene activation (157) and is consistent with previous studies showing that Prmt5 and MyoD could be co-immunoprecipitated from MyoD differentiated cell extracts (46).

Analysis of gene expression in these cells was quantified by real-time PCR. Controls are presented in the first column of Fig. 3D. MyoD was equivalently expressed in each of the cell lines where it was introduced; as previously reported, no MyoD was detected in mock or myogenin/Mef2D1b differentiated samples (55, 157). Myogenin was equivalently expressed in lines that were myogenin/Mef2D1b differentiated. We note that the levels of introduced myogenin were within 2-fold of the level normally induced by MyoD, thus introduced myogenin was not grossly overexpressed. As

previously reported (46), in MyoD-differentiated cells, myogenin expression was compromised in both of the Prmt5 antisense lines. Equivalent levels of Mef2D1b were present in the myogenin/Mef2D1b differentiated cells (Fig. 3D).

We then examined late gene expression. In wildtype MyoD differentiated cells, induction of MCK, desmin, and dystrophin, another late gene, was observed, while MyoD differentiated Prmt5 antisense-expressing cells failed to activate these late myogenic targets (Fig. 3D, right). In cells differentiated with myogenin/Mef2D1b, all of the cell lines were able to induce each of the late genes equivalently (Fig. 3D, right). These results, coupled with the ChIP data in Fig. 3B-C, indicate that Prmt5 and dimethylation of H3R8 are not directly required for the activation of myogenic late genes. Instead, Prmt5 is required for the activation of myogenin (46) and Fig. 3D) and thus is indirectly required for late gene expression.

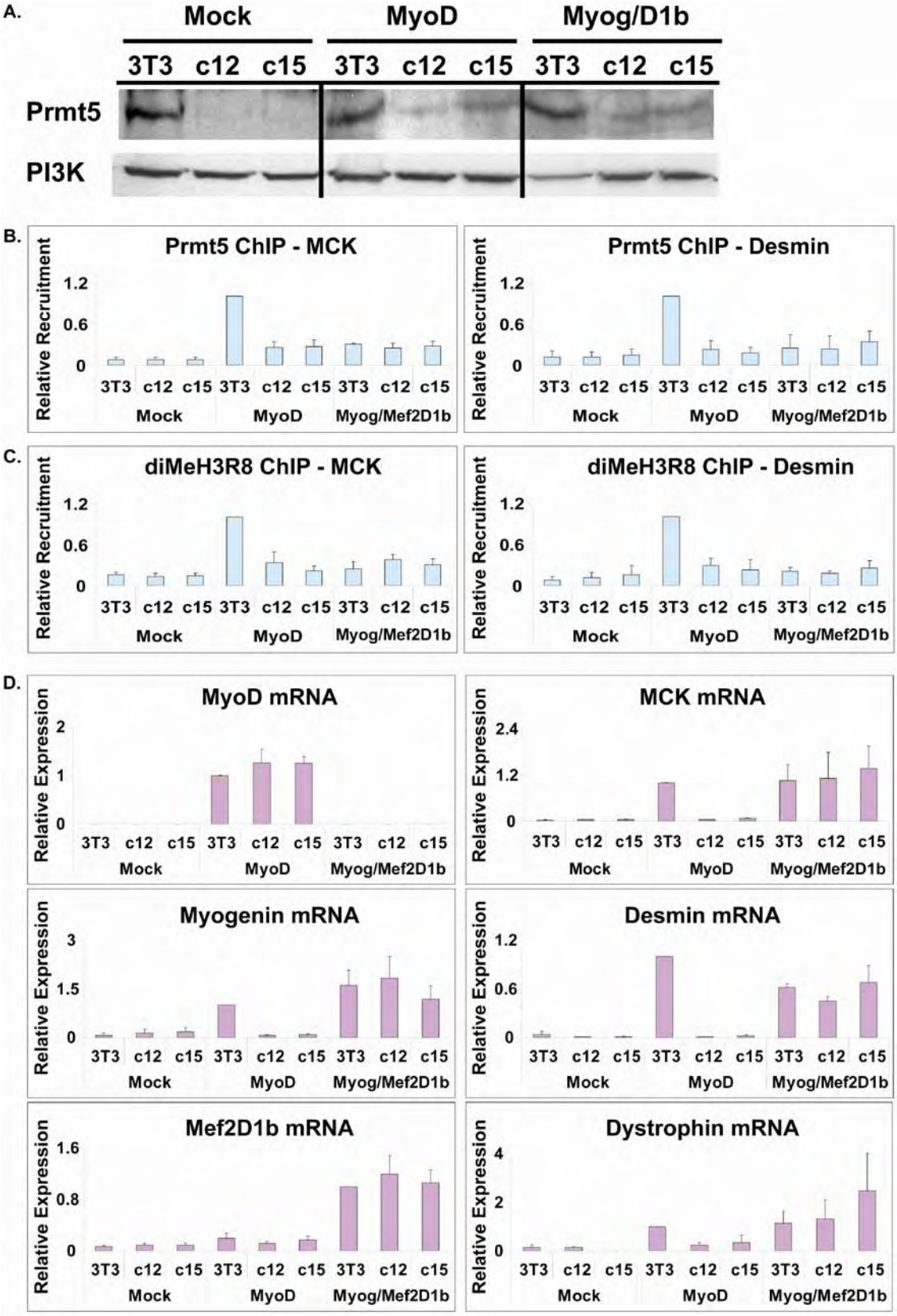
A.
Mock
MyoD
Myog/D1b
3T3 c12 c15 3T3 c12 c15 3T3 c12 c15
Prmt5
PI3K
B.
Prmt5 ChIP - MCK
Prmt5 ChIP - Desmin
Relative Recruitment
C.
diMeH3R8 ChIP - MCK
diMeH3R8 ChIP - Desmin
Relative Recruitment
D.
MyoD mRNA
MCK mRNA
Myogenin mRNA
Desmin mRNA
Mef2D1b mRNA
Dystrophin mRNA
Relative Expression
Mock
MyoD
Myog/Mef2D1b

Figure 3-3. Prmt5 binds to late myogenic target genes but is not required for gene activation. (A) Western blot demonstrating the extent of Prmt5 expression in wildtype (3T3) and Prmt5 antisense (c12, c15) cell lines that were mock differentiated or differentiated with MyoD or with myogenin + Mef2D1b (Myog/Mef2D1b) for 24 hours. Phosphoinositide 3-kinase (PI3K) levels are shown as a control. (B, C) ChIP experiments were performed using antibodies recognizing Prmt5 and diMeH3R8 and were analyzed by quantitative PCR (Q-PCR). Values are expressed relative to the values obtained for binding in MyoD differentiated NIH3T3 cells, which were set at 1. (D) mRNA expression analysis for the indicated genes was performed by reverse transcriptase PCR (RT-PCR) and quantified by Q-PCR. Quantification of transcripts was normalized to total amount of EF1-α mRNA. Values are expressed relative to the expression values obtained in MyoD differentiated NIH3T3 cells, which were set at 1, except for the evaluation for Mef2D1b expression, where the value obtained for myogenin/Mef2D1b differentiated cells was set at 1. Data in panels B-D are the average +/- standard deviation of 4 independent experiments.

Carm1/Prmt4 associates with gene regulatory sequences of late myogenic targets and is required for transcriptional activation at these loci

Since Prmt5 is not directly required for the activation of late myogenic targets, this prompted us to examine if Carm1/Prmt4, the other arginine methyltransferase physically present on myogenic late gene regulatory sequences, could be co-activating late myogenic genes. To address the requirement for Carm1/Prmt4 in late gene expression, we utilized immortalized MEFs derived from wildtype or Carm1/Prmt4 deficient mice (240). As expected, the cells were deficient for Carm1/Prmt4 under mock, MyoD-induced, and myogenin/Mef2D1b-induced differentiation conditions (Fig. 4A).

ChIP experiments were performed in mock, MyoD, or myogenin/Mef2D1b differentiated wild type (WT) and Carm1/Prmt4 knockout (KO) immortalized mouse embryo fibroblasts. Carm1/Prmt4 binding to the MCK and desmin promoters was observed in MyoD-differentiatated as well as in myogenin/Mef2D differentiated WT MEFs (Fig. 4B). A concomitant enrichment in the amount of dimethylated H3R17 was observed at both promoters (Fig. 4C). As expected, no detectable Carm1/Prmt4 binding or enrichment in di-methylated H3R17 was observed in the mock differentiated cells (Figs. 4B-C). In the KO MEFs, neither Carm1/Prmt4 nor dimethylated H3R17 was present, demonstrating that dimethylation of H3R17 at these loci is due to Carm1/Prmt4 (Figs. 4B-C). These findings demonstrate that Carm1/Prmt4 is able to directly bind late muscle promoter regions regardless of how gene activation is achieved; expression of either MyoD or myogenin/Mef2D1b is sufficient to promote binding and histone modification.

The binding of Carm1/Prmt4 to late gene regulatory sequences at 8 hours post-differentiation (Fig. 2) corresponds to the timing of binding of myogenin and Mef2 at these promoters, as well as the interaction of the Brg1 ATPase of SWI/SNF chromatin remodeling enzymes and concomitant increases in nuclease accessibility (157). The simultaneous occurrence of these events suggests that Carm1/Prmt4 serves as a coactivator of myogenin and Mef2D1b. We therefore performed re-ChIP analysis to determine whether myogenin and Carm1/Prmt4 were present together at late gene regulatory sequences. We immunoprecipitated Carm1/Prmt4 from cross-linked chromatin isolated from MyoD-expressing cells prior to or after differentiation was induced and subsequently immunoprecipitated the Carm1/Prmt4 associated chromatin with antibodies against either myogenin or MyoD. The results demonstrate that Carm1/Prmt4 and myogenin were co-localized on both MCK and desmin regulatory sequences in differentiated, but not undifferentiated cells (Fig. 4D). No co-localization of Carm1/Prmt4 with MyoD was observed (Fig. 4D), in agreement with previous observations that MyoD binding to late gene regulatory sequences diminishes at the onset of late gene expression (157). The data support the idea that Carm1/Prmt4 is simultaneously present with myogenin at myogenic late gene loci.

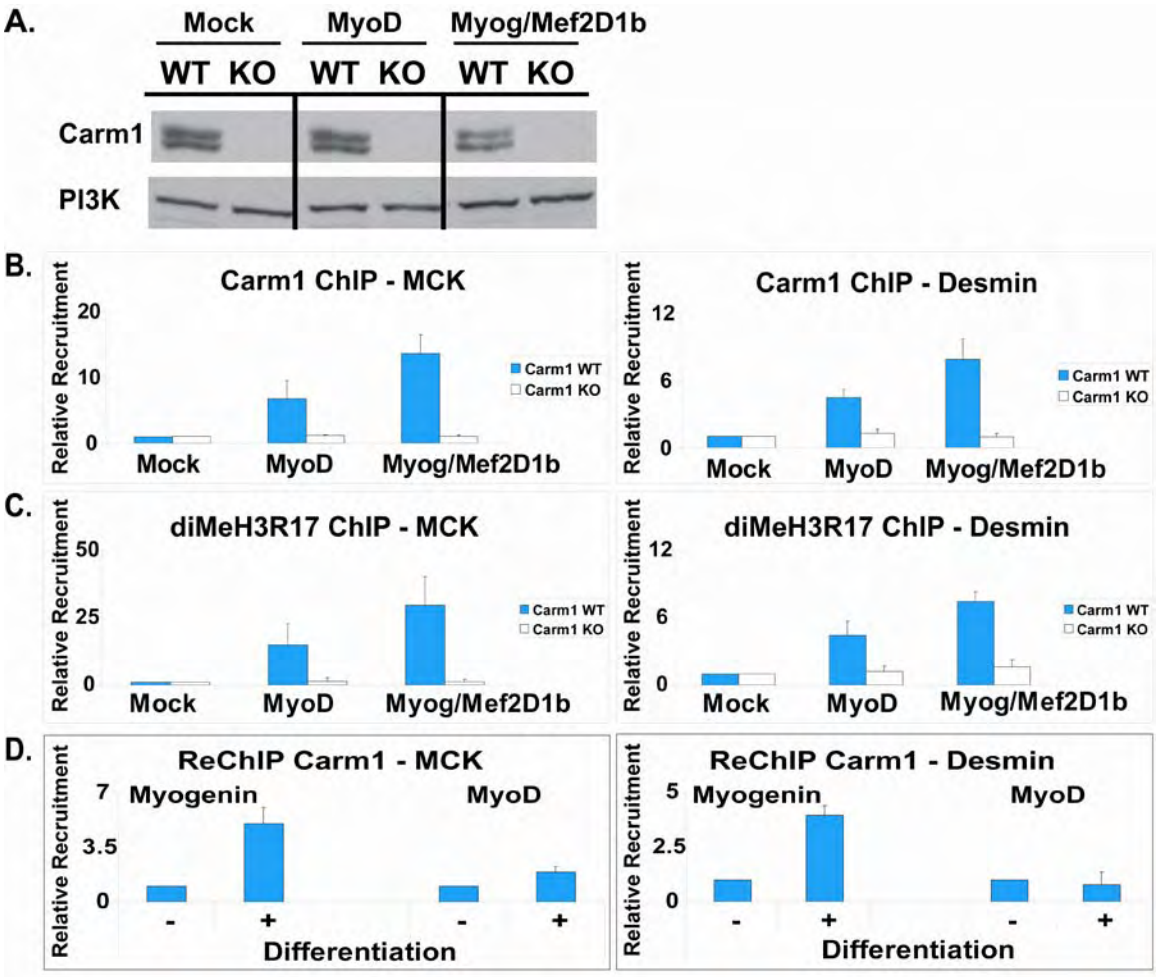
A.
Mock
MyoD
Myog/Mef2D1b
WT KO WT KO WT KO
Carm1
PI3K
B.
Carm1 ChIP - MCK
Relative Recruitment
20
10
0
Carm1 WT
Carm1 KO
Mock
MyoD
Myog/Mef2D1b
Carm1 ChIP - Desmin
12
6
0
C.
diMeH3R17 ChIP - MCK
50
25
0
diMeH3R17 ChIP - Desmin
D.
ReChIP Carm1 - MCK
Myogenin
MyoD
7
3.5
0
- + - +
Differentiation
ReChIP Carm1 - Desmin
5
2.5

Figure 3-4.

Figure 3-4. Carm1/Prmt4 and dimethylated H3R17 bind to late myogenic gene regulatory regions. (A) Western blot showing Carm1/Prmt4 protein levels in immortalized wildtype (WT) or Carm1/Prmt4 knockout (KO) mouse embryo fibroblasts (MEFs) that were mock differentiated or differentiated with MyoD or with myogenin + Mef2D1b (Myog/Mef2D1b) for 24 hours. Phosphoinositide 3-kinase (PI3K) levels are shown as a control. (B, C) ChIP experiments were performed using antibodies recognizing Carm1/Prmt4 and diMeH3R17 and were analyzed by quantitative PCR (Q-PCR). Values are expressed relative to the values obtained for binding in mock-differentiated WT MEFs, which were set at 1. (D) Re-ChIP experiments were performed and quantified by Q-PCR. Material immunoprecipitated with Carm1/Prmt4 antibodies were subsequently immunoprecipitated with either a MyoD or a myogenin antibody. Data in panels B-C represent the average +/- standard deviation of 5 independent experiments, while the data in panel D is the average +/- standard deviation of 4 independent experiments.

Gene expression analysis was then performed by real-time PCR to assess the functional significance of Carm1/Prmt4 binding. MyoD was induced and equivalently expressed in MyoD-differentiated WT and KO MEFs but was not detectable in mock or myogenin/Mef2D1b-differentiated samples (Fig. 5 - left column). Myogenin and Mef2D1b were equivalently expressed in myogenin/Mef2D1b-differentiated samples, and the levels of introduced myogenin were roughly equivalent to the levels normally induced by MyoD (Fig. 5 - left column). Interestingly, in MyoD-differentiated samples, myogenin expression was robust and was not compromised in KO MEFs (Fig. 5 - left column), indicating that Carm1 was not required for the activation of the myogenin gene.

When late gene activation was examined, MCK, desmin, and dystrophin gene expression was induced in MyoD and myogenin/Mef2D1b-differentiated WT MEFs, but not in mock-differentiated WT MEFs (Fig. 5 - right column). In contrast, induction of late myogenic genes was severely compromised in KO MEFs, regardless of whether the cells were differentiated with MyoD or with myogenin/Mef2D1b (Fig. 5 - right column). The lack of late gene expression in myogenin/Mef2D1b differentiated cells means that there is a direct requirement for Carm1/Prmt4 during late gene induction, because even when the myogenin and Mef2D1b regulators were provided to the cell, the lack of Carm1/Prmt4 prevented late gene expression. In combination with the analysis of myogenin expression in MyoD differentiated cells (Fig. 4D - left column), the data indicate that Carm1/Prmt4 is required for late gene expression but not for expression of the early myogenin gene.

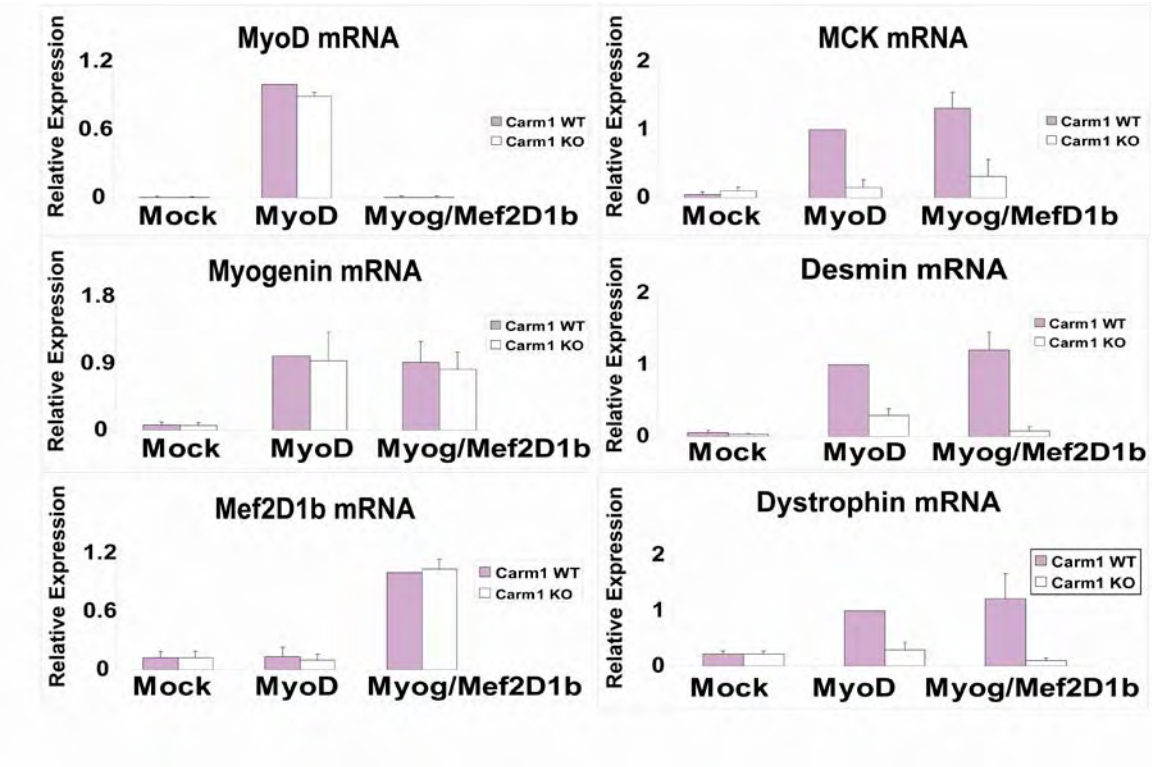
MyoD mRNA
Relative Expression
1.2
0.6
0
Mock
MyoD
Myog/Mef2D1b
Carm1 WT
Carm1 KO
MCK mRNA
Relative Expression
2
1
0
Mock
MyoD
Myog/MefD1b
Carm1 WT
Carm1 KO
Myogenin mRNA
Relative Expression
1.8
0.9
0
Mock
MyoD
Myog/Mef2D1b
Carm1 WT
Carm1 KO
Desmin mRNA
Relative Expression
2
1
0
Mock
MyoD
Myog/Mef2D1b
Carm1 WT
Carm1 KO
Mef2D1b mRNA
Relative Expression
1.2
0.6
0
Mock
MyoD
Myog/Mef2D1b
Carm1 WT
Carm1 KO
Dystrophin mRNA
Relative Expression
2
1
0
Mock
MyoD
Myog/Mef2D1b
Carm1 WT
Carm1 KO

Figure 3-5.

Figure 3-5. Carm1/Prmt4 is required for myogenic late gene expression mRNA expression analysis of the indicated genes by Q-PCR in immortalized wildtype (WT) or Carm1/Prmt4 knockout (KO) mouse embryo fibroblasts (MEFs) that were mock differentiated or differentiated with MyoD or with myogenin + Mef2D1b (Myog/Mef2D1b) for 24 hours. Transcript levels were normalized to the total amount of EF1-α mRNA. Values are expressed relative to the expression values obtained in MyoD differentiated WT MEFs, which were set at 1, except for the evaluation for Mef2D1b expression, where the value obtained for myogenin/Mef2D1b differentiated WT cells was set at 1. Data show the average +/- standard deviation of 5 independent experiments.

Carm1/Prmt4 binding to myogenic late gene regulatory sequences is independent of Prmt5

The data indicate that Carm1/Prmt4 is required for the induction of late myogenic gene expression whereas Prmt5 is not. This suggests that Carm1/Prmt4 binding should be independent of Prmt5 function. To address this question we examined Carm1/Prmt4 binding in the Prmt5 deficient cell lines using ChIP. The data in Fig. 6A demonstrate that Carm1/Prmt4 bound equivalently well in the presence and absence of Prmt5, whether differentiation was induced by MyoD or by myogenin/Mef2D1b. Although Prmt5 binding to late myogenic regulatory sequences precedes Carm1/Prmt4 binding, we also performed the converse experiment to determine whether Prmt5 binding required Carm1/Prmt4. Such an experiment would exclude the possibility that Prmt5 binding was dependent upon a Carm1/Prmt4 function that did not involve its ability to bind to myogenic late gene sequences. Examination of Prmt5 binding in MyoD differentiated WT and Carm1/Prmt4 null MEF lines showed that Prmt5 binding did not require Carm1/Prmt4 (Fig. 6B). Consistent with the data presented in Fig. 3B, Prmt5 did not bind to myogenic late gene regulatory sequences when differentiation was induced by myogenin/Mef2D1b due to the absence of MyoD (Fig. 6B). Thus the binding of each of the two distinct Prmts to myogenic late gene loci is independent of the other.

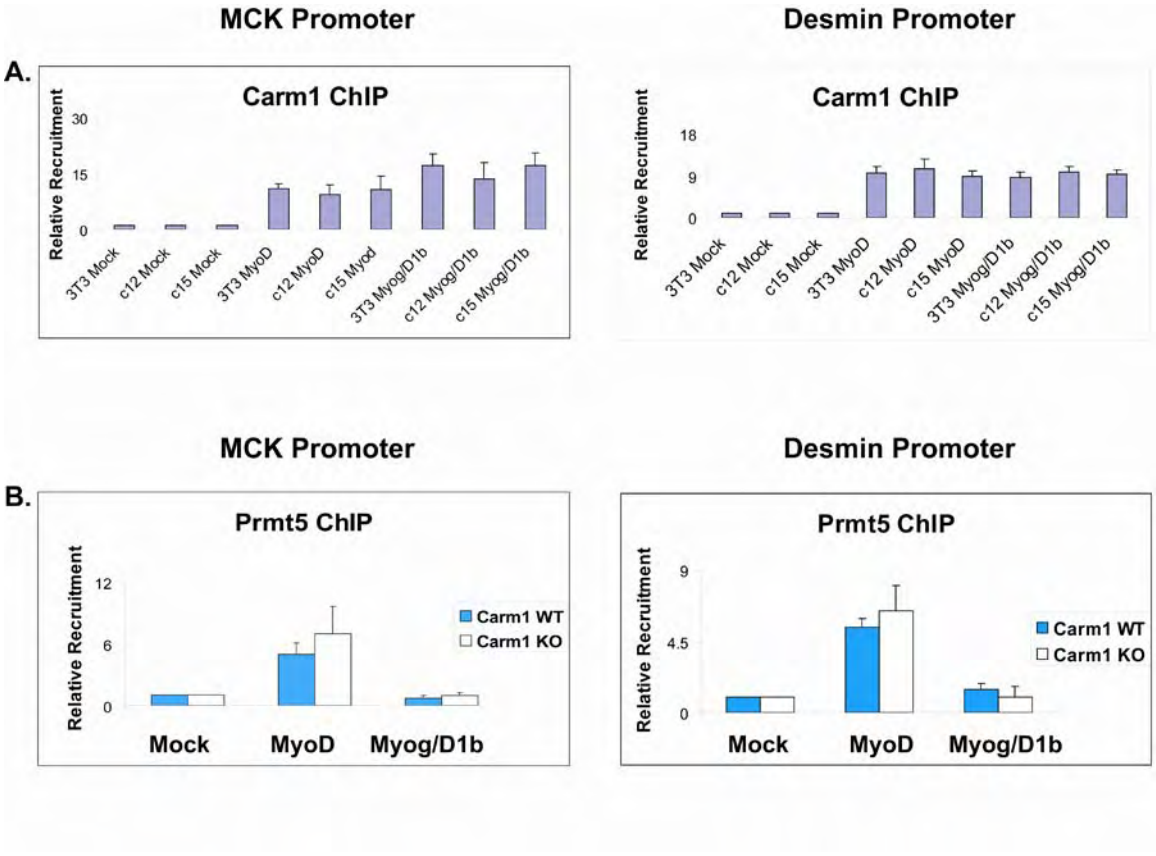
MCK Promoter
Desmin Promoter
A.
Carm1 ChIP
Relative Recruitment
30
15
0
3T3 Mock
c12 Mock
c15 Mock
3T3 MyoD
c12 MyoD
c15 Myod
3T3 Myog/D1b
c12 Myog/D1b
c15 Myog/D1b
Carm1 ChIP
Relative Recruitment
18
9
0
3T3 Mock
c12 Mock
c15 Mock
3T3 MyoD
c12 MyoD
c15 MyoD
3T3 Myog/D1b
c12 Myog/D1b
c15 Myog/D1b
MCK Promoter
Desmin Promoter
B.
Prmt5 ChIP
Relative Recruitment
12
6
0
Carm1 WT
Carm1 KO
Mock
MyoD
Myog/D1b
Prmt5 ChIP
Relative Recruitment
9
4.5
0
Carm1 WT
Carm1 KO
Mock
MyoD
Myog/D1b

Figure 3-6.

Figure 3-6. Binding of Carm1/Prmt4 at the regulatory regions of late myogenic targets is not dependent on Prmt5, while binding of Prmt5 to these sequences does not require Carm1/Prmt4. (A) NIH3T3 cells or c12 and c15 cells that express antisense against Prmt5 were mock differentiated or differentiated with MyoD or with myogenin + Mef2D1b (Myog/D1b) for 24 hours and were used for ChIP analysis using an antibody recognizing Carm1. (B) Immortalized MEFs derived from Carm1 wild-type (WT) or knockout (KO) mice were mock differentiated or differentiated with MyoD or with myogenin + Mef2D1b (Myog/D1b) for 24 hours and used for ChIP analysis using an antibody recognizing Prmt5. Values are expressed relative to the values obtained for binding in mock-differentiated cells, which were set at 1. Data in panels A and B represent the average +/- standard deviation of 3 independent experiments.

In vitro interactions between myogenic regulatory factors and Prmt5 and Carm1/Prmt4

We utilized GST fusions with Prmt5 and with Carm1/Prmt4 to investigate whether each Prmt was capable of directly interacting with the myogenic regulators implicated in controlling early and late myogenic gene expression. GST-Prmt5, GST-Carm1/Prmt4, and GST were expressed in and purified from bacteria (Fig. 7C) and incubated with ^{35}S-labeled, in vitro translated (IVT) MyoD, myogenin, or Mef2D1b. GST alone did not interact with any of the tested myogenic regulators (Fig. 7A-B). GST-Carm1/Prmt4 could interact with both IVT myogenin and with IVT Mef2D1b but only weakly interacted with IVT MyoD (Fig. 7A). These results are consistent with the re-ChIP data presented in Fig. 4D indicating that Carm1/Prmt4 and myogenin co-occupy myogenic late gene regulatory sequences and with the functional data presented in Fig. 5 showing that Carm1/Prmt4 was required for myogenin and Mef2D1b to activate myogenic late gene expression. The weak interaction with IVT MyoD is consistent with the observation that Carm1/Prmt4 was not required for activation of the myogenin gene at early times (Fig. 5), which is a MyoD dependent event.

In contrast, GST-Prmt5 interacted with IVT MyoD, myogenin, and Mef2D1b (Fig. 7B). The interaction between Prmt5 and MyoD is consistent with physical and functional data that demonstrated a requirement for Prmt5 in MyoD-mediated activation of the myogenin gene during myogenesis (46). It is also consistent with the observation that Prmt5 is present on myogenic late gene regulatory sequences at early times of differentiation prior to the expression of the late genes (Fig. 2A), a time when MyoD and HDAC2 are also present (157). Although interactions between Prmt5 and myogenin and

Mef2D1b were observed in vitro, differentiation mediated by myogenin/Mef2D expression did not involve recruitment of Prmt5 to the late gene loci (Fig. 3B). This suggests that one or more of the proteins may be modified in vivo, or that the interactions that were revealed in vitro are occluded in the context of the factor binding at myogenic regulatory sequences in differentiating cells.

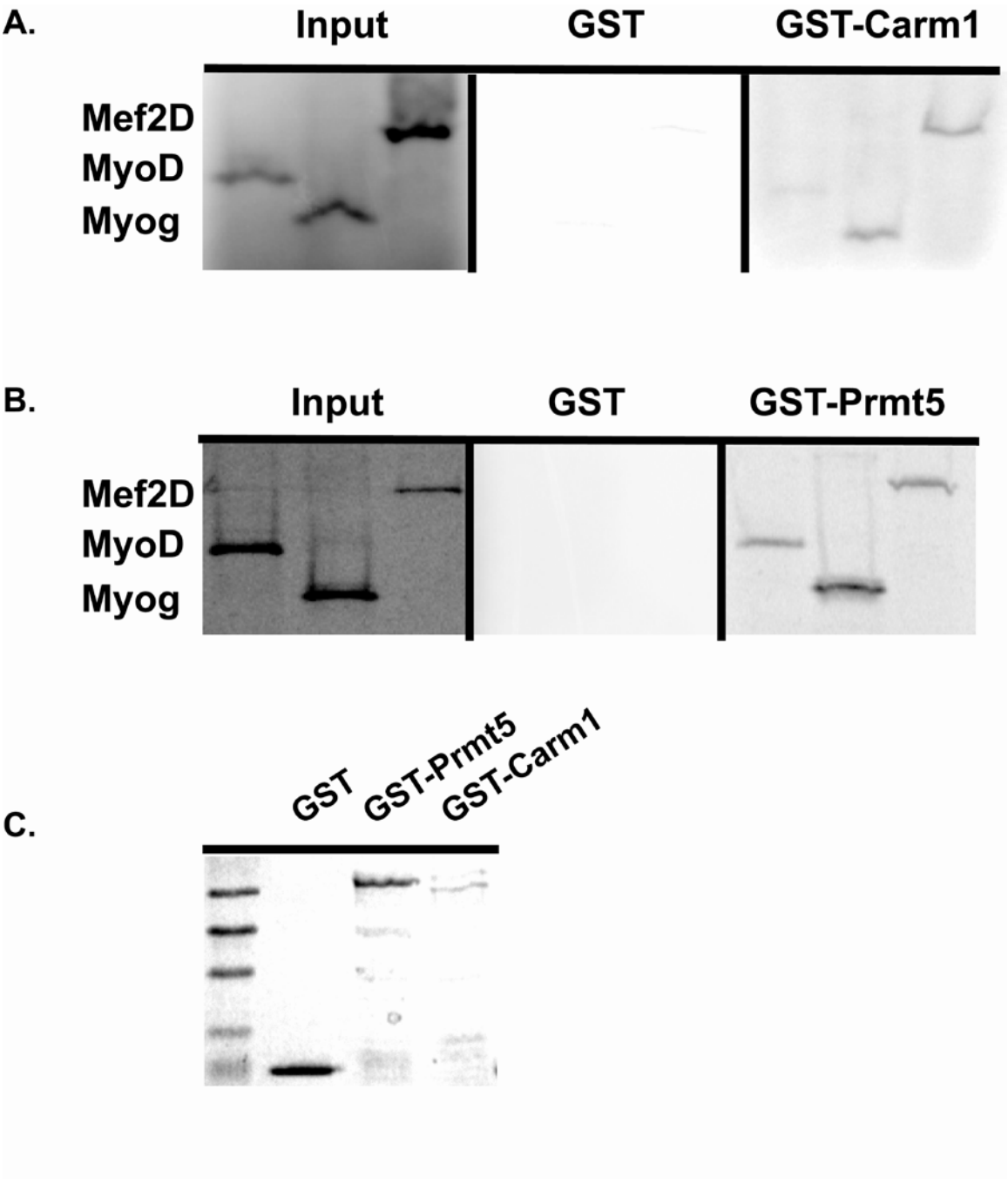
A.
Input
GST
GST-Carm1
Mef2D
MyoD
Myog
B.
Input
GST
GST-Prmt5
Mef2D
MyoD
Myog
C.
GST
GST-Prmt5
GST-Carm1

Figure 3-7.

Figure 3-7. In vitro interactions exist between Prmt5 and Carm1/Prmt4 and muscle regulatory factors. GST, GST-Prmt5 and GST-Carm1/Prmt4 were expressed and purified from BL21 cells by binding to glutathione beads. (A) Bead-bound GST and GST-Prmt5 were incubated with in vitro translated, full-length, ^{35}S-radiolabelled MyoD, myogenin, or Mef2D1b to determine if interactions exist between these factors. Following extensive washing, pelleted beads and any bound proteins were electrophoresed on an SDS-polyacrylamide gel, and the gels was dried and exposed to film. (B) The experiment in (A) was repeated with GST and GST-Carm1/Prmt4.
(C) Purified GST and GST fusion proteins were resolved on SDS-PAGE and visualized by Coomassie staining.

Carm1/Prmt4 binding at myogenic late gene regulatory sequences permits binding of the Brg1 ATPase of SWI/SNF chromatin remodeling enzymes and subsequent chromatin remodeling

Our earlier work established that transcriptional activation of late myogenic genes requires the SWI/SNF chromatin remodeling enzymes, and, in particular, the activity of the Brg1 ATPase subunit (157). The ChIP assays presented above revealed that Carm1/Prmt4 binds to the regulatory elements of late myogenic genes and is responsible for di-methylating H3R17, while gene expression analysis indicated that Carm1/Prmt4 was required for late gene expression. To probe the mechanism of the requirement for Carm1/Prmt4, we determined whether the loss of Carm1/Prmt4 affected Brg1 binding and function at late muscle genes. In both MyoD and myogenin/Mef2D1b differentiated Carm1/Prmt4 KO cells, we observed that the binding of Brg1 at the regulatory sequences of late myogenic targets was severely diminished (Fig. 8A). This reduction in Brg1 binding was not due to a reduction in the levels of Brg1 protein as shown by Western blot (Fig. 8B). Thus Carm1/Prmt4 is responsible for facilitating Brg1 binding. The association of an ATP-dependent chromatin remodeling enzyme with a regulatory sequence implies a localized chromatin structural change. To document any structural changes in chromatin at these loci in Carm1/Prmt4 WT and KO MEFs, a restriction enzyme accessibility assay (REAA) was performed. Upon initiation of differentiation with MyoD or myogenin/Mef2D1b, but not in mock-differentiated cells, there was an induction of restriction enzyme accessibility at Pvu II sites present in the MCK and

desmin promoter regulatory regions in the WT MEFs (Fig. 8C). We previously demonstrated that these changes in chromatin accessibility were Brg1 dependent (157).

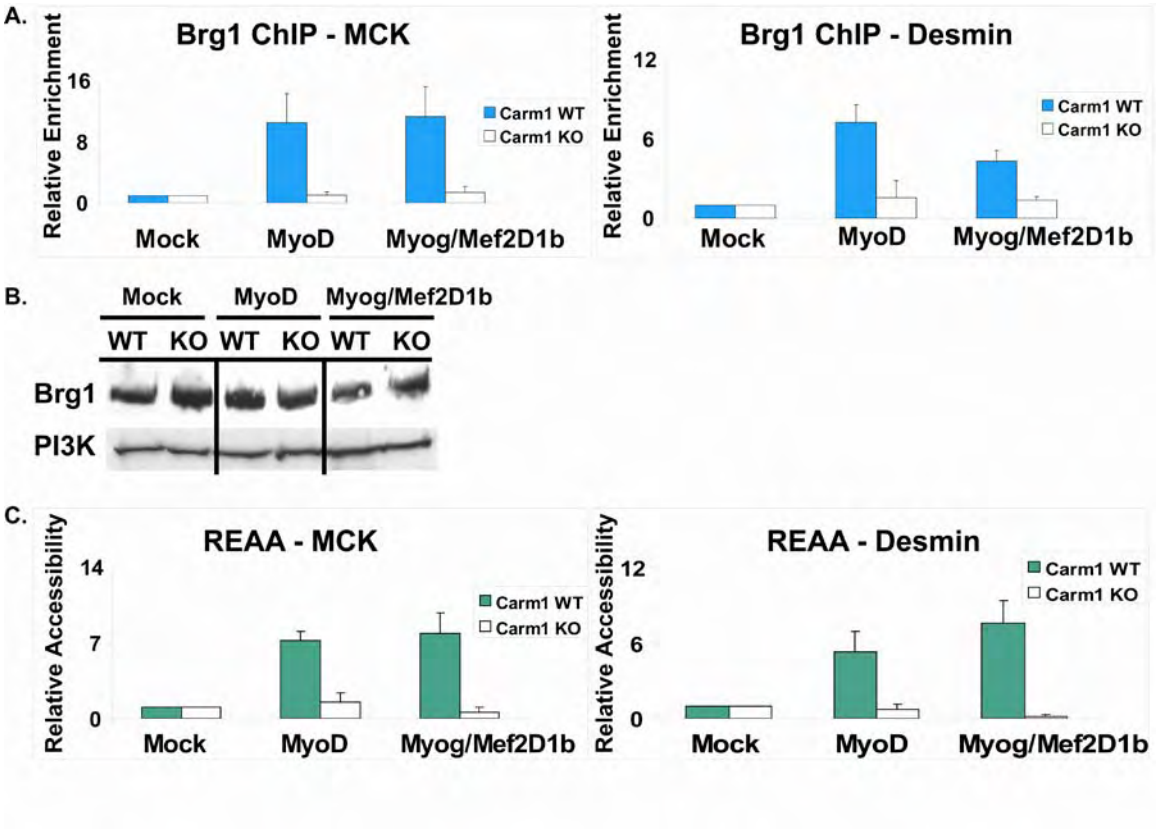
A.
Brg1 ChIP - MCK
Relative Enrichment
16
8
0
Carm1 WT
Carm1 KO
Mock
MyoD
Myog/Mef2D1b
Brg1 ChIP - Desmin
Relative Enrichment
12
6
0
Carm1 WT
Carm1 KO
Mock
MyoD
Myog/Mef2D1b
B.
Mock
MyoD
Myog/Mef2D1b
WT
KO
WT
KO
WT
KO
Brg1
PI3K
C.
REAA - MCK
Relative Accessibility
14
7
0
Carm1 WT
Carm1 KO
Mock
MyoD
Myog/Mef2D1b
REAA - Desmin
Relative Accessibility
12
6
0
Carm1 WT
Carm1 KO
Mock
MyoD
Myog/Mef2D1b

Figure 3-8.

Figure 3-8. Carm1/Prmt4 is required to facilitate the binding and function of the chromatin remodeling enzyme Brg1. Immortalized MEFs derived from Carm1 wild-type (WT) and knockout (KO) mice were mock differentiated or differentiated with MyoD or with myogenin + Mef2D1b (Myog/Mef2D1b) for 24 hours and were used for (A) ChIP analysis using an antibody recognizing Brg1, (B) Western blot analysis to examine Brg1 and PI3K protein levels in each sample, or (C) restriction enzyme accessibility assay (REAA) to assess the extent of chromatin accessibility at Pvu II restriction sites in the indicated gene regulatory regions. Values presented in panels (A) and (C) are expressed relative to the values obtained for binding or accessibility in mock-differentiated WT MEFs, which were set at 1. Data in panels A and C represent the average +/- standard deviation of 5 independent experiments.

However, in Carm1/Prmt4 KO MEFs, no increase in accessibility was observed (Fig. 8B), and the promoter chromatin structure at these loci remained in a more inaccessible state. These results demonstrate that the Carm1/Prmt4 methyltransferase is required for myogenic late gene expression because it facilitates binding of the Brg1 ATP-dependent chromatin remodeling enzyme and subsequent chromatin remodeling at these regulatory sequences. Therefore the histone modifying enzyme is required for the activity of the ATP-dependent chromatin remodeling enzyme. In conjunction with our earlier study, the results indicate a common molecular explanation for why different arginine methyltransferases are required for transcriptional activation at different stages of the skeletal muscle differentiation process.

DISCUSSION

Shared function by different Prmts at different stages of myogenesis

Since both Prmt5 and Carm1/Prmt4 had been implicated in myogenesis (35, 46), we sought to determine whether these different arginine methyltransferases might cooperate in the activation of specific myogenic loci. Although we demonstrated that both enzymes were physically located at regulatory sequences controlling the expression of myogenic late genes and that both enzymes modified histones at these loci, we also demonstrated that only Carm1/Prmt4 was necessary for late gene activation. Thus while the contribution made by Prmt5 at myogenic late gene loci remains to be defined, it is clearly not a direct requirement for late gene expression.

We also noted that the absence of Carm1/Prmt4 had no impact on the ability of MyoD to induce the expression of myogenin (Fig. 5), which contrasts the previous results obtained studying differentiation under conditions where Prmt5 levels were reduced (46). Thus, even though late gene expression was dependent upon Carm1/Prmt4, expression of the early gene, myogenin, was not. Instead of cooperativity at specific loci, what we observed was a sequential requirement for Prmt5 and Carm1/Prmt4 that correlated with the temporal class of gene being activated. To be specific, Prmt5 was required to facilitate activation of the early gene myogenin, whereas Carm1 was required for late myogenic gene induction.

Our results contradict a conclusion of an earlier study where antisense constructs were used to reduce the levels of Carm1/Prmt4 (35). These investigators observed that reduction of Carm1/Prmt4 inhibited differentiation but attributed the effect to a decrease

in the induction of myogenin. The reasons for the discrepancy between this report and our data are undetermined; our results clearly show that myogenin is robustly induced in the absence of Carm1/Prmt4 (Fig. 5).

Having defined a series of protein:DNA interactions that occur at late gene regulatory sequences (157), we sought to determine which of these events might be compromised by the absence of Carm1/Prmt4 as a means to explain the lack of late gene expression observed in the absence of Carm1/Prmt4. The results showed that interaction of Brg1, the ATPase of SWI/SNF chromatin remodeling enzymes previously demonstrated to be required for myogenesis, was compromised at late gene regulatory sequences (Fig. 6A). The functional consequence of this loss was then demonstrated by the lack of chromatin remodeling at these loci (Fig. 6C). We therefore conclude that Carm1/Prmt4 is required to promote Brg1 binding and chromatin remodeling at late myogenic genes.

On a more general scale, the results indicate that an arginine methyltransferase is required for an ATP-dependent chromatin remodeling enzyme to function. Our prior demonstration that Prmt5 facilitates Brg1 and SWI/SNF enzyme function at the myogenin promoter during gene activation (46) is extended by the observation that Carm1 is required at late myogenic gene regulatory regions for the same reason, suggesting this may be a general mechanism. Combined, these studies indicate that Prmt5 and Carm1/Prmt4, two distinct arginine methyltransferases, promote the same step of gene activation, which is the recruitment of the SWI/SNF ATP-dependent chromatin remodeling enzyme. Prmt5 and Carm1/Prmt4 are not known to methylate the same sites

on histones; Prmt5 dimethylates H3R8 and H4R3 (163, 164), while Carm1/Prmt4 dimethylates H3R17 and H3R26 (199), however, both enzymes are capable of modifying non-histone substrates as well (39, 70, 110, 117, 123, 236). Importantly, Prmt5 and Carm1/Prmt4 can methylate the same non-histone substrates, including CA150, SmB, PABP1, U1C and SF3b4 (37), although it is still not clear whether the same arginine residues are methylated in these common substrates (37). The exact mechanism by which Prmt5 and Carm1/Prmt4 promote Brg1 interaction at different gene regulatory sequences during myogenesis remains to be determined, but one attractive hypothesis is that the dimethylation of different histone residues makes the chromatin a better substrate for the ATP-dependent remodeling enzyme.

Another question that remains unclear is why different arginine methyltransferases would be required at different times of myogenesis to facilitate the same step in the activation process. One possible explanation is that the combination of activators and cofactors present at early and late myogenic gene regulatory sequences are different (discussed further below) and that the specific arginine methylations mediated by each enzyme are specific for promoting gene activation by the different sets of regulatory proteins in ways that remain to be defined. An additional possibility is that the arginine methyltransferases modify different transcriptional regulatory proteins in addition to modifying histone tails. Finally, it must be noted that despite the presence of Carm1/Prmt4 and dimethylated H3R17 at late gene regulatory sequences both in tissue culture and in vivo, Carm1/Prmt4 deficient mice, which are perinatal lethal for undetermined reasons, show no gross defect in skeletal muscle appearance (240). This

implies that there is either a functional defect in one or more muscles that contribute to breathing or feeding, or that there are undefined redundant mechanisms in vivo to compensate for the deficiency of Carm1/Prmt4. Given the widespread redundancies between MyoD, Myf5, and Mrf4 during development (reviewed in (20, 176)), additional redundant mechanisms to ensure skeletal muscle formation and function are possible.

Mechanisms relating to Prmt5 and Carm1/Prmt4 function

We previously determined that Prmt5 functioned directly in the induction of the myogenin gene (46). It is well established that MyoD is critical in the activation of the myogenin promoter (reviewed in (20, 76, 195, 217)); demonstration by re-ChIP analysis that MyoD and Prmt5 are co-localized to myogenin promoters in primary cells that are actively transcribing the myogenin gene supports the idea that Prmt5 acts as a co-activator for MyoD (46). Additional experiments indicating that MyoD and Prmt5 can be co-immunoprecipitated from differentiated cells further support this conclusion (46). Here we report that Prmt5 can directly interact with MyoD in an in vitro interaction assay, providing further mechanistic explanation for the cooperativity exhibited by these factors. In addition, we show that Prmt5 is localized to myogenic late gene regulatory sequences at the onset of differentiation, prior to the initiation of late gene expression. Previous work indicates that MyoD is also present at late gene loci before activation (157); it is accompanied by a class I HDAC, which has been shown to maintain MyoD in a transcriptionally inactive state in myoblast cultures (134, 177). We propose that MyoD

targets Prmt5 to gene regulatory sequences, regardless of whether it is functioning in an activating or repressing capacity.

In contrast, Carm1/Prmt4 binding to late gene loci correlates with the time of late gene activation. In both developing embryonic skeletal muscle tissue and MyoD differentiated fibroblasts, the time of late gene activation is marked by coincident binding of myogenin, Mef2, and the SWI/SNF ATPase, Brg1 and changes in chromatin accessibility (157). The addition of Carm1/Prmt4 to this complex of regulators that functions at the time of transcription initiation suggests that Carm1/Prmt4 acts as a co-activator of myogenin and/or Mef2 proteins. Transient transfection studies imply cooperativity between Carm1/Prmt4 and Mef2, and Carm1/Prmt4 interacts with the Mef2C isoform in in vitro interaction assays (35). The demonstration by re-ChIP analysis that Carm1/Prmt4 is co-localized with myogenin, but not MyoD, at late gene regulatory sequences (Fig. 4D) also supports this hypothesis. The additional demonstration that Carm1/Prmt4 can directly interact with the Mef2D1b isoform and with the myogenin protein in vitro (Fig. 7A), but only weakly interacts (Fig. 7A) or does not interact (35) with MyoD in vitro suggests that targeting of Carm1/Prmt4 occurs via myogenin and/or Mef2 proteins.

Both Prmt5 and Carm1/Prmt4 were shown to co-immunoprecipitate and co-purify with Brg1 (165, 237) and coordinated activity between Brg1 and Prmt5 (46, 164, 165) and between Brg1 and Carm1/Prmt4 (237) has previously been demonstrated. Collectively the data presented here and in previous reports (19, 46, 54) support a model (Fig. 9) where MyoD initially binds indirectly to the myogenin promoter via interaction

with Pbx1 and also binds to sites at late myogenic gene regulatory sequences, thereby facilitating and recruitment of Prmt5 to both classes of genes. The recruitment of Prmt5 at the myogenin gene mediates dimethylation of H3R8 and is necessary for the subsequent recruitment of Brg1 based SWI/SNF chromatin remodeling enzymes, leading to chromatin remodeling and expression of the myogenin gene. Upon accumulation of myogenin protein, myogenin and Mef2 proteins bind to cognate binding sites at late gene regulatory sequences, displacing MyoD and the HDAC, as shown previously (157) and recruiting Carm1/Prmt4. Carm1/Prmt4 recruitment mediates dimethylation of H3R17 and facilitates the recruitment of Brg1 based SWI/SNF chromatin remodeling enzymes, leading to chromatin remodeling and expression of the late myogenic genes, terminal differentiation, and the formation of mature skeletal muscle tissue.

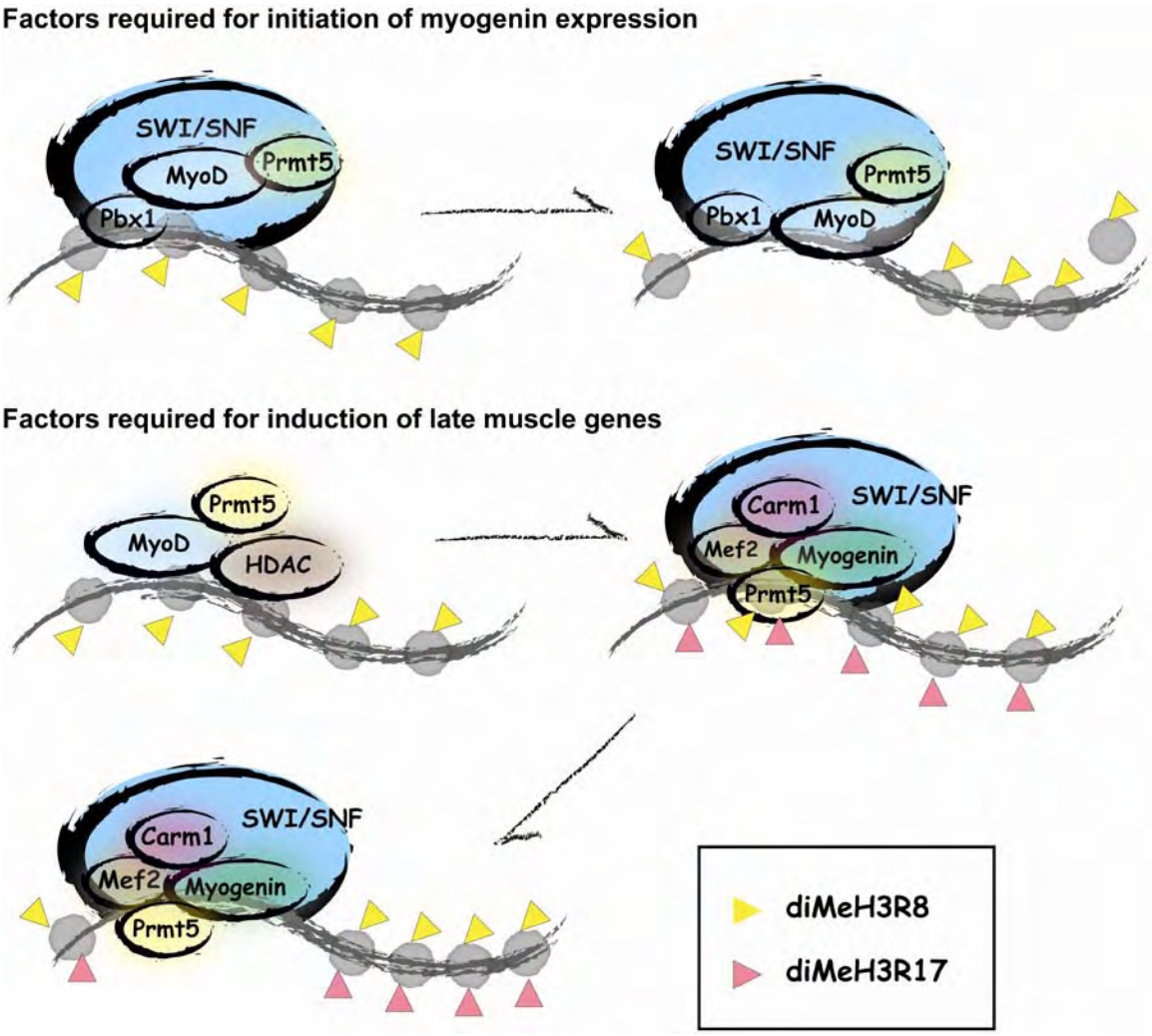
Factors required for initiation of myogenin expression
SWI/SNF
Prmt5
MyoD
Pbx1
SWI/SNF
Prmt5
Pbx1
MyoD
Factors required for induction of late muscle genes
Prmt5
MyoD
HDAC
Carm1
SWI/SNF
Mef2
Myogenin
Prmt5
SWI/SNF
Carm1
Mef2
Myogenin
Prmt5
diMeH3R8
diMeH3R17

Figure 3-9.

Figure 3-9. A schematic model illustrating Prmt function at the myogenin and at representative late gene regulatory sequences. Prmt5 mediates dimethylation of H3R8 and facilitates Brg1 based SWI/SNF chromatin remodeling enzyme interaction and function at the myogenin promoter at early times of myogenic differentiation while Carm1/Prmt4 mediates dimethylation of H3R17 and facilitates Brg1 based SWI/SNF chromatin remodeling enzyme interaction and function at myogenic late genes.

MATERIALS AND METHODS

Cell Culture

NIH3T3 cells, Prmt5 antisense-expressing cell lines (164), and immortalized MEFs from wild type or knockout Carm1 embryos (240) were cultured in Dulbecco's Modified Eagle Media supplemented with 10% calf serum. Prmt5 antisense cell lines were maintained in 2.5ug/mL puromycin. Cells were grown to ~50% confluence then transdifferentiated into the skeletal muscle lineage by ectopic expression of MyoD or myogenin in combination with Mef2D1b, using the pBABE retroviral vector system (149, 150), as previously described (53, 56, 157, 190) . The viral inoculum was applied to the subconfluent cells for 24 hours, during which time the cells became confluent. Cells were then differentiated using DMEM supplemented with 2% horse serum + 10ug/mL insulin. Mock infected cells were also treated with DMEM + 2% horse serum, and all samples were maintained in the differentiation media for up to 24 hours. Samples were harvested at indicated times for RNA, protein, and ChIP analysis.

RNA Isolation and RT-PCR

RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcriptase reactions performed to generate cDNA used 1 ug of RNA as previously described (46, 54, 157) . Amplification of transcripts was quantified by Q-PCR using the Opticon Engine (MJ Research) and primers previously described (46, 54, 157).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed and quantified as described previously using primers that were also previously described (46, 54, 157, 158). Primers spanning the desmin and MCK promoters and enhancers were used with no significant differences between binding to enhancer or promoter sequences observed. The immunoprecipitation step utilized rabbit polyclonal antisera raised against Brg1 (52), dimethylated H3R8 and Prmt5 (164, 165) and commercial antibodies raised against Prmt5 (Becton Dickinson - 611539), Carm1/Prmt4 (Bethyl Labs – A300-421A, Upstate – 07-080), and dimethylated H3R17 (Upstate -07-214). As an additional negative control, every sample was analyzed for the presence of the IgH enhancer sequences; no specific enrichment of IgH sequences was ever observed. Re-ChIP analysis was performed as previously described (146) with antibodies against Prmt5, MyoD (12) and myogenin (Santa Cruz - sc576).

Westerns

Samples were harvested at the indicated time points by scraping into 1mL of PBS followed by brief centrifugation to obtain a cell pellet. Carm1 westerns were performed by using cell lysates from immortalized MEFs using the ReliaBlot protocol (Bethyl Labs), while all other cell pellets were resuspended in NP-40 Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 20% glycerol, 1 mM dithiothreitol), with freshly added protease inhibitors (1 ug/mL of pepstatin A, 4 ug/mL of leupeptin, 1 mM phenylmethylsulfonyl fluoride), and lysed by brief sonication.

Following centrifugation at 10K for 10 minutes, cell debris was discarded and lysate concentrations were quantified by spectrophotometry. 50ug of protein were loaded and electrophoresed on an SDS gel, then transferred overnight onto nitrocellulose membranes. Membranes were blocked for 1 hour in 5% milk in 1X TBST, and incubated in primary antibody diluted in 5% milk in 1X TBST overnight. Antibodies used included Brg1 antisera (52), Prmt5 antisera (165), Carm1 (Bethyl Labs), and PI3K (Upstate). Membranes were then washed 3X for 5 minutes each in 1X TBST, incubated in secondary antibody diluted in 5% milk in 1X TBST, washed 3X for 5 minutes each and visualized by ECL (Amersham).

Tissue Isolation and Nuclei Preparation

Isolation of nuclei from liver and myofibers was performed as described previously (46, 158). Briefly, skeletal muscle tissue from the hindlimb of 4-6 week old BL6 mice was dissected and minced on ice into $1mm^3$ pieces. Minced samples were pooled and digested with 110U of collagenase supplemented with 1mM $CaCl_2$ in PBS at 37º C for 1 hour with agitation. Separation of satellite cells and myotubes was achieved by use of a 70uM filter (Becton Dickinson). Separated populations of cells were resuspended in lysis buffer (10mM HEPES-KOH (pH7.3), 10mM KCl, 5mM $MgCl_2$, 0.5mM DTT, 0.2mM PMSF, 3ug/mL cytochalasin B, 10ug/mL leupeptin) and nuclei were released by homogenization followed by dounce homogenization. Integrity of nuclei was ascertained by light microscopy. This nuclei mixture was overlayed onto a sucrose gradient and centrifuged as previously described (46, 157, 158). Following centrifugation, nuclei were

crosslinked for ChIP and snap frozen using liquid nitrogen. All samples were then stored at -80º C until use.

Restriction Enzyme Accessibility Assay

Restriction enzyme accessibility analysis was performed as described previously (54, 157). Briefly, nuclei were released from cells by dounce homogenization and DNA was quantified by spectrophotometry. 100ug of DNA was subjected to limited digestion with PvuII for 1 hour at 37º C. Following digestion, DNA was ligated to a linker previously described (54, 157). Ligation mediated PCR was used to quantify the amount of nuclease accessible DNA using primers corresponding to linker DNA and specific gene loci as previously described (54, 157). Results were normalized to input genomic DNA using Q-PCR.

Plasmid Construction

pCDNA3.1-MyoD was constructed by isolating the EcoR1 fragment encoding the MyoD cDNA from pEMSV-MyoD (50) and cloning it into EcoR1 digested pCDNA3.1 (Invitrogen).

GST Pull-down Experiments

Competent BL21 *E. Coli* was transformed with pGEX-2TK, pGEX-Prmt5 (165), and pGEX-Carm1 (34). Cells were grown to OD=0.8 and induced with 1.5mM IPTG (Invitrogen # 15529019) for 6 hours at 37ºC. Cells were pelleted and resuspended in

STE Buffer (20 mM Tris pH 7.6, 5 mM $MgC1_2$, 100 mM NaCl, 1 mM EDTA) containing 4mg/mL Lysozyme and incubated on ice for 15 minutes. 5 mM DTT, 0.5 mM PMSF and 1% (w/v) aprotinin were added sequentially and followed by brief vortexing. 1.5% Sarkosyl was added to the cells followed by brief vortexing. Lysis of bacterial cells was accomplished by sonication and the protein lysate was centrifuged at 14.5K for 15 minutes at 4°C. The sonicated lysate was incubated with a slurry of Glutathione beads resuspended in PBS for 30 mins at 4°C while rocking. Beads were washed for 5 minutes 5X with STE buffer and resuspended in a 50% slurry. Beads were resuspended in sample buffer and subjected to SDS PAGE and followed by Coomassie staining to quantify immobilized GST or GST-fusion proteins.

pCDNA3.1-MyoD, pCS2-Myogenin (a kind gift from Dr. S. Tapscott), and pCDNA1.1-MEF2D1b (a kind gift from Dr. E. Olson) were incubated with the TNT Quick Coupled Transcription/Translation System (Promega # 1171, 2081) to generate ^{35}S-Methionine labeled proteins. In order to preclear, radiolabeled proteins were resuspended in 500uL of NETN and incubated with 35uL of glutathione beads for 45 minutes at 4°C while rocking. Beads were discarded and glutathione-immobilized GST, GST-Prmt5 and GST-Carm1 fusion proteins were then incubated with ^{35}S-radiolabelled full length MyoD, Myogenin and Mef2D1b for a minimum of 2 hrs at 4°C while rocking. Beads were washed for 5 minutes 5X with NETN Buffer, resuspended in sample buffer, boiled and subjected to SDS-PAGE. Gels were dried and were visualized using a phosphoimager (Molecular Dynamics).

CHAPTER IV

General Discussion

We have demonstrated that Prmt5 is functionally required for transcriptional regulation of myogenic differentiation because it facilitates ATP-dependent chromatin remodeling at myogenin promoter sequences. Binding of Prmt5 and dimethylation of histone substrates at the myogenin promoter are necessary for the targeting of Brg1, and remodeling of chromatin at this locus to enable binding of transcription factors such as MyoD. Prmt5 has been described primarily as a repressor though few groups have described its role as an activator. One report indicated that dose-dependent reduction of Prmt5 results in reduced NFAT and IL-2-dependent gene expression (186). A purified multi-protein complex containing Prmt5 and p44 has been shown to mediate androgen receptor-dependent gene expression though Prmt5-dependent transcription activation did not require methyltransferase activity. Collectively these findings indicate that the mechanism by which Prmt5 influences transcription activation is quite complex.

Prmt5 has been purified in a multi-protein repressor complex containing Brg1 mSin3A and HDAC2, and this complex serves to regulate the expression of c-Myc target genes such as carbamoyl-phosphate synthase-aspartate carbamoyltransferase-dihydroorotase (*cad)* and nucleolin (*nuc)*(165). This complex also regulates the genes nonmetastatic 23 (*NM23)* and suppressor of tumorigenicity 7 (*ST7*)(164). Prmt5-mediated methylation of Spt5, a component of the transcription elongation complex that

is associated with the active hyperphosphorylated form of the polymerase, reduced its affinity for the complex and thus inhibited transcription elongation (117).

By virtue of the fact that Prmt5 is able to associate with a variety of protein complexes, it is likely that these interactions dictate its role as a repressor or, in our hands, an activator. It is possible that many unidentified coregulators of Prmt5 exist, and these factors direct Prmt5 activity to specific subsets of genes or augment its interaction with multi-protein transcription regulatory complexes. Recent reports have revealed that such a coregulator exists and it is aptly termed cooperator of Prmt5 (COPR5). This factor bound tightly and specifically to Prmt5, permitting preferential binding to histone H4 at a subset of Prmt5 target genes (118).

Since we have established that Prmt5 is necessary for the activation of early myogenic targets, it was of interest that binding of Prmt5 and methylation of histone substrates did not require the function of Brg1, and were needed to facilitate Brg1 binding, suggesting that Prmt5 binds before Brg1. It is possible that methylated histone substrates are better substrates for Brg1-dependent chromatin remodeling. In fact, acetylated histones precede and assist Brg1 targeting to the myogenin promoter prior to gene activation (54). It is not unreasonable to suggest that different histone modification events may be cooperating at the myogenin promoter to facilitate loading of Brg1. For example, hyperacetylated histones have been shown to provide higher affinity substrates for methyltransferases. At the estrogen-inducible pS2 promoter, the HAT CBP is bound to and acetylates histone H3 at the promoter providing a better substrate context for Carm1-mediatead histone H3 methylation (48). Alternatively, the function of

methyltransferases may be necessary for subsequent histone modifications. siRNA-mediated knockdown of PRMT1 resulted in near complete ablation of methylated histone H4 arginine 3 (H4R3) at the ß-globin locus. Furthermore, domain wide loss of histone H3 acetylation, accompanied by increased H3 methylation, was observed, indicating that histone methylation may be required for subsequent histone modifications such as acetylation (100).

Alternatively, the association of Prmt5 and dimethylation of histones substrates at myogenic regulatory regions may be required at early time points, prior to the onset of early muscle genes in order to facilitate the recruitment of HATs and acetylation of histones. Prior studies (54) have indicated that histone acetyltransferases occupy the myogenin promoter, acetylate histones and MyoD in order to facilitate binding of SWI/SNF complex. Binding of these chromatin remodeling factors occurs hours before the onset of myogenin gene expression. Our preliminary data suggests that Prmt5 is present at the myogenin promoter prior to the onset of differentiation and stable MyoD binding (data not shown). Prior to the onset of differentiation, Prmt5 is bound to the myogenin promoter prior to the association of HATs or SWI/SNF, and is accompanied by increases in H3R8 and H4R3 dimethylation. When Prmt5 is downregulated in the antisense-expressing cells, its requisite histone methylation marks disappear and acetylation of histones H3 and H4 is severely diminished. Furthermore, as histone acetylation is required to allow binding of SWI/SNF complex, binding of Brg1 is also compromised when Prmt5 is knocked down. Prmt5 is likely bound to the myogenin promoter via its interaction with the Pbx-bound MyoD protein. These results indicate

that an early role for Prmt5 exists. Specifically, Prmt5 and/or dimethylated H3R8 and/or H4R3 are required to facilitate the binding of HATs, acetylation of histones and ultimately the binding of the SWI/SNF complex, which is required to remodel chromatin and permit stable binding of myogenic transcription factors such MyoD.

These results however, do not clarify if Prmt5 itself or its enzymatic activity are required for subsequent events at the regulatory regions of the muscle gene myogenin. ChIPs performed in cells expressing a mutant version of Prmt5, which contains a mutation or deletion of its enzymatic SAM domain, rendering it functionally inert, would be useful in determining if dimethylation of histones is essential for the recruitment of HATs and SWI/SNF or if the protein itself functions as a scaffold protein that binds both HATs and SWI/SNF complex. Coimmunoprecipitations using either wt or mutant Prmt5 would reveal if an associations with HATs and Brg1 exist, or if these interactions are dependent on methyltransferase activity.

We have established that the regulation of early and late myogenic target genes is mediated by two distinct PRMTs. These Prmts function independently during various stages of differentiation but are ultimately required to facilitate binding of Brg1 and consequently, to elicit ATP-dependent chromatin remodeling. It is puzzling why two different PRMTs are needed for essentially one function. Our previous studies have shown that an interaction between Prmt5, Brg1 and MyoD exists. MyoD has also been observed at the regulatory sequences of myogenin prior to the onset of gene expression via its interaction with the constitutively bound heterodimer Pbx1/Meis. This interaction appears to specifically “mark” the myogenin locus for activation as HAT binding, histone

acetylation and ATP-dependent chromatin remodeling closely follow the initial non-consensus binding of MyoD to Pbx1/Meis (54).

In comparison, it has been reported that MyoD associated with late muscle gene promoters in a Brg1 independent manner and was responsible for recruiting HDAC activity, prior to the onset of late activation (157). Late gene activation occurs at approximately 8 hours and coincides with the disappearance of both MyoD and HDAC2. As expected, gene activation is concurrent with binding of Carm1, myogenin and its cofactor Mef2d1b, and recruitment of Brg1. In the case of late muscle gene regulatory elements, MyoD is not sufficient to elicit binding of Brg1. MyoD is required to facilitate the activation of myogenin and Mef2d, both of which appear to be essential for recruitment of Brg1. Genes induced during skeletal myogenesis are classified into two groups, early and late. The former do not require any additional rounds of protein synthesis and are dependent upon factors such as MyoD and/or Myf5. Myogenin and MRF4, products of MyoD-mediated transcriptional activation, are subsequently required for the activation of late muscle genes such as desmin and muscle creatine kinase.

MyoD appears to have a distinct role at early and late myogenic gene regions. While MyoD is required at early genes in order to facilitate recruitment of Brg1, it acts in a repressive capacity at the regulatory sequences of late muscle genes. MyoD recruitment to late gene promoter elements and its association with HDAC activity indicate that it may be responsible for maintaining late muscle targets in a repressed state until myogenin and Mef2d1b are present and able to recruit Brg1. Ectopic overexpression of myogenin and Mef2d1b are sufficient to induce significant and

precocious late muscle gene activation, hours earlier than when cells are differentiation of MyoD (157). Though Prmt5 is present and regulates dimethylation of histones at late muscle gene promoters, precocious gene activation does not occur because myogenin and Mef2d1b are required to recruit Brg1 and initiate gene activation. As an interaction exists between Prmt5 and MyoD, its role at late muscle gene sequences may also be somewhat altered in comparison with its requirement as an activator at early gene promoters. According to the ChIP data that we present, Prmt5 is not required for the recruitment of Brg1 at the promoter regions of late genes.

Our results have shown that throughout a time course of differentiation, Prmt5 is bound to and dimethylates histones at the regulatory regions of late muscle genes. Prmt5 association with these promoters was observed at t = 0h, prior to the onset of gene activation at t = 8h. It is apparent that Prmt5 is bound to these gene sequences via its interaction with MyoD, which we know to be associated with HDAC activity and possessing similar kinetics to Prmt5. *In vitro* binding experiments using GST and GST-Prmt5 fusion proteins and S-35 radio-labeled MyoD, myogenin and Mef2D, revealed that Prmt5 was able to interact directly with all three proteins.

Though coimmunoprecipitations of Prmt5 and endogenous muscle regulatory proteins have not been performed in primary myoblasts, Prmt5 recruitment to the promoter sequences of late muscle genes occurs via its interaction with MyoD. At the onset of gene activation, MyoD and HDAC2 disappear and are replaced by the binding of myogenin, Mef2D1b and Brg1. Though MyoD is no longer present at these regulatory sequences, Prmt5 is able to remain bound to late muscle promoters via its interaction with

myogenin and Mef2D. It is unclear what the sustained association of Prmt5 with late muscle gene sequences is indicative of, though we have ascertained that Prmt5 binding occurs via its interaction with either myogenin or Mef2D.

In order to eliminate the possibility that Prmt5 or its methyltransferase activity could be necessary for subsequent recruitment of Carm1, ChIPs were performed in Prmt5 antisense-expressing cells and revealed that though Prmt5 proteins levels were knocked down, Carm1 binding and dimethylation of histones was intact. Furthermore, the binding and methyltransferase activity of Carm1 were not required for Prmt5 binding and activity.

The role of Prmt5 at the regulatory sequences of late muscle genes is complex. Its association with MyoD and HDAC activity prior to the onset of gene activation implies it behaves in a repressive capacity. However, sustained binding of Prmt5 and its accompanying modification mark throughout the time course of differentiation suggests that while it is not essential for late gene activation, it is involved in the induction process and its constitutive binding may be to combat the activity of arginine demethylase activity. In order to determine if this is the case, additional ChIPs may be performed to determine the status of methylated histones at the regulatory sequences of late muscle targets when Prmt5 protein is knocked down. Additionally, ChIPs could be performed to determine if JMJD6, a recently identified protein arginine demethylase, exhibited increased occupancy at late myogenic promoter regions when Prmt5 protein levels were reduced. Though Prmt5 is not absolutely required to facilitate Carm1 binding or recruitment of Brg1 to these late muscle gene sequences, it is decidedly important for priming these promoter regions for key activation events.

Evidence (data now shown) indicates that in the adipogenic differentiation program, Prmt5 appears to have a dual role as both repressor and activator. Prior to adipocyte marker activation, Prmt5 is required to suppress lipogenic transcripts since a knockdown of Prmt5 protein levels results in precocious lipid accumulation and lipid biosynthetic gene induction. Association of Prmt5 at lipogenic gene regulatory sequences was observed in concert with dimethylated histones. In contrast, during the differentiation process, Prmt5 binds to and is responsible for dimethylating histones at adipogenic promoter regions. Reductions in Prmt5 and dimethylated histones are followed by diminished Brg1 binding at these promoters and ultimately elicit a significant decrease in adipocyte gene transcription, indicating that Prmt5 is also an activator.

Further, evidence indicates that MyoD and myogenin have distinct roles at specific promoters, such that MyoD is required for robust activation of early transcripts while myogenin is necessary for enhancement of late gene expression. Thus the interaction between Prmt5 and MyoD may dictate which subset of promoter sequences are directly regulated by Prmt5, specifically that Prmt5 is recruited by MyoD and is functionally required for early gene transcription events but is involved in repressing precocious activation of late muscle transcripts.

We have shown that Carm1 at late myogenic targets occurs concomitantly with the onset of gene expression. Others have demonstrated that transcription of late muscle-specific targets is accompanied by concurrent binding of myogenin, Mef2d1b and Brg1 (157). A specific interaction has been shown to exist between Carm1 and Mef2 family

members but not MyoD or myogenin (35), though in our hands Carm1 is able to interact with Mef2D and myogenin. The muscle-specific isoform Mef2d1b has been shown to synergistically activate late myogenic gene expression in combination with Brg1 and myogenin as ChIP analysis places these proteins at late muscle-specific promoter sequences concurrently with gene activation (157). The nucleosomal methylosome activator complex (NUMAC) is a multi-protein complex which contains Carm1 and Brg1, and is assembled at the regulatory regions of activated estrogen-dependent genes (237). These findings suggest that Mef2-mediated targeting of Carm1 influences the subset of genes that are regulated by this PRMT. Furthermore, as we have shown that no interaction exists between Carm1 and MyoD, this is a key element in distinguishing the function of Carm1 at the regulatory sequences of muscle genes, from that of Prmt5, which depends greatly on MyoD for the specificity of its interactions with muscle specific cis-elements. Therefore, we have shown that these Prmts posses unique interactions with MRFs, that themselves have distinct transactivation functions during the different stages of muscle differentiation. The binding of Prmt5 or Carm1 with their respective partners and any associated complexes will define their functional status at early and late stages of muscle differentiation.

Our results show that late myogenic gene activation is ablated in cells where Carm1 is absent. Strangely, the knock out mouse model does not present with any overt skeletal muscle phenotypes though there do appear to be defects in adipocyte gene expression (239). Histological analysis of wild type and null Carm1 MEFs reveal no major structural defects though reduced muscle fiber density was observed in conjunction

with reduced individual myotube size and skeletal muscle presented with centrally localized nuclei indicating newly synthesized or repaired fibers reminiscent of regenerating muscle.

Muscle regeneration is characterized by 2 phases: degenerative and regenerative. The initial degenerative phase is accompanied by activation of mononucleated cells, principally inflammatory cells and myogenic cells (182, 218). It has been postulated that factors released by injured muscle cells activate resident inflammatory cells causing them to signal circulatory inflammatory cells which subsequently activate myogenic cells (71, 162). Muscle degeneration is then followed by activation of muscle repair processes characterized by increased cellular proliferation (180, 223, 226). Activated myogenic cells serve as sources of new myonuclei which fuse to new or existing myofibers during the repair process (31, 85). Newly formed myofibers can be identified morphologically by their small caliper and centrally localized nuclei, similar to what was observed by histology. These findings suggest that there some defect exists during terminal differentiation or fusion of newly regenerated myofibers, but does not affect whole tissue function in the animal.

Though others have shown defects in muscle-specific gene expression when Carm1 was reduced the mouse model dictates that Carm1 is dispensable for this process. It is apparent that some compensatory mechanism exists *in vivo* to complement the absence of Carm1. It is possible that other PRMTs may be functionally redundant. Studies knocking down both Prmt5 and Carm1 specifically in skeletal muscle tissue, would be useful in determining if functional overlap exists between these proteins,

though there is no precedence to implicate Prmt5 functionally compensates for the lack of Carm1.

Our findings have demonstrated that PRMT function is required to facilitate ATP-dependent chromatin remodeling during myogenic differentiation. Another caveat is promoter specificity is conferred by association of these Prmts with distinct MRFs and these interactions will ultimately determine the functional status of these Prmts in the context of gene activation. We have focused on molecular events necessary to elicit transcription activation during differentiation. As we have seen binding of Prmt5 and Carm1 *in vivo,* it would be intriguing to examine if either PRMT was involved in the activation and self-renewal process of satellite or possibly the repair and regeneration dynamics of myofibers. It would also be of interest to determine the role of PRMTs during higher order chromatin reorganization events in response to differentiation signals as the role of histone modifying proteins remains largely unexplored. It is apparent that much more remains to be elucidated concerning the role of arginine methylation during cellular differentiation events.

Appendix A

Brg1 is Required for Transcription Regulation of Neuromuscular Junction Gene Activation

Exquisite temporal regulation of transcription activation is a hallmark of cell differentiation processes. Many reports have demonstrated that lineage specific transcription factors cooperate with chromatin remodeling enzymes, specifically ATP-dependent nucleosome remodelers and histone-modifying enzymes, to initiate gene induction. Using a cell line capable of tetracycline-inducible expression of dominant negative Brg1, the catalytic subunit of the Swi/Snf enzyme, we examine the role of ATP-dependent chromatin remodeling enzymes during activation of neuromuscular junction target genes such as acetylcholine receptor (AChR) •, ß, •, and muscle specific kinase (MuSK). Subsets of myogenic targets can be activated by distinct muscle regulatory factors (MRFs) and recent work has shown that the combination of myogenin & Mef2d1b is sufficient to activate late stage muscle-specific genes as well as NMJ transcripts. Chromatin immunoprecipitation (ChIP) conducted using Brg1 antisera indicate that Brg1 is indeed present at the regulatory sequences of these loci. Expression of dominant-negative Brg1 impedes the ability of Swi/Snf to bind at these promoter regions, and corresponds to a failure to activate NMJ gene transcription. Analysis of chromatin structure by restriction accessibility assay in the absence of Swi/Snf confirmed that chromatin at NMJ gene loci was not remodeled and remained in an inaccessible and therefore repressive state. To elucidate the relationship between Brg1 and MRFs during NMJ gene regulation *in vivo,* ChIPs were performed using samples isolated from skeletal

muscle adult mice to analyze the binding patterns of these proteins. Interestingly, MyoD was enriched at NMJ regulatory sequences in satellite cells but not in mature myofibers. In contrast, myogenin, which has been shown to be sufficient to activate late myogenic gene targets and NMJ transcripts, was robustly localized to NMJ promoter regions in myofibers but not in satellite cells. Brg1 was present at NMJ loci in both satellite cells and myofibers. As NMJ target genes are only induced following terminal differentiation of the myofiber, these results suggest that myogenin specifically targets the activity of Swi/Snf during the later stages of myogenic differentiation, specifically to regulate genes required for neuromuscular junction formation.

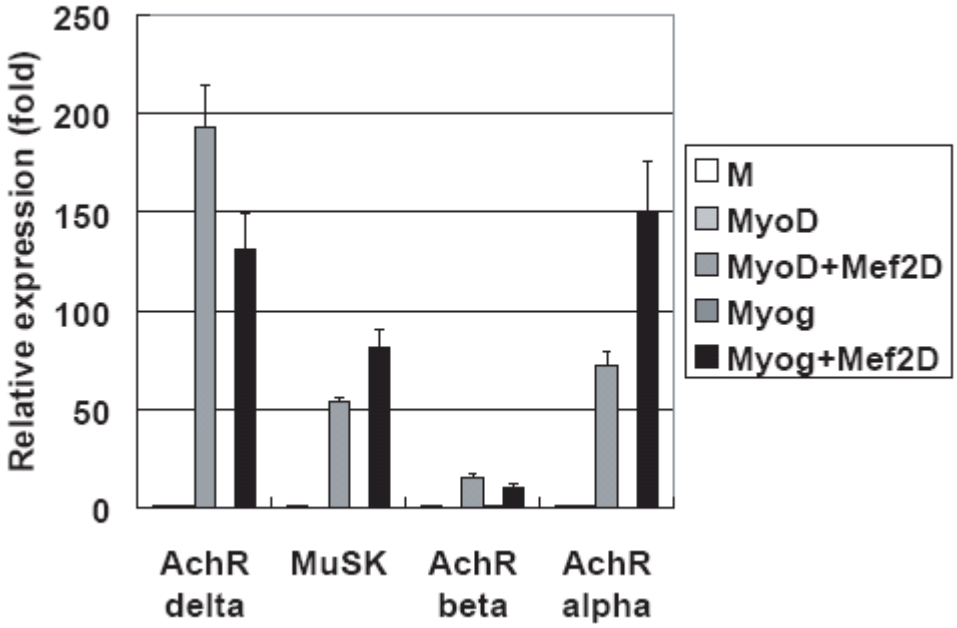
250
200
150
100
50
0
Relative expression (fold)
M
MyoD
MyoD+Mef2D
Myog
Myog+Mef2D
AchR delta
MuSK
AchR beta
AchR alpha

Figure A1.

Figure A1.

Skeletal muscle differentiation was induced in cells by the exogenous expression of myogenic regulatory factors (MRFs); specifically, NIH 3T3 fibroblasts were infected with retrovirus encoding MyoD, myogenin or the muscle specific isoform Mef2d1b. Cells were mock differentiated with the empty retroviral vector as a negative control. Robust transcription activation of neuromuscular junction (NMJ) gene targets, such as acetylcholine receptor (AChR) •, ß, • or muscle-specific kinase (MuSK), was observed in cells that were differentiated with MyoD & Mef2d1b or Myogenin & Mef2d1b. NMJ transcripts were undetectable in cells differentiated using MyoD or myogenin alone, indicating the activity of the MRF myogenin, in combination with the muscle specific coactivators Mef2d1b, is sufficient to induce NMJ gene activation. As expected, levels of NMJ transcripts were undetectable in mock-differentiated samples.

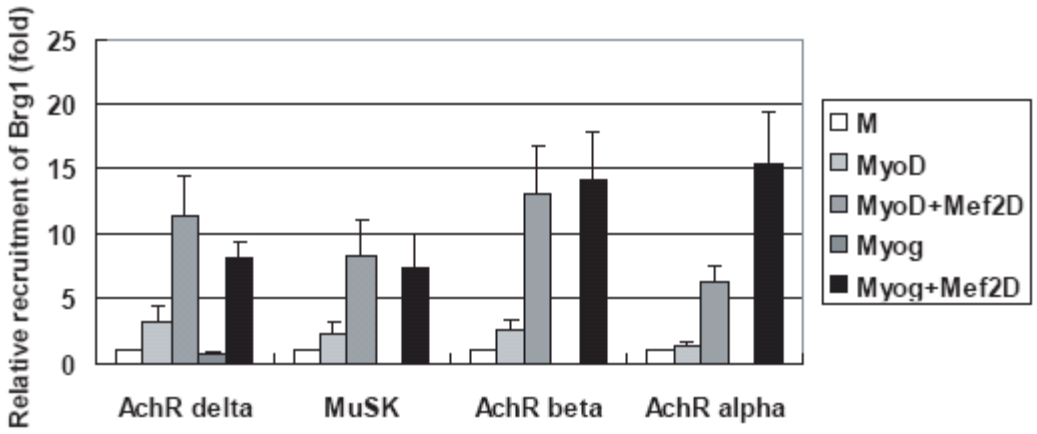
Relative recruitment of Brg1 (fold)
25
20
15
10
5
0
AchR delta
MuSK
AchR beta
AchR alpha
M
MyoD
MyoD+Mef2D
Myog
Myog+Mef2D

Figure A2.

Figure A2.

Cellular differentiation was achieved by the exogenous expression of MyoD, myogenin, Mef2d1b, and combinations of MyoD & Mef2d1b, or myogenin & Mef2d1b. Mock-differentiated cells were used as a negative control. Samples were crosslinked and chromatin immunoprecipitation was performed as described previously. Brg1-antisera was used to immunoprecipitate Brg1-containing complexes present at the gene regulatory regions of NMJ targets acetylcholine receptor (AChR) •, ß, • and muscle-specific kinase (MuSK). Binding of Brg1 was significantly enriched at the regulatory regions of all NMJ target genes analyzed in cells differentiated using MyoD & Mef2d1b or myogenin & Mef2d1b, indicating that the binding of Brg1 occurs in a myogenin & Mef2d1b-dependent fashion and coincides with transcription activation.

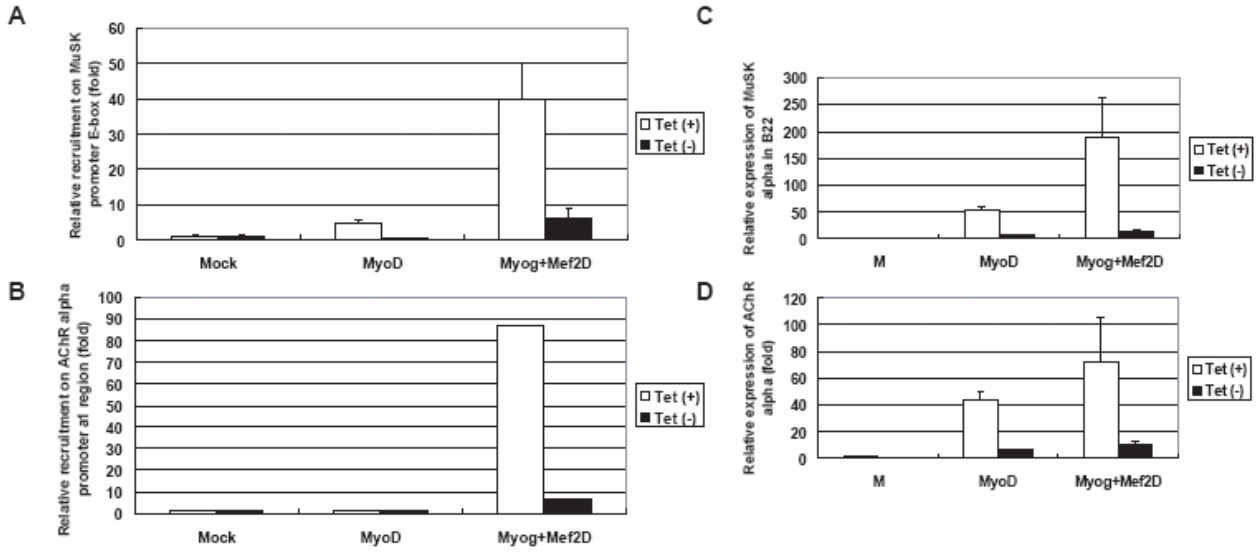
A
Relative recruitment on MuSK promoter E-box (fold)
60
50
40
30
20
10
0
Mock
MyoD
Myog+Mef2D
Tet (+)
Tet (-)
B
Relative recruitment on AChR alpha promoter at region (fold)
100
90
80
70
60
50
40
30
20
10
0
Mock
MyoD
Myog+Mef2D
Tet (+)
Tet (-)
C
Relative expression of MuSK alpha in B22
300
250
200
150
100
50
0
M
MyoD
Myog+Mef2D
Tet (+)
Tet (-)
D
Relative expression of AChR alpha (fold)
120
100
80
60
40
20
0
M
MyoD
Myog+Mef2D
Tet (+)
Tet (-)

Figure A3.

Figure A3.

As described previously, modified NIH 3T3 fibroblasts were maintained in the presence of tetracycline to prevent the expression of dominant negative Brg1, while the removal of tetracycline permits the expression of dominant negative Brg1. Differentiation was accomplished by retroviral infection with vectors encoding MyoD or myogenin & Mef2d1b. Cells were also mock-differentiated using empty retroviral vector. Samples were harvested for ChIP and gene expression analysis via quantitative PCR (Q-PCR). Brg1-antisera was used to immunoprecipitate Brg1-containing complexes present at the promoter sequences of NMJ loci AChR • and MuSK. Significant enrichment of Brg1 binding was observed at these regulatory regions in cells differentiated using the combination of myogenin & Mef2d1b. Notably, association of Brg1 with NMJ promoters was reduced in cells expressing dominant negative Brg1. However, no appreciable binding was seen in MyoD-differentiated samples. As expected, no significant binding was seen in mock-differentiated cells. Analysis of gene expression indicates that transcription activation of NMJ targets is heavily reduced in cells expressing dominant-negative Brg1, indicating that the binding and ATP-ase activity of Brg1 are required to elicit gene activation of NMJ genes.

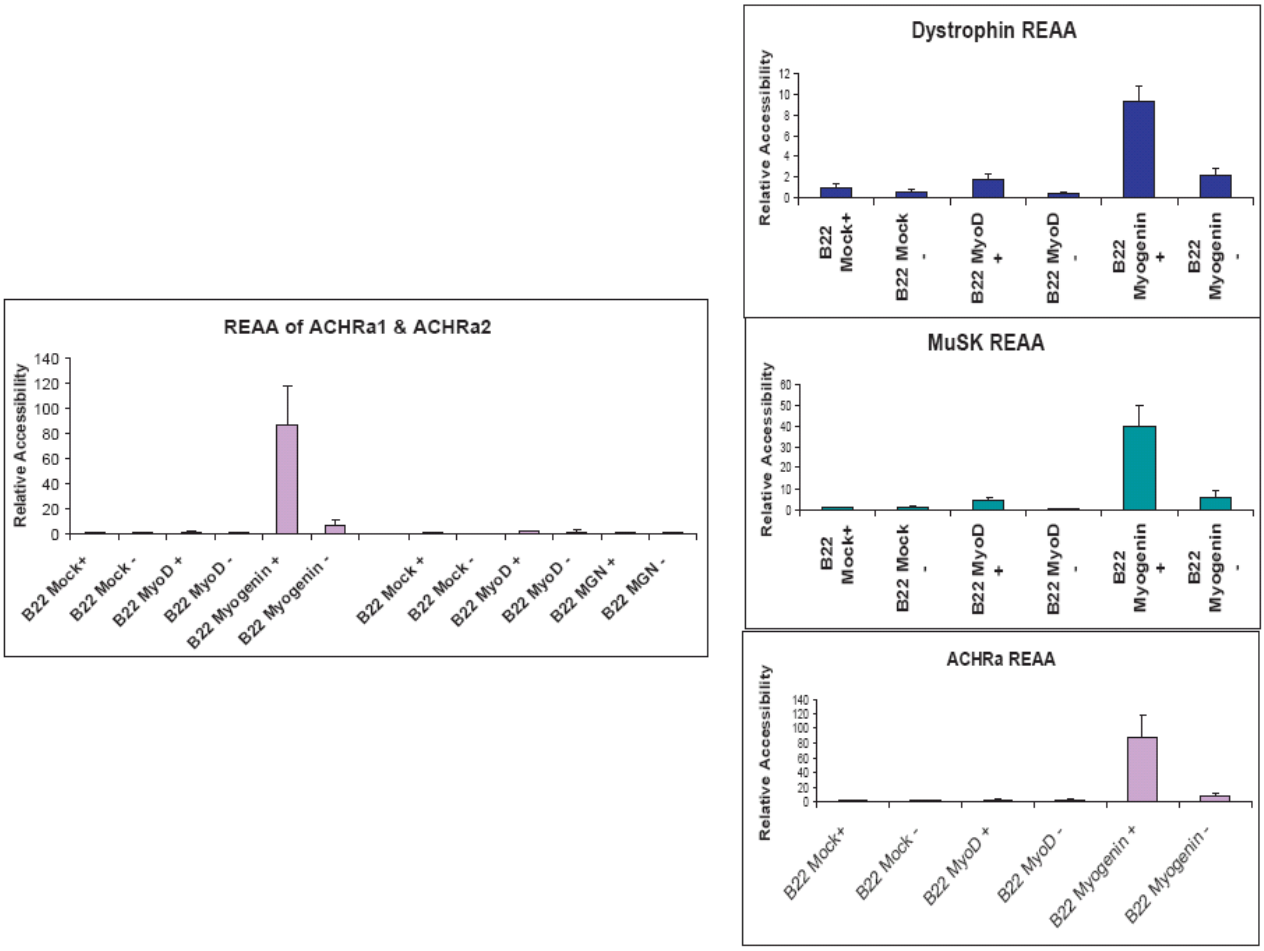
REAA of ACHRa1 & ACHRa2
Relative Accessibility
140
120
100
80
60
40
20
0
B22 Mock+
B22 Mock -
B22 MyoD +
B22 MyoD -
B22 Myogenin +
B22 Myogenin -
B22 Mock +
B22 Mock -
B22 MyoD +
B22 MyoD -
B22 MGN +
B22 MGN -
Dystrophin REAA
Relative Accessibility
12
10
8
6
4
2
0
B22 Mock+
B22 Mock -
B22 MyoD +
B22 MyoD -
B22 Myogenin +
B22 Myogenin -
MuSK REAA
Relative Accessibility
60
50
40
30
20
10
0
B22 Mock+
B22 Mock -
B22 MyoD +
B22 MyoD -
B22 Myogenin +
B22 Myogenin -
ACHRa REAA
Relative Accessibility
140
120
100
80
60
40
20
0
B22 Mock+
B22 Mock -
B22 MyoD +
B22 MyoD -
B22 Myogenin +
B22 Myogenin -

Figure A4.

Figure A4.

The state of chromatin at NMJ loci was assessed using restriction enzyme accessibility assay (REAA) in differentiated cells in the presence and absence of tetracycline. Chromatin was highly accessible in cells differentiated using myogenin & Mef2d1b. However, nuclease accessibility was diminished in cells expressing dominant negative Brg1. No detectable increase in chromatin accessibility was observed in MyoD-differentiated or mock-differentiated cells. The ATP-ase activity of Brg1 is required for the alteration of chromatin structure at NMJ loci in order to promote transcription activation. The values represent data from 3 independent experiments.

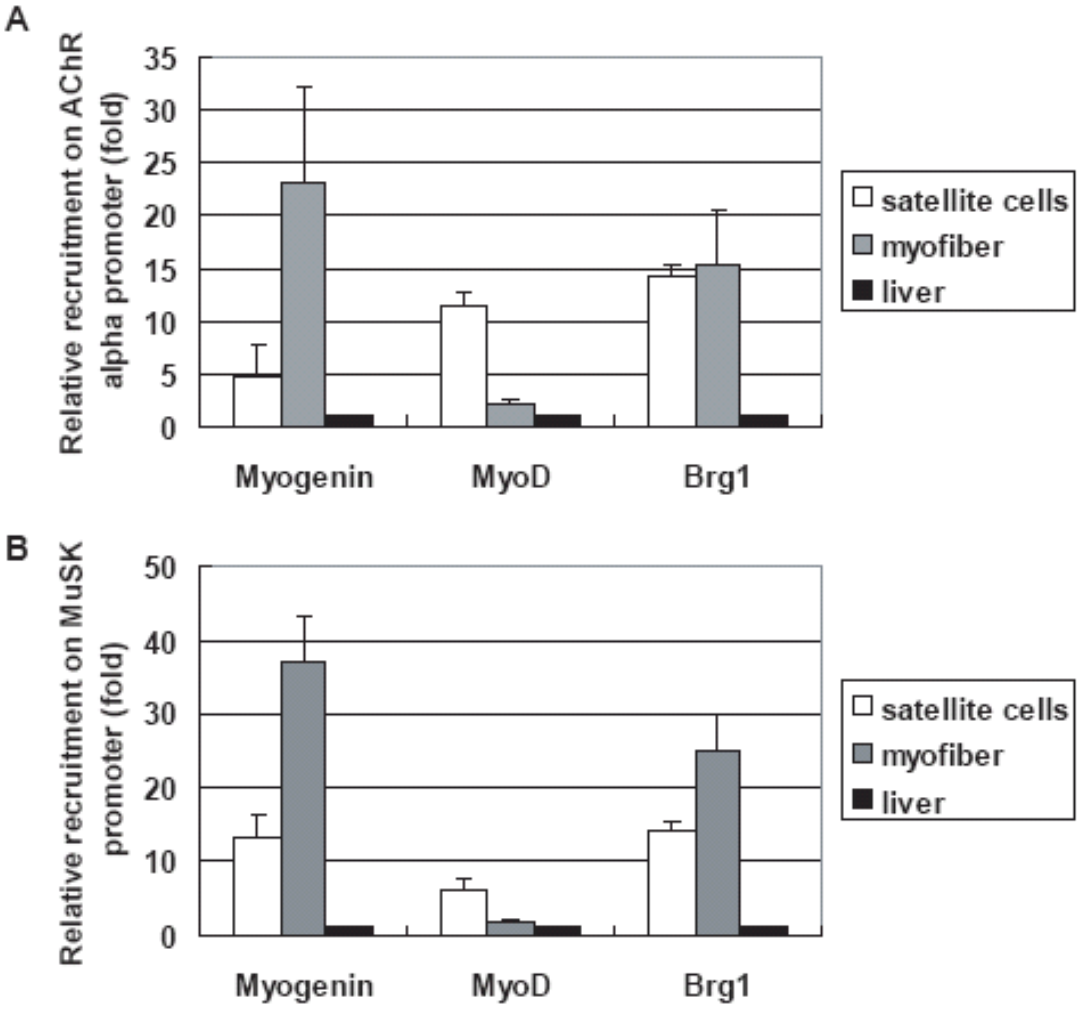
A
Relative recruitment on AChR alpha promoter (fold)
35
30
25
20
15
10
5
0
Myogenin
MyoD
Brg1
satellite cells
myofiber
liver
B
Relative recruitment on MuSK promoter (fold)
50
40
30
20
10
0
Myogenin
MyoD
Brg1
satellite cells
myofiber
liver

Figure A5.

Figure A5.

Skeletal muscle tissue isolated from mice was used to purify a population of satellite cells and myofibers, which were then prepared for ChIP analysis. Liver was isolated and used for negative control. Antibodies recognizing myogenin, MyoD and Brg1 were used to immunoprecipitate DNA sequences bound by these proteins. MyoD-binding was increased at the regulatory regions of NMJ genes in satellite cells but not in myofibers or liver. However, myogenin binding was robustly induced at promoter sequences of NMJ loci in myofibers but not in satellite cells or liver. Brg1 was present at these promoter regions in both satellite cells and myofibers but not in liver. The distinct binding pattern of MyoD and myogenin indicates that both MRFs contribute to the regulation of NMJ target genes but myogenin is the primary transcription factor responsible for directing transcription activation in a Brg1 dependent manner.

Appendix B

Comparative *in silico* analysis identifies bona fide MyoD binding sites within the Myocyte Stress 1 gene promoter

The molecular mechanisms governing the regulation of skeletal muscle specific expression of Myocyte stress I (MSI), a striated muscle actin binding protein required for the activity of the myocardin related transcription factor (MRTF)/serum response factor (SRF), are largely unknown. Analysis of the regulatory sequences of MS1 was conducted in C2C12 myoblasts and using *in silico* comparative techniques. Our contributions included a time course of myoblasts and differentiated C2C12 cells that were used to show that MS1 gene expression was upregulated in a differentiation-specific manner in C2C12 muscle cells. *In silico* analysis of the promoter sequences within the 1.5kbp residing 5' upstream of the start site revealed two conserved myogenic regulatory domains. Cotransfection of C2C12 muscle cells with plasmids over-expressing MRFs and *ms1* promoter driven luciferase reporter indicated that MS1 was transactivated by MyoD. Mutagenesis and EMSA demonstrated that MyoD binding occurred at two distinct E-boxes, E1 and E2. We also demonstrated, through chromatin immunoprecipitation analysis, binding of MyoD at these respective E-boxes corresponded with the onset of gene expression. Thus tissue specific and differentiation dependent induction of *ms1* mRNA is mediated by temporal binding of MyoD at distinct evolutionary conserved E-boxes within 1.5kbp 5' upstream of the start site.

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Research article

Comparative *in silico* analysis identifies *bona fide* MyoD binding sites within the Myocyte Stress 1 gene promoter

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Abstract

Background: Myocyte stress 1 (MS1) is a striated muscle actin binding protein required for the muscle specific activity of the evolutionary ancient myocardin related transcription factor (MRTF)/serum response factor (SRF) transcriptional pathway. To date, little is known about the molecular mechanisms that govern skeletal muscle specific expression of MS1. Such mechanisms are likely to play a major role in modulating SRF activity and therefore muscle determination, differentiation and regeneration. In this study we employed a comparative *in silico* analysis coupled with an experimental promoter characterisation to delineate these mechanisms.

Results: Analysis of MS1 expression in differentiating C2C12 muscle cells demonstrated a temporal differentiation dependent up-regulation in *ms1* mRNA. An *in silico* comparative sequence analysis identified two conserved putative myogenic regulatory domains within the proximal 1.5 kbp of 5' upstream sequence. Co-transfecting C2C12 myoblasts with *ms1* promoter/luciferase reporters and myogenic regulatory factor (MRF) over-expression plasmids revealed specific sensitivity of the *ms1* promoter to MyoD. Subsequent mutagenesis and EMSA analysis demonstrated specific targeting of MyoD at two distinct E-Boxes (E1 and E2) within identified evolutionary conserved regions (ECRs, α and β). Chromatin immunoprecipitation (ChIP) analysis indicates that co-ordinated binding of MyoD at E-Boxes located within ECRs α and β correlates with the temporal induction in *ms1* mRNA.

Conclusion: These findings suggest that the tissue specific and differentiation dependent up-regulation in *ms1* mRNA is mediated by temporal binding of MyoD at distinct evolutionary conserved E-Boxes within the *ms1* 5' upstream sequence. We believe, through its activation of *ms1*, this is the first study to demonstrate a direct link between MyoD activity and SRF transcriptional signalling, with clear implications for the understanding of muscle determination, differentiation and regeneration.

Background

During mammalian embryogenesis, the development of skeletal muscle is mediated by a co-ordinated series of events that begins with commitment of mesodermal precursor cells to the skeletal muscle lineage, followed by myoblast fusion and the subsequent progression of a programme of muscle specific gene expression [1-3]. A specialised group of transcription factors control this process of myogenic specification and differentiation. These factors, designated the myogenic regulatory factors (MRFs), include four basic helix-loop-helix (bHLH) E-Box binding proteins: MyoD, Myf5, Myogenin and MRF4 [4]. During development MyoD and Myf5 dictate myoblast specification while Myogenin and MRF4 regulate terminal differentiation [5,6]. In collaboration with the MRFs, the MADS-box myocyte enhancer factor (MEF) family of proteins contribute to the programme of muscle specific gene expression [7,8].

Serum response factor (SRF), a MADS box transcription factor related to the MEFs, also regulates skeletal muscle gene expression through binding of a DNA sequence known as the serum response element (SRE) or CArG box [9-11]. In addition to binding and regulating numerous muscle specific promoters [12,13], perturbation of SRF activity severely impairs myoblast fusion and differentiation [14-16]. Confirming an important role for myogenic SRF activity, a conditional skeletal muscle specific knockout of SRF results in severe skeletal muscle myopathy that results in perinatal lethality [17].

SRF activity is dependent on its interaction with a range of cell-type specific and signal responsive co-factors [18]. Myocardin, the founding member of a family of extraordinarily powerful myogenic SRF co-activators [19], has been shown to be necessary and sufficient for cardiac and smooth muscle specific gene expression [9,20,21]. Unlike myocardin, the myocardin-related transcription factors (MRTFs), MRTF-A (also known as MAL/MKL1/BASC) and MRTF-B (MKL2), are expressed in skeletal muscle in addition to multiple other cell types [22-24].

A requisite role for the MRTFs in skeletal muscle development has been inferred from experiments in cultured muscle cells, in which RNAi mediated knock-down of MRTF-A repressed SRF-dependent gene expression resulting in impaired myoblast fusion and subsequent formation of multinucleated myotubes [25]. Transgenic mice expressing a dominant-negative form of MRTF-A displayed a phenotype reminiscent of the skeletal muscle SRF knock out mice supporting an important role for the MRTFs in the control of muscle fiber growth and maturation [17]. In contrast to myocardin, which is constitutively nuclear, the MRTFs shuttle between the cytoplasm and the nucleus with nuclear accumulation required for SRF transactivation. Muscle specific mechanisms, which promote MRTF nuclear accumulation, represent important regulatory pathways in the process of myogenic differentiation via the MRTF/SRF signalling axis [9].

We, and others, have previously identified a novel striated muscle specific actin binding protein, myocyte stress 1 (MS1, also known as STARS) [26,27] which has the ability to synergistically activate SRF-dependent transcription through a Rho-A dependent mechanism. Kuwahara and colleagues subsequently demonstrated that STARS (mouse homologue of MS1) activates SRF dependent transcription by inducing the nuclear accumulation of MRTF-A and -B through a Rho-A dependent mechanism [28]. STARS perturbation via RNAi resulted in a significant attenuation of muscle specific SRF activity suggesting that endogenous STARS is an important component of the muscle specific MRTF/SRF transcriptional pathway [28]. In support of this we have shown that ectopic MS1 expression results in an increased expression of characterised MRTF/SRF target genes (Koekemoer AL and Chong NW, unpublished) [29]. Interestingly, we have recently shown that morpholino knockdown of zebrafish *ms1* (*zms1*) resulted in severe musculoskeletal deformities with curvature and shortening of the longitudinal axis [30]. This data supports the previous studies and demonstrates that MS1 is a central component of the evolutionary ancient muscle specific MRTF/SRF signalling axis.

Despite the important role of MS1 in skeletal muscle formation and function, little is known about the molecular mechanisms governing its expression. The proximal 1.5 kbp 5'-flanking sequence has recently been shown to be able to direct LacZ expression in adult cardiac and skeletal muscle, with two MEF2 responsive motifs within the proximal flanking sequence essential for the observed cardiac specificity [31]. However, the factors, motifs and regulatory mechanisms governing the skeletal muscle specific expression profile remain unknown. Understanding such mechanisms will give us an exquisite insight into how the MRTF/SRF signalling axis is regulated during myogenesis in addition to expanding our knowledge of the genetic circuits involved in mygenic differentiation.

In this study we investigated the transcriptional regulation of the *ms1* gene during myogenic differentiation using the C2C12 myoblast cell line as an established model system. We have shown that two myogenic E-Boxes, located within evolutionary conserved regions in the *ms1* promoter, play distinct roles in recruiting MyoD and subsequently activating the *ms1* promoter during myogenic differentiation.

Results

Ms1 *transcript is differentially expressed during myogenic differentiation*

Ms1 expression is restricted to striated muscle with early developmental expression during myogenesis in both vertebrate and invertebrate models [27,30]. In order to evaluate *ms1* expression during myoblast differentiation *in vitro*, cultured C2C12 cells, a myoblast cell line established from the leg muscle of the C3H mouse [32], were used. C2C12 is a myoblast cell line, which remains proliferative in the presence of high concentrations of fetal bovine serum. Upon serum depletion, these myoblasts differentiate and fuse with each other into myotubes [33]. Quantitative RT-PCR was conducted using 1 μg of total RNA obtained from subconfluent C2C12 myoblasts (MB) and from C2C12 myotubes differentiated for 3 days (MT). As shown in Figure 1A, there is a significant increase in *ms1* transcript in differentiated C2C12 cells compared to confluent proliferating C2C12, suggesting a differentiation dependent up-regulation of *ms1* mRNA.

The temporal expression profile of specific genes expressed during myogenic differentiation can give us an exquisite insight into both the function of the gene and the regulatory processes governing its expression [34,35]. We have therefore measured the temporal expression profile of *ms1* transcript during the controlled differentiation of C2C12 myoblasts over a three-day period. RT-PCR using RNA isolated on consecutive days during differentiation shows that *ms1* transcript is significantly induced within the first day of the differentiation process with a maintained increase in expression over the subsequent three days (Figure 1B). *Ms1* can thus be regarded as an early wave myogenic transcript [36], with this temporal profile having implications for its role and regulation during myogenic differentiation.

Analysis of the ms1 *5' upstream DNA sequence*

The transcriptional up-regulation of *ms1* during myogenic differentiation suggests its expression might be targeted by differentiation promoting transcription factors. During myogenesis, differentiation is under the control of E-Box binding myogenic basic helix-loop-helix (MyoD, Myogenin, Myf4 and Myf5) and MADS domain Mef2 proteins [37], with all myogenic genes containing binding motifs for these transcription factors in their promoters and associated regulatory loci. We therefore, by means of comparative sequence analysis, proceeded to analyse the rat *ms1* 5' upstream sequence for enrichment of myogenic transcription factor binding motifs. Using the VISTA software [38] we compared 5 kbp of 5' upstream sequence from rat, with the orthologous locus in human. This compares sequences that shared a common ancestor over 60 million years ago and it has been shown empirically that conserved non-coding sequences, also known as evolutionary conserved regions (ECRs), identified between these spe-

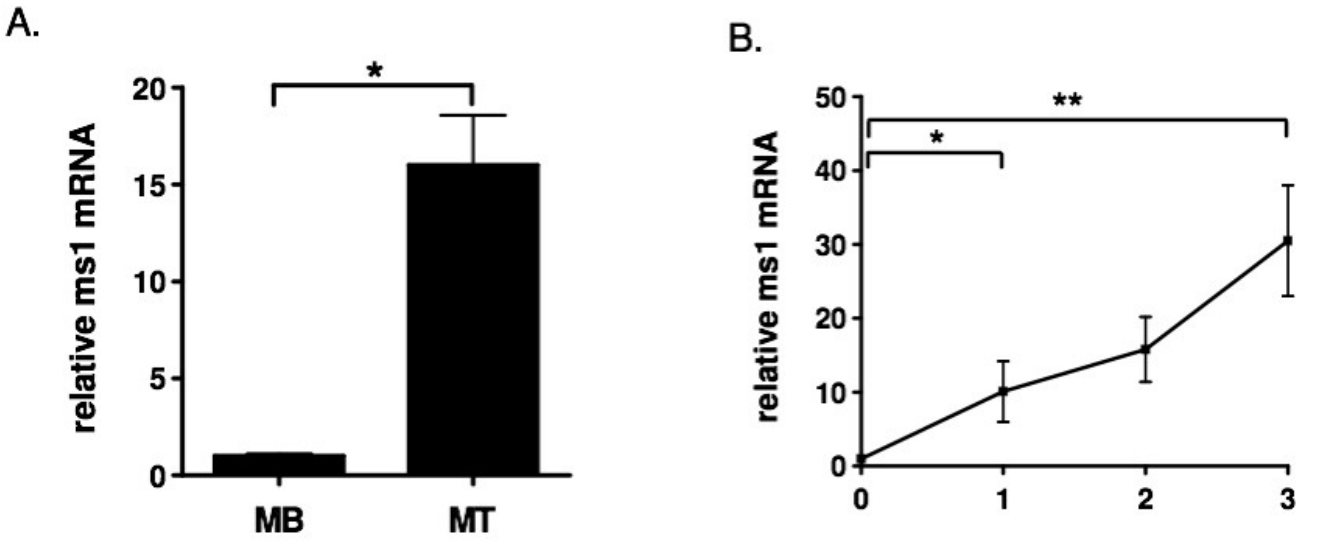

Figure 1

Quantitative real-time PCR analysis of *ms1* gene expression during the myogenic differentiation of C2C12 cells. (A) Total RNA was isolated from C2C12 sub-confluent myoblasts (MB) and myotubes (MT: 3 days post differentiation). The RNA was subjected to reverse transcription followed by quantitative PCR with mouse *ms1* and EF1α specific primers. (B) Total RNA was isolated from C2C12 cells during myogenic differentiation (day 0 to day 3) and subjected to quantitative PCR. Expression level at day 0 was arbitrarily set at 1. Values are represented as means ± SE of at least three different experiments. (*P < 0.05, **P < 0.05).

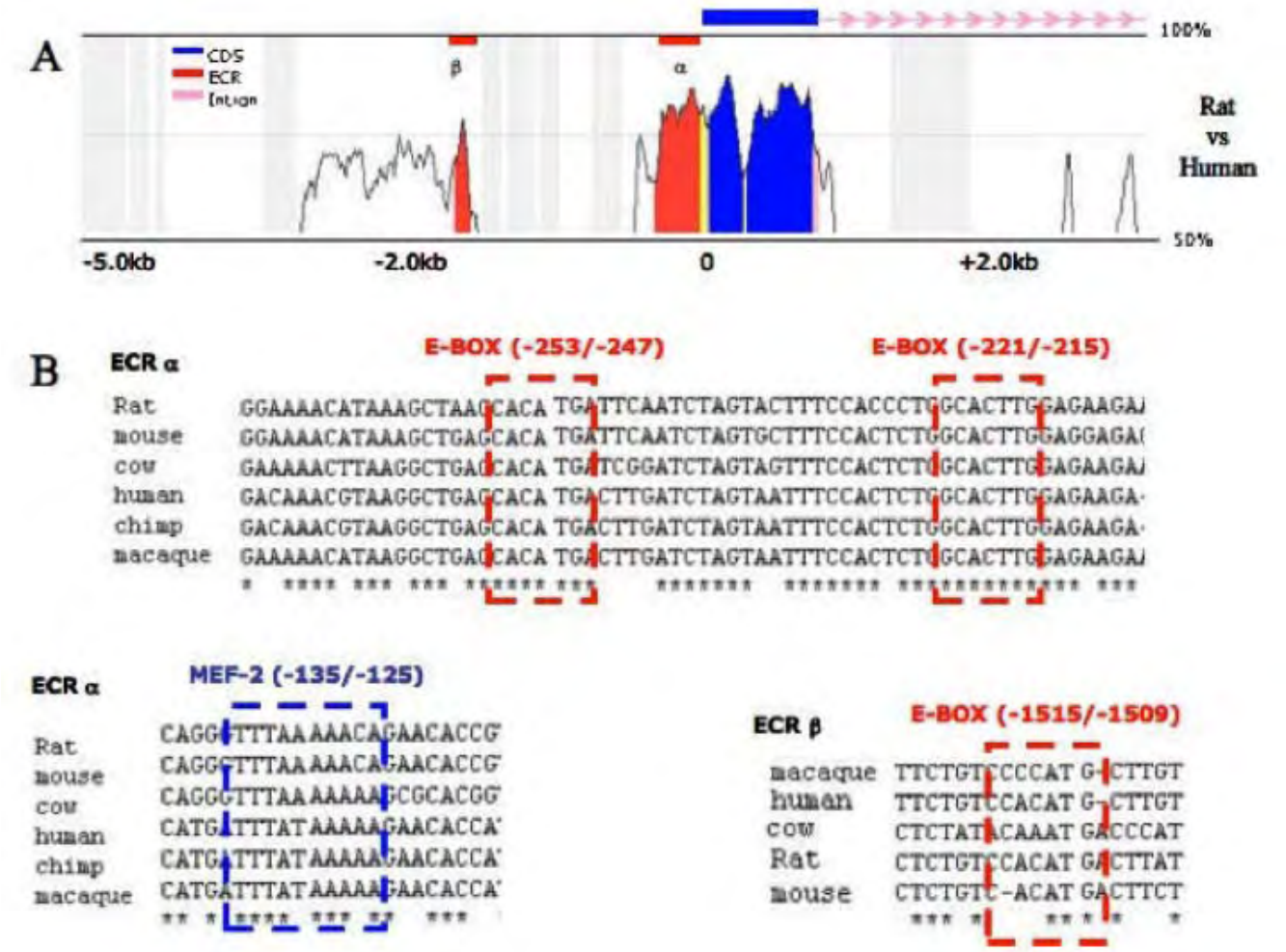

Figure 2

***in silico* analysis of *ms1* putative promoter**. (A) Phylogenetic comparative analysis of *ms1* 5'-region from *Homo sapiens* and *Rattus norvegicus* (reference sequence), as obtained by VISTA, setting ECR at ≥ 100 bp and conservation at ≥ 80%. (B) Alignment of ECR α and β, containing several putative transcription factor-binding sites, in *Rattus norvegicus, Mus musculus, Bos taurus, Homo sapiens, Pan troglodytes and Macaca mulatta*. Sequences were obtained from the ENSEMBL genome database and aligned using CLUSTAL W.

cies represent ideal candidates as functional transcription factor binding motifs and regulatory domains [39].

Our *in silico* analysis (identifying sequences with 80% conservation over a minimum of 100 bp window, Figure 2A) identified two ECRs, α and β (represented in red), within the flanking 5 kbp of 5' upstream sequence. ECR a encompassed the proximal 400 bp upstream of the transcription start site (TSS) and we propose represents the proximal promoter. Within this proximal ECR a putative TATA box (TATT) was found with optimal interspacing from TSS [40], hence suggesting that this constitutes the core promoter. Of particular interest, two E-Box sequences (-253/-247 bp and -221/-215 bp) and a Mef2 motif (-135/-125) were identified within this ECR. Further sequence comparisons, aligning orthologous sequences obtained from ENSEMBL, confirms full sequence conservation of these identified myogenic motifs across multiple species (*Rattus norvegicus, Mus musculus, Bos taurus, Homo sapiens, Pan troglodytes, Macaca mulatta*), supporting a conserved functional role for these motifs (Figure 2B).

In addition to the proximal ECR, a fully conserved E-Box was also identified in the distal ECR β, located 1.5 kbp upstream of the TSS (Figure 2A). This ECR may represent a skeletal muscle enhancer, with such enhancer's common upstream of other myogenic genes including desmin and muscle creatine kinase [41,42]. In summary our com-

parative analysis suggests that the *cis* hardwiring required for myogenic specific expression is contained within the 2 kbp 5'-upstream sequence with myogenic motifs identified in ECRs α and β.

Cell specific activity of the ms1 *promoter*

On the basis of the comparative *in silico* analysis, a 1645 bp fragment of rat genomic DNA (-1585/+60) was obtained by PCR. This DNA fragment, encompassing the α and β ECRs, was sub-cloned into the promoterless pGL3 Basic reporter plasmid and the activity of the resulting P-

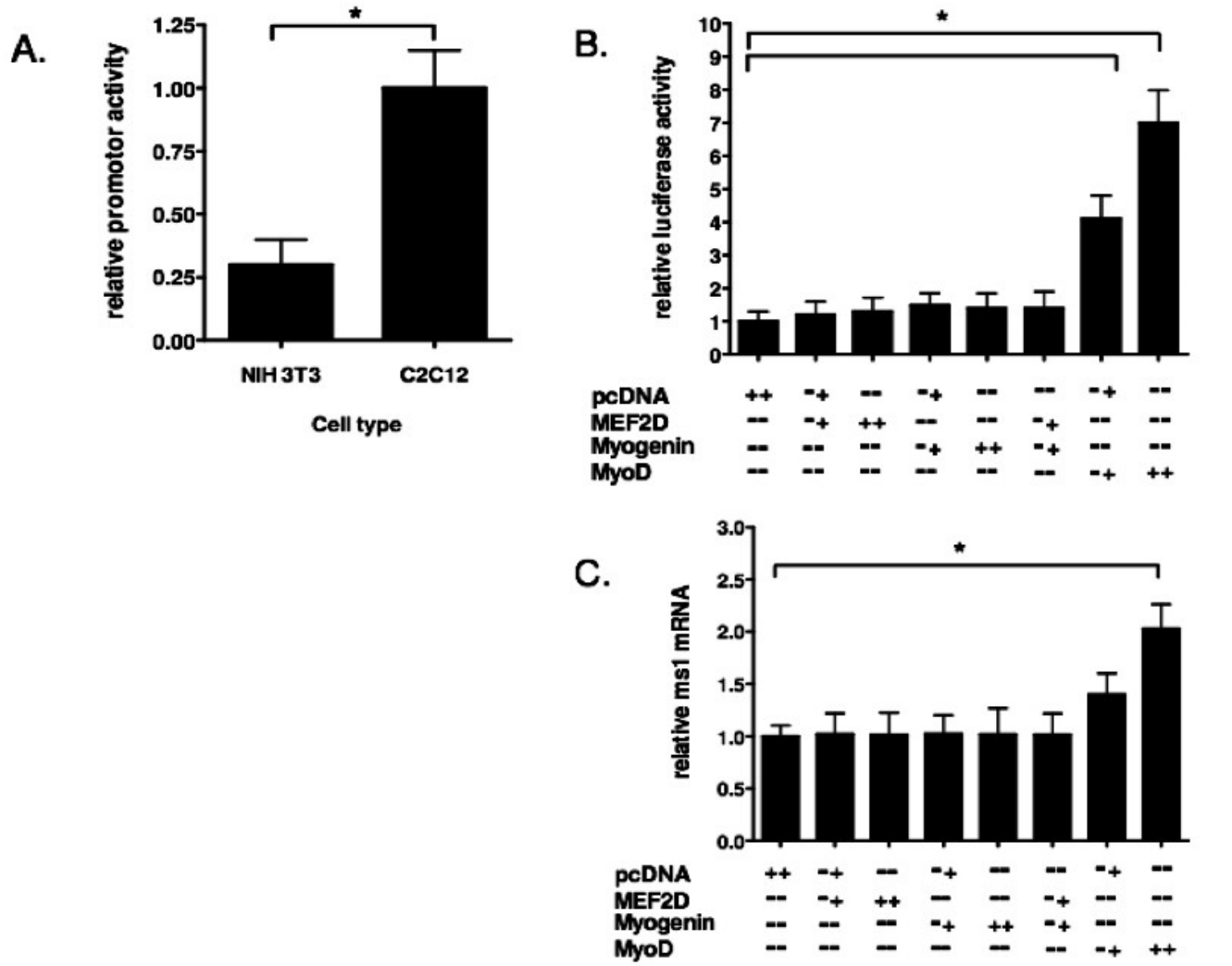

Figure 3

Analysis of *ms1* promoter function in myogenic cell lines. (A) Subconfluent NIH3T3 fibroblasts and C2C12 myoblasts were transiently transfected with a *ms1* promoter reporter construct spanning from -1585 relative to transcription start site and extending to nucleotide +60. Promoter fragment was cloned into the pGL3-Basic luciferase vector. Activity relative to pGL3-B alone was determined in each cell line 48 hours later, at which point the cells were still subconfluent. The relative activity of the promoter fragment (vs pGL3-B) in each cell line was then determined with relative activity in C2C12 assigned arbitrary value of one (B) Vectors (**+**:0.3 μg, **++**:0.6 μg) expressing Mef2D, myogenin and MyoD were co-transfected in combination with the *ms1* prompter reporter construct (P-1585/+60) into C2C12 myoblasts as described in (A). Luciferase activity in cells transfected with pcDNA and *ms1* promoter reporter (P-1585/+60) was arbitrarily set at 1 fold activation. (C) Subconfluent H9c2 myoblasts were transiently transfected with the MRF over-expression vectors (**+**:0.5 μg, **++**:1 μg). 48 hours post transfection, at which point the cells were 80% confluent, total RNA was isolated, reverse transcribed and the expression levels of TATA binding protein (TBP) and *ms1* were determined by real time PCR. *Ms1* expression in each sample was normalised to that of TBP. Statistically significant differences are indicated by *P < 0.05.

1585/+60 construct was analysed *in vitro*. The P-1585/+60 wild type promoter reporter (Figure 3A) is approximately four times more active in the C2C12 myoblasts than in the NIH 3T3 mouse fibroblasts. This data suggests that there is sufficient myogenic *cis* information encompassed within the promoter reporter to drive cell-specific activity in a myogenic cellular environment.

Myogenic factors can modulate the ms1 *promoter*

Since putative E-Box and Mef2 binding motifs are located within ECR α and β, the sensitivity of these motifs to the over-expression of their cognate binding proteins was determined. The rat *ms1* promoter (P-1585/+60) was transfected into C2C12 myoblasts in the presence or absence of the specific MRFs and Mef2D. In the presence of MyoD, *ms1* promoter activity was dose dependently increased seven fold compared with control (empty pcDNA3.1 vector). Myogenin alone or in combination with Mef2D, shown to synergistically activate other myogenic promoters [43], did not significantly activate the *ms1* promoter (Figure 3B). Mef2D alone had no overall activating effect on the *ms1* promoter.

To determine whether these effects were observed at the endogenous *ms1* promoter, MRF and Mef2D proteins were ectopically expressed in H9c2 cells, a rat myoblast cell line which can enter the skeletal muscle differentiation programme and expresses an array of skeletal muscle specific contractile and calcium handling proteins [44]. The expression level of endogenous *ms1* mRNA in both control (empty vector alone) and MRF transfected H9c2 cells was determined by quantitative real-time PCR. MyoD over-expression (1.0 μg) significantly increased *ms1* mRNA levels by two fold (Figure 3C). Myogenin and Mef2D alone, or in combination, had no effect on endogenous levels of *ms1* transcript, suggesting MyoD is the primary myogenic activator of the endogenous *ms1* promoter in a myoblast cellular context. These results suggest that MyoD can target the *ms1* promoter to enhance its activity, both *in vitro* and *in vivo*.

Site directed mutagenesis of the ms1 *promoter*

MyoD activates target promoters via heterodimerisation with ubiquitous E2A proteins (E12, E47, E2-5, [45]), which allows a stable DNA binding complex to bind the E-Box sequence (consensus sequence, CANNTG). In order to asses the contribution of the three conserved E-Boxes (in ECR α and β) in mediating MyoD sensitivity, we executed site directed mutagenesis of their binding sites within the *ms1* promoter reporter construct (Table 1, Figure 4A).

Reporter constructs containing mutated sequences for the three E-Boxes, singular and in combination (Figure 4A), were co-transfected with the MyoD over-expression plasmid into C2C12 myoblasts. Mutating E1 (P-1585/+60ΔE1) reduced promoter sensitivity to ectopic MyoD expression by 50% (from 7- to 3.5-fold; Figure 4B), suggesting this E-Box (located in ECR β) is important for promoter sensitivity to MyoD. The additional mutation in E2 (P-1585/+60ΔE1/ΔE2) further attenuated promoter sensitivity to 2.5 fold (~67% decrease) (Figure 4B). The combined triple E-Box mutant (P-1585/+60ΔE1/ΔE2/ΔE3) did not result in a further decrease in promoter sensitivity to MyoD compared to the double mutant (Figure 4B). These results suggest that both E1 and E2, but not E3, are required for MyoD activation of the rat *ms1* promoter.

The putative TATA box (TATT) was also mutated exchanging the adenine, at second position for a guanine (TATT to TGTT). This resulted in a dramatic 95% decrease in promoter activity (Figure 4C) in C2C12 myoblasts. Comparable loss of activity was observed in H9c2 myoblasts and NIH 3T3 fibroblasts (data not shown) suggesting loss of activity is not cell type specific. This suggests that this proximal TATT sequence represents a *bona fide* TATA box.

In vitro *binding of MyoD at distal and proximal E-Box sequences*

The present mutagenesis analysis suggests that E1 and E2, but not E3, play an important role in mediating MyoD sensitivity to the rat *ms1* promoter. To further elucidate the biological importance of the E1 and E2 sequences, we synthesised specific oligonucleotides (Table 1) containing the E-Box elements present in ECR α and β, E1, E2 and E3. These digoxigenin (DIG)-labelled double-stranded oligonucleotides were then incubated with a cold MyoD binding consensus E-Box sequence control in EMSA experiments with whole cell extracts from C2C12 myoblasts. As shown in Figure 5, incubation of C2C12 whole cell extracts with both MyoD consensus and E1, E2 and E3 sequences results in a specific DNA-Protein band shift.

The E1 and E2 shifted bands were successfully competed with excess unlabelled MyoD consensus sequence, suggesting these probes were bound by MyoD protein. In contrast, E3 could not be competed with unlabelled excess of MyoD consensus suggesting other E-Box binding proteins are shifting E3 *in vitro*. All three E-Box shifted bands were competed with unlabelled excess of self, confirming shifted bands were specific to each sequence (data not shown).

In agreement with our mutagenesis analysis, our EMSA data suggests that MyoD can target E1 and E2, but not E3. Other E-Box binding proteins expressed in C2C12 myoblasts are able to bind E3. Future experiments are aimed at determining their identity.

Table 1: Oligonucleotide Sequences for PCR and EMSA*

Cloning PCR	
P-1585-SacI	5'-TATTCAATGCTTAGTCCTGC-3'
P+60-HindIII	5'-CCAAGCTTCAGGCTACCTGTTTCTTCTC-3'
Site Directed Mutagenesis PCR	
ΔT ATA Fw	5'-CACCCTTTCACACCCTGCTTCT**G**TTTAAATCCCAGGCAACTC-3'
ΔT ATA Rv	5 '-GAGTTGCCTGGGATTTAAA**C**AGAAGCAGGGTGTGAAAGGGTG-3'
ΔE1 Fw	5 '-CACTGAACAGGTGCTGTTTCTCTGTC**GTTAAG**ACTTATCCTTTCAG TTCTCTTAAAA-3'
ΔE1 Rv	5 '-TTTTAAGAGAACTGAAAGGATAAGT**CTTAAC**GACAGAGAAACAGCA CCTGTTCAGTG-3'
ΔE2 Fw	5'-CTTTCCACCCTGGC**G**C**GG**GGAGAAGAAAGGAG-3'
ΔE2 Rv	5'-CTCCTTTCTTCTCC**CC**G**C**GCCAGGGTGGAAAG-3'
ΔE3 Fw	5 '-CAAGGAAAACATAAAGCTAAGC**G**C**GG**GATTCAATCTAGTACTTC-3'
ΔE3 Rv	5 '-GAAGTACTAGATTGAATC**CC**G**C**GCTTAGCTTTATGTTTTCCTTG-3'
Quantitative PCR	
MS1 Fw	5'-GTGACAGCATAGACACAGAGGAC-3'
MS1 Rv	5'-CACTGCTGCCCACCTGCCTT-3'
EF1-α Fw	5'-AGCTTCTCTGACTACCCTCCACTT-3'
EF1-α Rv	5'-GACCGTTCTTCCACCACTGATT-3'
ChIP Quantitative PCR	
E1-1526	5'-CACATTTTTATCTGGTCTAATACACTG-3'
E1-1482	5'-ATTTTTAATAGAACTGAAAAGAGAAGTCA-3'
E2-281	5'-TAAGGTCAAGGAAAACATAAAGCTA-3'
E2-190	5'-ACGGATATGTTCCCTCCTCTCTC-3'
EMSA	
E-Consensus	5'-CCCTTGGAACATCTGTCGATGCTG-3'
E1-MS1	5'-TTCTCTGTCCACATGACTTATCCT-3'
E2-MS1	5'-ACCCTGGCACTTGGAGAAGAA-3'
E3-MS1	5'-AGCTAAGCACATGATTCAATC-3'

*Mutated oligonucleotides are indicated in bold and underlined.

Direct binding of MyoD to the endogenous ECRs within the ms1 *promoter*

Our results suggest that the E1 and E2 sites are essential for *ms1* reporter gene function. We then utilised chromatin immunoprecipitation (ChIP) to determine whether MyoD is physically recruited to the endogenous ECRs *in vivo*, and determine the temporal dynamics of MyoD recruitment during C2C12 differentiation.

An ECR-specific quantitative PCR (Figure 6A) was performed on formaldehyde-crosslinked, sheared chromatin isolated during C2C12 differentiation, which was immunoprecipitated with MyoD and IgG specific antibodies. As shown in Figure 6B, MyoD appears to be constitutively bound at the E2 domain during differentiation, thus MyoD binding precedes the induction of *ms1* transcript (Figure 1B). Interestingly MyoD is not bound to E1 until day 1 (Fig. 6C), which coincides with transcriptional induction of *ms1* during the differentiation process. A five-fold enrichment in relative binding of MyoD is present at day 1 compared to day 0, with this level of enrichment maintained at E1 during the subsequent three days. These data suggest that MyoD targets both the E1 and E2 domains *in vivo* during C2C12 differentiation. However, temporal binding at E1 coincides with differentiation dependent transcriptional induction. We speculate that the ECR β represents a differentiation-dependent skeletal muscle enhancer, with temporal binding required for differentiation-dependent transcriptional induction of the *ms1* promoter.

Discussion

Understanding the mechanisms through which SRF activity is regulated during myogenesis is important if we want to expand our knowledge of the gene regulatory pathways and networks that drive skeletal muscle determination

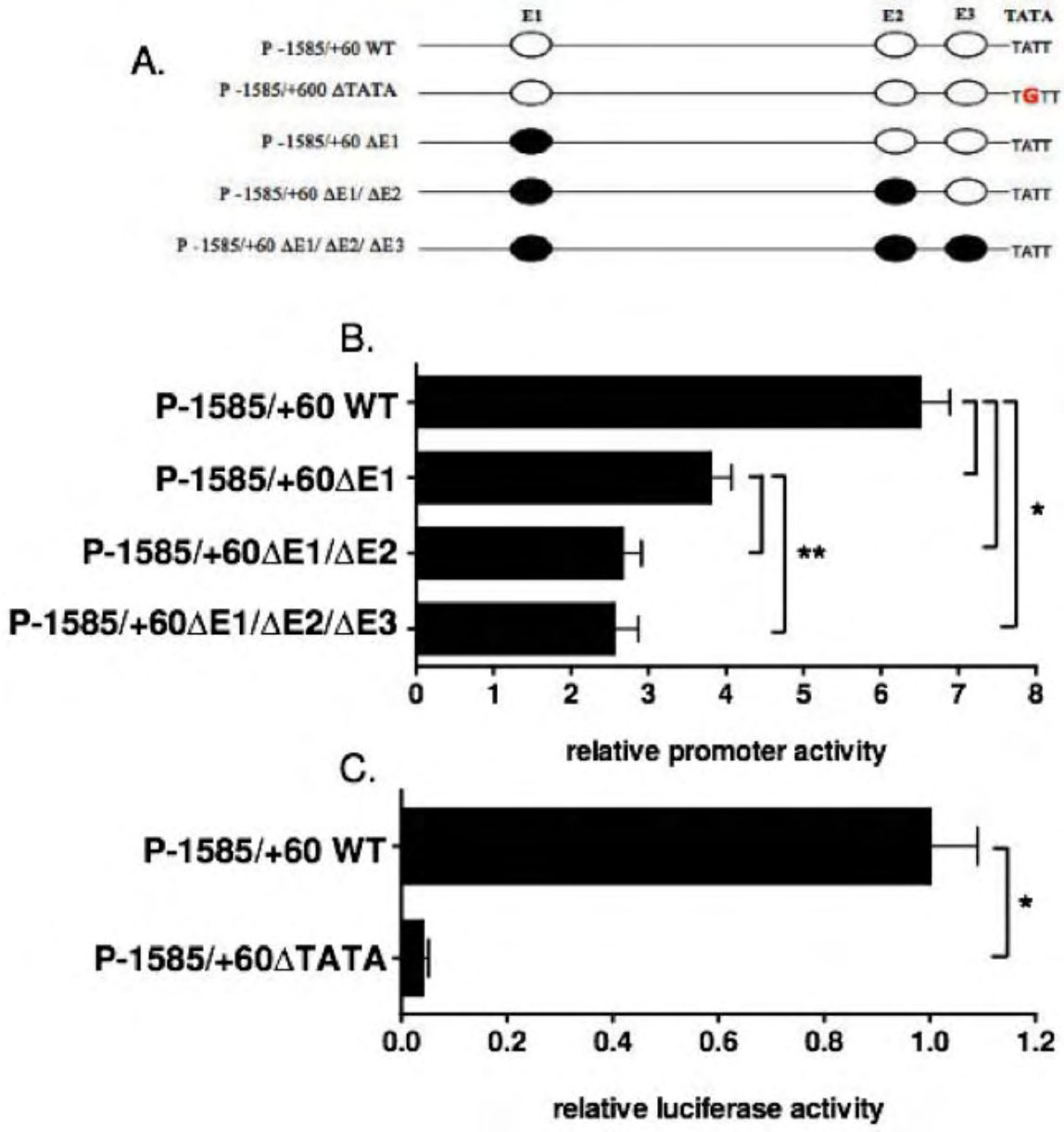

Figure 4

***Ms1* promoter sensitivity to exogenous MyoD with targeted mutations of putative MyoD binding E-Box sequences**. (A) Schematic representation of the reporter gene constructs used in luciferase assays. Wild type and mutant E-Box sequences are represented in white and black ovals respectively. (B) The E-Box sequences 1,2 and 3 (E1, E2 and E3) contained within the wild type P-1585/+60 construct were subjected to site directed mutagenesis. The subsequent single, double and triple E-Box mutant constructs were transiently co-transfected with MyoD into subconfluent C2C12 myoblasts for luciferase assays that were harvested 48 hours later, when the cells were 80% confluent, Luciferase activity, representing promoter sensitivity to ectopic MyoD expression (fold activation vs pcDNA), was inhibited by approximately 50% in the single E1 mutant with the double E1/E2 mutant resulting in a 67% reduction in activity with respect to wild type promoter sensitivity. Triple E1/E2/E3 mutant maintained the same level of MyoD sensitivity compared to the double E1/E2 mutant. (C) The putative TATA box sequence was subjected to site directed mutagenesis and transiently expressed in C2C12 myoblasts as described above and assayed for luciferase activity. TATA mutation resulted in a 95% reduction in luciferase activity with respect to wild type P-1585/+60 construct. The results are expressed as mean ± SE of at least three different experiments, in triplicate for each construct. Statistically significant differences compared to the appropriate WT construct are indicated by *$P < 0.05$ and **$P < 0.05$.

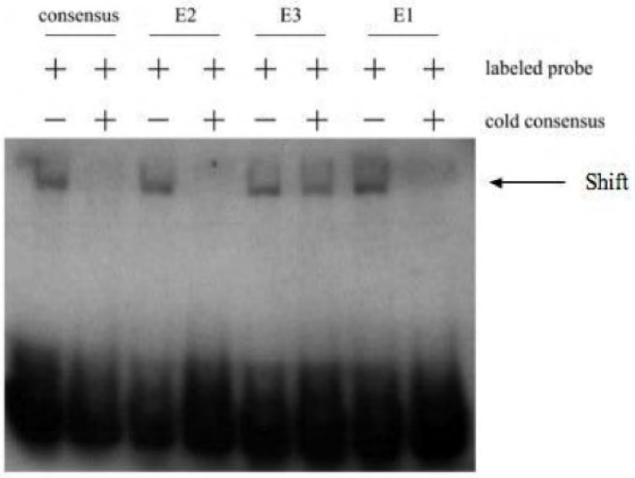

Figure 5
EMSA analysis of E1, E2 and E3 in the ms1 promoter. DIG-labelled oligonucleotide probes for the MyoD E-Box binding consensus, E1, E2 and E3 binding sites were incubated with whole cell protein extracts made from subconfluent C2C12 myoblasts. Competition experiments were performed using a 200-fold excess of unlabeled MyoD E-Box consensus probe. Arrow indicates the resulting bandshifts.

and differentiation. Targeting and activation of muscle specific genes by SRF is dependent on specific association with the powerful co-activators, MRFT-A and -B, and Rho-A signalling. We, and others, have recently shown the actin binding protein, MS1 (STARS), to be both required and sufficient for muscle specific activation of the RhoA/MRTF/SRF signalling axis. Therefore, understanding the transcriptional regulatory mechanisms governing *ms1* expression will give us a key insight into how the MRTF/SRF axis is regulated during myogenic differentiation.

As a first step towards understanding the transcriptional mechanisms governing *ms1* expression in muscle differentiation, we analysed *ms1* expression in differentiating C2C12 cells. A robust induction in *ms1* expression was observed during the first day of differentiation suggesting *ms1* is an early 'wave' myogenic transcript [34]. Fernandez and colleagues [48] have reported that C2.7 myoblast fusion and differentiation is dependent on SRF, as a consequence of its role in regulating MyoD expression. In addition, MyoD and SRF have been shown to physically interact and syngerstically activate target promoters, with consensus SREs enriched in *bona fide* MyoD target promoters [35]. Considering this, one would expect SRF activity to coincide with MyoD during early myogenic differentiation. We propose this early expression of *ms1* drives muscle specific activation of the MRTF/SRF axis, coupling this pathway with MyoD expression and activity.

An *in silico* comparative sequence analysis suggested that the proximal 1.5 kbp 5'-upstream sequence would be capable of driving muscle specific transcription. Within this region, two evolutionary conserved regions were identified, both of which were enriched with ultra conserved binding motifs for key myogenic regulatory factors. The proximal ECR also contained a conserved TATA box located at the correct distance from transcription start site suggesting it constitutes the core promoter. This 1.5 kbp promoter fragment was significantly more active in a muscle versus a non-muscle cell type (Figure 3A) supporting the *in silico* derived hypothesis. It is of interest that in a recent study the proximal 1.5 kbp 5'-upstream region of the mouse STARS gene was able to direct transgenic lacZ expression in adult skeletal muscle *in vivo*, thus supporting our *in vitro* data [31].

We speculated that the ultra conserved myogenic regulatory motifs encompassed within this promoter fragment would be important for muscle specific activity. A significant increase in promoter reporter activity was observed with the ectopic expression of the MRF, MyoD. However myogenin and Mef2D were not able to activate the *ms1* promoter reporter in this myoblast cellular environment. This pattern of sensitivity was also the same at the level of endogenous *ms1* transcription.

The observed specific sensitivity to MyoD complements the endogenous *ms1* expression profile during C2C12 differentiation. MyoD is responsible for myogenic gene activation during early stages of differentiation [2,35], the time at which *ms1* transcription is induced. Myogenin and Mef2 proteins are themselves subsequently induced by MyoD, and in a combinatorial manner drive expression of late myogenic genes as well as consolidating and maintaining expression of early myogenic genes [43,49]. In addition this specific sensitivity may suggest that only MyoD and not myogenin can target the myogenic E-Boxes within the *ms1* promoter in a myoblast cellular environment. This is not uncommon, for example, MyoD targets myogenic E-Boxes within the chicken MLC-1 promoter, which cannot be bound by myogenin [50].

It is of interest that Mef2C binding to the Mef2 consensus sequence identified in our comparative analysis (ECR α) has been shown to mediate basal and stress inducible cardiac specific promoter activity both in *in vitro* and *in vivo* (Kuwahara et al, 2007). In addition, skeletal muscle specific deletion of the Mef2C isoform at early, but not late times of embryogenesis, results in mice with disorganised myofibers that are perinatal lethal (Potthoff et al, 2007). Our data (Fig. 3) suggest that this Mef2 motif in the *ms1* promoter is not required for the initial MyoD mediated induciton of ms1. However, our data do not preclude the possibility that Mef2D may contribute to ms1 expression

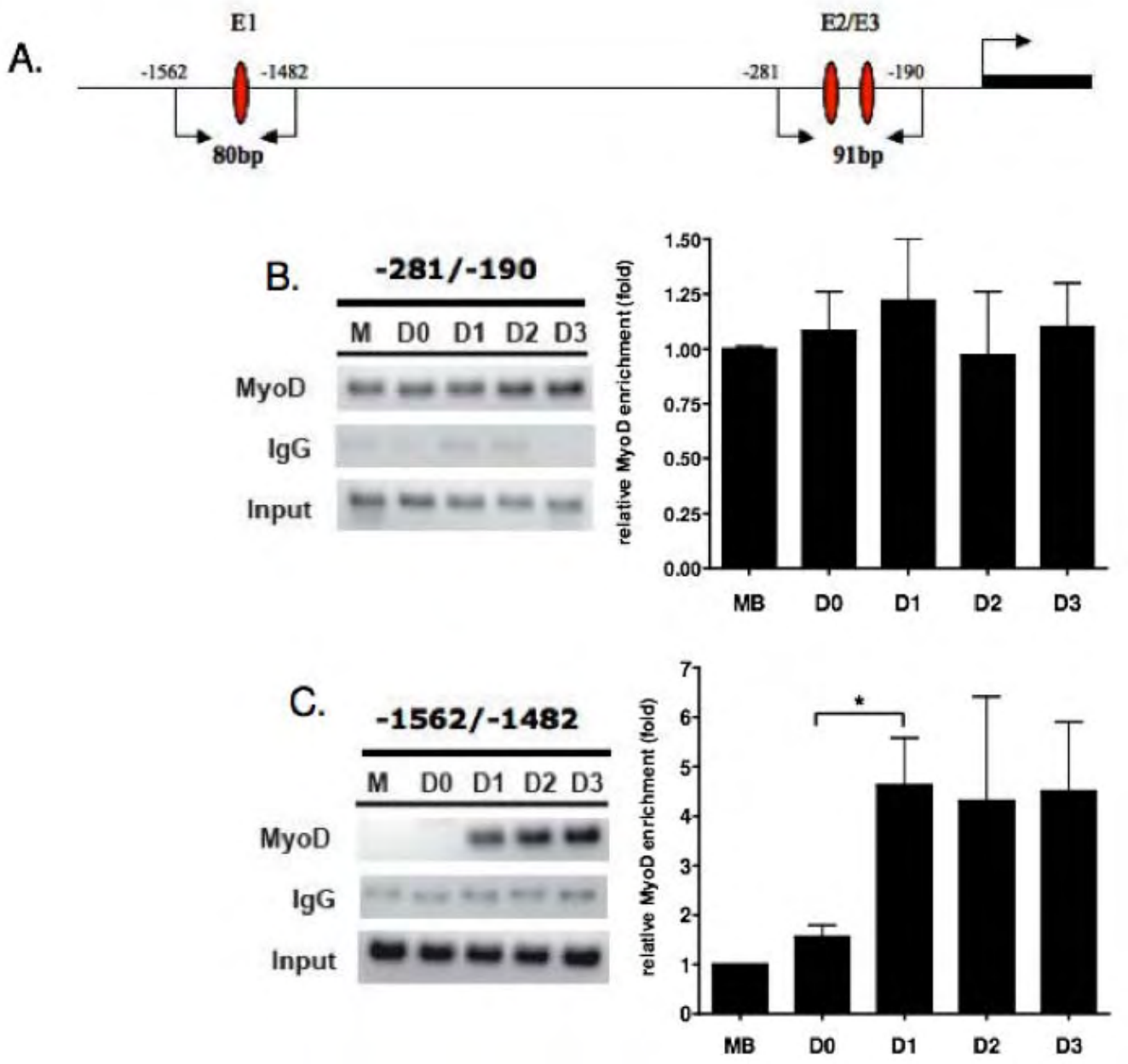

Figure 6

Chromatin immunoprecipitation assay performed on differentiating C2C12 cells to evaluate in vivo MyoD binding at the *ms1* promoter. (A) A schematic map of the amplified DNA fragments (product size) and the primer locations encompassing E1, E2 and E3. TSS position is also illustrated. Proteins were cross-linked to the DNA (in C2C12 cells during myogenic differentiation) with formaldehyde, DNA was sheared by sonication, and Abs directed against IgG or MyoD were added to precipitate any protein-DNA complexes. The precipitated complexes were pre-cleared with protein A beads. Samples analyzed included proliferating, subconfluent myoblasts (M), confluent myoblasts harvested prior to the induction of differentiation (D0), myoblasts subjected to differentiation conditions for 24 hours (D1), and differentiating myotubes at 48 hours (D2) or 72 hours (D3) post-differentiation. Quantitative real time PCR were performed on isolated DNA using primers encompassing the proximal (B) and distal (C) E-Box sequences (E2/E3 and E1 respectively). Amplification was quantified and normalised to the input of each sample. The results are expressed as mean ± SE of at least three different ChIPs. Statistically significant differences in fold enrichment are indicated by *P < 0.05. Representative PCR reactions were stopped in the linear amplification range and run on agarose gel for visualisation.

later in myogenesis or that other isoforms of Mef2 may contribute to ms1 activation.

We hypothesised that the observed MyoD sensitivity was via specific targeting of MyoD to the ultra conserved E-Box's, E1, E2 and E3, located within the two evolutionary conserved regions. Indeed, via site directed mutagenesis and electromobility shift assays, we demonstrated that E1 and E2, but not E3 appear to be targeted by MyoD. Surprisingly a significant level of MyoD sensitivity was retained in the triple E-Box mutant. This may suggest the presence of other non-conserved E-Boxes targeted by MyoD within the promoter reporter or alternatively be a result of the up-regulation of other myogenic transcription factors by MyoD, which subsequently target and activate the *ms1* promoter at other motifs. It is of interest to note the presence of a conserved SRE within the proximal ECR, which can be bound by SRF in cardiomyocytes *in vivo* (Ounzain S, unpublished). MyoD upregulation is predicted to increase the activity of SRF [48], so it is possible for SRF to target and increase activity of *ms1* promoter independent of MyoD binding at E1 and E2. It is also interesting to consider the up-regulation of the muscle specific isoform of Mef2D (Mef2D1b), which we suspect may target the *ms1* promoter in late stages of differentiation.

Many muscle specific genes are activated at different times during the myogenic differentiation process. Numerous studies suggest that this differential expression of each target gene is a product of specific temporal binding of MyoD at distinct E-Boxes within the *cis* regulatory domains of the gene, which itself is coupled to chromatain modification and remodelling [3]. We therefore used ChIP to measure *in vivo* binding of MyoD at the *ms1* E1 and E2 domains during C2C12 differentiation. Our analysis shows that MyoD is constitutively bound at the proximal E2 domain during differentiation, with binding preceding the induction of *ms1* transcript at day 1 (Figure 6B). MyoD is capable of binding target sequences prior to gene activation, acting in a repressive manner via the specific recruitment of repressive chromatin remodelling complexes. Interestingly, *ms1* transcriptional induction at day 1 (Figure 1B) coincides with MyoD binding at E1, located within the ECRβ (Figure 6C). This suggests MyoD targeting at E1 is required for transcriptional activation of *ms1* and we therefore propose that ECRβ could represent a differentiation-dependent skeletal muscle enhancer.

Prior to activating transcription, MyoD associated with HDACs serves to mark myogenic genes for subsequent differentiation cues and thus activation [51]. It is conceivable that MyoD binding at the *ms1* promoter prior to differentiation primes the promoter in a 'poised' myogenic state. The recruitment of HDACs by MyoD causes the local chromatin environment to be compacted and will prevent the association of MyoD and other activating factors with specific cognate binding sites. However appropriate differentiation signals can stimulate MyoD to toggle between HDAC and HAT recruitment in addition to association with differentiation-specific myogenic factors [3]. This facilitates binding of MyoD with other E-Boxes (E1) and allows the formation of activating transcriptional complexes.

MyoD acetylation has recently been implicated as a central mediator of the temporal activation of muscle specific gene expression during myogenesis [47]. We therefore cannot rule out the possibility that during C2C12 differentiation an acetylated MyoD form with increased DNA binding efficiency [52] is capable of binding the E1 domain, resulting in the temporal activation of *ms1* transcription. It is of interest that preliminary data indicate that trichostatin A, a HDAC inhibitor, is able to increase ms1 transcript abundance in H9c2 myoblasts (Ounzain S, unpublished).

Taking the present data together, we propose a model whereby MyoD binding at the *ms1* proximal ECR in myoblasts represses *ms1* transcription via the recruitment of HDACs. This proximal binding is essentially priming the *ms1* promoter, placing it in a poised state for sensing appropriate differentiation cues. Upon differentiation MyoD associates with HATs and SWI/SNF, which subsequently causes remodelling of the local chromatin environment, allowing MyoD to bind E1 within the distal ECR or alternatively, temporally acetylated MyoD binds the E1 domain. Further targeting of HATs and specifically SWI/SNF complexes (at E1) can facilitate binding of TBP and other factors involved in polymerase II pre-initiation complex formation and promote transcriptional elongation [53-58]. We thus speculate that temporal targeting of MyoD at E1 is required to establish the optimum environment for Pol II action and robust transcription.

In summary our data suggests that MS1 is a key component of a MyoD generated feed-forward regulatory circuit, where factors induced by MyoD (like *ms1*) feed-forward to regulate late MyoD activity (via SRF) at subsequent target genes, therefore acting to temporally pattern the timing of gene expression during skeletal myogenesis. This MyoD-MS1-SRF feed-forward network would serve to consolidate and amplify the myogenic cascade. Indeed SRF itself acts in combination with MyoD to activate many downstream genes, thus, through the specific regulation of *ms1*, MyoD is able to synchronize SRF activity with its own and thus collaborate to mediate the temporal activation of downstream genes.

We believe this is the first study to demonstrate a direct link between MyoD activity and SRF transcriptional signalling, with *ms1* serving as the nodal point to integrate these two central myogenic regulatory networks. It is of interest that in cardiomyocytes MS1 serves a similar function in that it integrates the Mef2 and SRF signalling networks, providing a link for crosstalk between them [31]. This is thus a conserved emerging paradigm for MS1 function both in cardiac and skeletal muscle. In addition we have data to suggest that MS1 is capable of integrating the GATA4 cardiogenic network with SRF activity [59].

This study also has implications for myogenic disease phenoptypes. IGF-1 and IL-4, both central mediators of post-natal skeletal muscle regeneration are regulated by SRF in response to stress [60]. Therefore understanding the molecular mechanisms regulating *ms1* expression may allow us to identify and develop therapeutic strategies for the up-regulation of *ms1* gene expression in a disease phenotype, which would facilitate regeneration via stimulation of SRF activity and resulting up-regulation of IL-4 and IGF-1.

Conclusion

Identification of direct transcriptional targets of MyoD and de-convolution of the transcriptional regulatory networks that operate in muscle cells represent an essential target if we are to understand not only how muscle differentiates but also how it responds to stress and damage, therefore allowing regeneration. We have demonstrated that via temporal binding of MyoD at distinct E-Boxes within the *ms1* promoter, *ms1* potentially serves to integrate the MyoD and SRF myogenic regulatory circuits, thus driving a feed-forward auto-regulatory circuit that consolidates and amplifies the myogenic phenotype. We believe this is the first study to describe a direct link between MyoD activity and SRF signalling, with *ms1* allowing cross talk to occur between these two independent myogenic networks. This implicates MS1 as a key factor involved in myogenic differentiation and potentially regeneration.

Methods

Cell cultures

The H9c2 rat myoblast and NIH3T3 mouse fibroblast cell lines were grown in a humidified atmosphere containing 5% CO_2 at 37°C in DMEM supplemented with 10% FCS, 2 mM glutamine, streptomycin and penicillin (each at 10 g/liter). C2C12 mouse skeletal myoblasts were cultured in DMEM supplemented with 20% FBS, 100 units/ml penicillin and 100 ug/ml streptomycin and maintained at 37°C in 5% CO2. The differentiation of C2C12 myoblasts into myotubes was achieved by the addition of differentiation medium (DMEM supplemented with 2% horse serum) at confluence for up to 4 days with medium change every 2 days.

Plasmid constructs

The rat MS1 gene sequence was obtained from GenBank (NC_005106) and was used to design primers that would amplify the 5' flanking region. Two oligonucleotides, P-1585-SacI and P+60-HindIII (Table 1), were designed to amplify a portion of DNA sequence starting -1585 bp upstream of the transcription start site (+1), with primers tailed with restriction sites for *SacI* and *HindIII* restriction enzymes respectfully. In addition to template (rat genomic DNA, WKY strain) and primers P-1585-SacI and P+60-HindIII, the reaction contained 0.2 mM dNTPs, Expand polymerase buffer and 5 units of *Taq* Expand high fidelity polymerase (Roche). Reaction was subjected to 35 cycles of amplification (45 s at 94°C, 45 s at 59°C and 90 s at 72°C). The PCR product was cloned into the vector pGEM-T Easy (Promega) and sequenced to ensure fidelity of amplification. The verified plasmid was then cut with SacI/HindIII and the released -1585 promoter fragment was purified and cloned into the pGL3-Basic (Promega) reporter vector, *SacI*/*HindIII* digested. The subsequent construct was designated P-1585/+60WT. The MyoD and Myogenin expression plasmids were provided by Dr Andrew Lassar [62,63] and the Mef2D expression plasmid was a gift from Dr Eric Olson (University of Texas, South Western Medical centre) [64].

Site directed mutagenesis

The P-1585/+60WT construct was subjected to site directed mutagenesis to mutate the TATA, E-Box1, 2 and 3 binding sites, using the Quik Change II site-directed mutagenesis kit (Stratagene). The mutagenic primers used to generate the TATA and E-Box mutations are reported in Table 1. Site-directed mutagenesis was performed according to the manufacturers instructions. Mutated fragments were then re-cloned into the Sac I/Hind III sites of the corresponding pGL3-Basic vectors. Double (ΔE1/ΔE2) and triple-site (ΔE1/ΔE2/ΔE3) mutation constructs, were generated by consecutive rounds of mutagenesis. The resulting plasmids were confirmed by DNA sequencing.

Transient transfections and luciferase assays

C2C12 and NIH 3T3 cells were transfected using the cationic transfection reagent, Jet Pei (QBiogene), according to the manufacturers protocol. Cells were seeded in six well plates. Twenty-four h post plating; the cells were co-transfected with 0.5 μg of promoter-luciferase construct and equimolar amounts for the other plasmids used (total of 0.6 μg). The total amount of DNA was kept constant using empty vector (pcDNA3.1). To normalise for transfection efficiency, the pRL-TK (Promega) expression plasmid containing *Renilla* luciferase (20 ng per well) was co-transfected. *Firefly* and *Renilla* luciferase activities were

measured at 48 h post-transfection using the Dual-Glo™ Luciferase assay system (Promega) and a Lumat LB9507 luminometer (Berthold Technologies). All plasmids were purified using Qiagen columns (Qiagen) and at least two preparations per plasmid were tested. The transfection efficiency was normalised using the *Renilla* luciferase activity levels and each transfection was performed in triplicate and repeated in a minimum of three independent experiments.

RNA isolation and quantitative real time RT-PCR

H9c2 cells seeded in six well plates were transfected with up to 1.5 μg of expression plasmid using JetPei (as above). Total amount of DNA was kept constant using empty vector (pcDNA3.1). Forty-eight h post transfection or 24 h with TSA, total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA (1 μg) was reverse transcribed to cDNA using oligo (dT) and Superscript II Reverse Transcriptase (Invitrogen). Rat *ms1* mRNA expression was analysed using quantitative PCR with fluorescent-labelled TaqMan probes (Rat *ms1* primers and probe, Cat No. Rn00598518_m1, Applied Biosystems). TBP was used as the internal control (Cat No. Mm00446973_m1, Applied Biosystems). PCR amplifications were performed in duplicate in 25 μl containing 2 μl cDNA template in 2× PCR master mix (Applied Biosystems). Amplification conditions as follows: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C for 15 s followed by 60°C for 1 min. Reactions were performed and products detected using an ABI-Prism HT 7900 sequence detector (Applied Biosystems). The level of expression of *ms1* mRNA was normalised to TBP expression. For C2C12 myoblasts, cells were cultured and harvested for RNA extraction according to the time course of differentiation. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturers instructions. Total RNA (2 μg) was reverse transcribed with Superscript III (Invitrogen). Quantitative PCR was performed with Qiagen HotStart Taq Master Mix and SYBR Green I as described previously (Schmittgen and Zakrajsek, 2000) using primers for *ms1* and EF1-α (Table 1). Amplifications were performed in a DNA Engine Opticon System (MJ Research) and quantified. *Ms1* mRNA levels were normalised to EF1-α mRNA levels.

Electromobility shift assays (EMSA)

Whole cell extracts were prepared from cultured C2C12 myoblasts using the CelLytic™-M cell lysis extraction reagent (Sigma) according to the manufacturers instructions. The protein concentrations were determined using the Bradford assay (Bio Rad). Whole cell extracts were incubated for 15 min with 0.5 pmol of the appropriate digoxigenin (DIG)-labelled double-stranded oligonucleotide probe (Table 1) in a 20 μl reaction containing binding buffer (Roche), 1 μg poly (dI-dC) (Roche) and 0.1 μg poly L-lysine (Roche). The probes were end-labelled with DIG-11-ddUTP (Roche). The reaction was stopped by the addition of 5 μl loading dye (Roche). For competition experiments, a 200-fold excess of unlabeled double-stranded oligonucleotide (Table 1) was added to the reaction. The samples were then run on a 4% non-denaturing polyacrylamide gel using 1× TGE buffer (50 mM Tris, 380 mM Glycine and 2 mM EDTA), and transferred to a positively charged nylon membrane (Amersham Biosciences). DIG-labelled oligonucleotides were visualised by incubation with alkaline phosphatase-labelled $F(ab)_2$ anti-DIG Ab, followed by chemiluminescence reaction with 100 μg/ml CPSD substrate (Roche).

Chromatin immunoprecipitation assay (ChIP)

C2C12 ChIPs were performed using MyoD (de la Serna et al 2005) and IgG (Santa Cruz) antibodies. The ChIP was performed as described previously [57], except that immune complexes were eluted with 0.1 M NaHCO3 and 1% SDS, and following reversal of cross-links, the eluate was digested by proteinase K digestion and purified using the Qiagen PCR Purification kit (28106). 4 μl of DNA eluted from the column was used for PCR. Inputs consisted of 1% chromatin before immunoprecipitation. PCRs were performed with Qiagen HotStart Taq Master Mix using primer sets reported in Table 1. Amplification was quantified by a DNA Engine Opticon System (MJ Research), then normalised to the input of each sample. PCR reactions run on agarose gels were stopped in the linear range and visualised using SYBR Green I.

Comparative DNA analysis

Comparative sequence analysis of human and rat was performed using web-based software available at the Lawrence Berkley Laboratory genome website (VISTA). Orthologous sequences from *Mus musculus, Bos taurus, Homo sapiens, Pan troglodytes and Macaca mulatta* were obtained from the ENSEMBL genome data base and aligned using CLUSTAL W.

Data Analysis

Data are expressed as means ± SE (represented as error bars). Comparisons were made using the Student's *t* test, considering $P < 0.05$ statistically significant.

Abbreviations

MRF: myogenic regulatory factor; SRF: serum response factor; MRTF: myocardin related transcription factor; STARS: striated muscle activator of Rho signalling; MEF2: myogenic enhancer factor 2; ECR: evolutionary conserved region; TSS: transcription start site; DIG: digoxigenin; EMSA: electromobility shift assay; ChIP: chromatin immunoprecipitation assay; TSA: trichostatin A; HDAC: histone deacetylase; HAT: histone acetyltransferease; SRE: serum response element; MLC-1: myosin light chain 1;

MS1: myocyte stress 1; IGF-1: insulin growth factor 1; IL-4: interleukin 4; DMEM: dulbecco's modified eagle medium; FCS: fetal calf serum; TBP: TATA binding protein; TGE: tris glycine EDTA; EDTA: ethylenediaminetetraacetic acid; SDS: sodium dodecyl sulfate.

Authors' contributions

SO planned the study, carried out the experimentation and drafted the manuscript with input from CSD, ANI and NWC. CSD performed RT-PCR and ChIP on C2C12 cells as well as contributing to the analysis and interpretation of the data. NJS participated in the co-ordination of the study. ANI contributed to the planning and co-ordination of the study in addition to analysis and interpretation of the data. NWC supervised the study and participated in drafting of the manuscript. All authors read and approved the final manuscript.

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Appendix C

Myogenin and the SWI/SNF ATPase Brg1 Maintain Myogenic Gene Expression at Different Stages of Skeletal Myogenesis

Though transcriptional regulation during skeletal muscle specification is well understood, there is less information regarding the maintenance of the differentiated state. The MRF MyoD and the mammalian SWI/SNF ATP-dependent remodeling enzymes are required for the induction of myogenesis in cell culture systems and the developing embryo. Myogenin, another member of the MRF family is necessary for activation of late myogenic transcripts that induce terminal differentiation. Our results revealed that myogenin was able to bind to its own promoter during the late stages of embryonic muscle development. Our contributions entailed the isolation of primary muscle satellite cells and mature myofibers from adult tissue for use during gene expression and ChIP analysis. Interestingly, in primary adult muscle satellite cells, MyoD and Brg1 localize to the myogenin promoter. In contrast, in mature myofiberes, myogenin and Brg1 were preferentially colocalized to the myogenin promoter, indicating that two distinct MRFs were required for coordinate myogenin expression. Further, we demonstrate that in the absence of MyoD, myogenin was able to occupy its own promoter, targeted the Brg1 ATPase to modify chromatin structure and thereby facilitated its own gene expression. *In vivo* electroporation of dominant negative Brg1 into skeletal muscle of newborn mice revealed that myogenin expression was abrogated and continued production of myogenin protein was reliant on Brg1 activity. Therefore, cooperation between myogenin and Brg1 was required to maintain the skeletal muscle phenotype *in vivo.*

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Myogenin and the SWI/SNF ATPase Brg1 Maintain Myogenic Gene Expression at Different Stages of Skeletal Myogenesis*

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Many studies have examined transcriptional regulation during the initiation of skeletal muscle differentiation; however, there is less information regarding transcriptional control during adult myogenesis and during the maintenance of the differentiated state. MyoD and the mammalian SWI/SNF chromatin-remodeling enzymes containing the Brg1 ATPase are necessary to induce myogenesis in cell culture models and in developing embryonic tissue, whereas myogenin and Brg1 are critical for the expression of the late genes that induce terminal muscle differentiation. Here, we demonstrate that myogenin also binds to its own promoter during the late stages of embryonic muscle development. As is the case during embryonic myogenesis, MyoD and Brg1 co-localize to the myogenin promoter in primary adult muscle satellite cells. However, in mature myofibers, myogenin and Brg1 are preferentially co-localized to the myogenin promoter. Thus, the myogenin promoter is occupied by different myogenic factors at different times of myogenesis. The relevance of myogenin in the continued expression from its own promoter is demonstrated in culture, where we show that myogenin, in the absence of MyoD, is capable of maintaining its own expression by recruiting the Brg1 ATPase to modify promoter chromatin structure and facilitate myogenin expression. Finally, we utilized *in vivo* electroporation to demonstrate that Brg1 is required for the continued production of the myogenin protein in newborn skeletal muscle tissue. These findings strongly suggest that the skeletal muscle phenotype is maintained by myogenin and the continuous activity of Brg1-based SWI/SNF chromatin-remodeling enzymes.

During skeletal muscle differentiation, MyoD plays a critical role in initiating the onset of muscle-specific gene expression in cooperation with the Mef2 family of activators and with the SWI/SNF chromatin-remodeling enzyme, Brg1, which alters chromatin structure at myogenic loci in a manner that facilitates transcription (1, 2). An important target of MyoD is myogenin, a related basic helix-loop-helix myogenic regulatory factor (MRF)[2] (3, 4). In addition to MyoD and myogenin, there are two additional members of the basic helix-loop-helix MRF family, Mrf4 and Myf5 (5, 6). Genetic analyses of mice deficient for one or for different combinations of the MRFs have demonstrated that complex relationships exist between members of this family. In particular, there is significant redundancy of function in numerous parameters relating to the initiation of myogenesis (reviewed in Ref. 7). In contrast, gene knock-out analyses indicate that terminal differentiation specifically requires myogenin, as mice deficient for myogenin formed myoblasts but did not develop mature skeletal muscle tissue (8–10). Thus, myogenin is the only MRF that is absolutely required for skeletal muscle differentiation during embryogenesis, and this requirement is manifested not for the initiation of myogenesis but instead for events associated with tissue formation.

Recently, we demonstrated that myogenin, in cooperation with Mef2D and Brg1, directed the expression of myogenic late genes that specify the skeletal muscle phenotype in cell culture and in embryonic tissue (11). We hypothesized that during the time of terminal differentiation and in post-differentiated skeletal muscle, myogenin might also bind to its own promoter and cooperate with Brg1 to regulate its own expression. This idea is further supported by prior cell culture experiments demonstrating that ectopic expression of myogenin in 10T1/2 cells could induce expression from the endogenous myogenin locus by an undefined mechanism (12, 13). Here we provide evidence to support this hypothesis by examining the occupancy and function of myogenin at its own promoter during embryonic and adult skeletal muscle differentiation and in culture and by functionally evaluating the requirement for Brg1 in myogenin expression in differentiated tissue.

EXPERIMENTAL PROCEDURES

Preparation of Mouse Embryonic Tissue—Conditions were identical to those published previously (11).

Nuclei Isolation from Mouse Skeletal Muscle Satellite Cells and Myofibers—Unless noted, all procedures were performed at 4 °C. Skeletal muscle from the upper hind limbs of 4- to 6-week-old C57/BL6 mice was minced to ~1 mm^3 and digested with 110 units/ml collagenase type II (Invitrogen) in phosphate-buffered saline containing 1 mM $CaCl_2$. Samples were incubated at 37 °C for 1 h with agitation (14, 15) and filtered

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[2] The abbreviations used are: MRF, myogenic regulatory factor; ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein.

using a 70-μm cell strainer (352350; BD Biosciences) (16). Flowthrough material was enriched for satellite cells, while material that did not pass through the filter was enriched for myofibers. Aliquots of the separated fractions were taken for RNA isolation; the remainder was pelleted by centrifugation and resuspended in 7 volumes of lysis buffer (10 mM HEPES-KOH, pH 7.3, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 3 μg/ml cytochalasin B, 10 μg/ml leupeptin). Following a 30-min incubation at 4 °C, nuclei were released by Dounce homogenization using the A pestle. Nuclei release was checked by Hoechst 33258 staining, and cell debris was removed by centrifugation. Nuclei were first resuspended in 2.5 volumes of 10 STM buffer (5 mM $MgCl_2$, 10 mM triethanolamine, pH 7.5, 5 mM $MgCl_2$, 10% sucrose, 10 μg/ml leupeptin) and then 2 volumes of 2.0 M sucrose/10 mM Tris-HCl/5 mM $MgCl_2$. The samples were overlaid onto 750 μl of 2.0 M sucrose/10 mM Tris-HCl/5 mM $MgCl_2$ in a Beckman ultracentrifuge tube (number 344057) and spun at 116,140 $\times$ *g* for 1 h at 4 °C. The pellet was resuspended in 500 μl of lysis buffer +0.1% Nonidet P-40. 1.0% formaldehyde at room temperature was added for 5 min for cross-linking, and, following centrifugation at 13,500 $\times$ *g*, pellets were frozen in liquid nitrogen. Thawed samples were resuspended in lysis buffer and sonicated.

Cell Culture—The B22 cell line inducibly expressing ATPase-deficient, dominant negative Brg1 (17) and retroviral infection (11, 18, 19) was previously described. Cells were cultured for 4 days in the presence of tetracycline (dominant negative Brg1 repressed) or in the absence of tetracycline (dominant negative Brg1 expressed) and were infected at 50% confluence with the indicated retrovirus for 30 h. Low serum differentiation medium was then added (time 0) to induce myogenic differentiation (20). Control samples were mock infected but still subjected to the differentiation protocol.

Quantitative Reverse Transcriptase Polymerase Chain Reaction Analysis—Reverse transcriptase PCR and quantitative PCR conditions were previously described (11). Primers for MyoD, myogenin, desmin, MCK (21), and EF1-α (11) were described. Endogenous myogenin (Fig. 3*A*) was amplified in the 3′-non-coding region with 5′-CAA GTG TGC ACA TCT GTT CTA GTC TC-3′, 5′-GTA TCA TCA GCA CAG GAG ACC TTG GT-3′. Quantitative PCR reactions shown on agarose gels were stopped in the linear range and visualized using SYBR Green I (Molecular Probes).

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation (ChIP) assays were performed as described (11, 22). ChIP PCRs were performed with Qiagen HotStart Taq Master mix using the primer sets described for the myogenin promoter and IgH enhancer (22). Antibodies used include myogenin (sc-576; Santa Cruz) and affinity-purified polyclonal rabbit antibodies raised against glutathione *S*-transferase fused to full-length MyoD (22) and against glutathione *S*-transferase fused to a unique portion of Brg1 (17). PCR amplification was quantified using a DNA Engine Opticon system (MJ Research).

Restriction Enzyme Accessibility Assay (REAA)—REAA and detection of restricted DNA by a modified version of ligation-mediated PCR were described previously (11, 22).

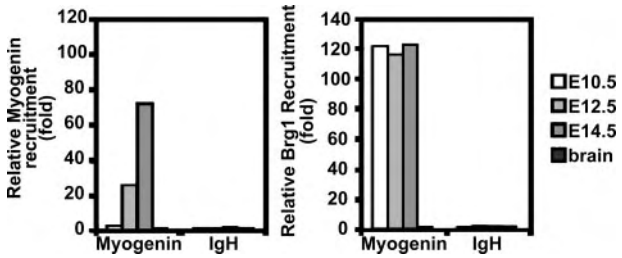

FIGURE 1. **Myogenin and Brg1 bind to the myogenin promoter during embryogenesis.** ChIP assays for myogenin (*left*) and Brg1 (*right*) were performed at the indicated stages of embryonic development. Relative recruitment was defined as the ratio of amplification of the PCR product relative to 1% of input genomic DNA. Values obtained from brain tissue were defined as 1. Quantification represents the average of two independent experiments. Variation between experiments did not exceed 20% for any sample. The IgH enhancer region, which contains an E box that does not bind MyoD, was amplified as control for ChIP specificity.

In Vivo Electroporation—*In vivo* electroporation was performed on CL57/BL6 mice at postnatal day 2 (P2) (23, 24). Expression vectors encoding GFP (0.2 μg/μl) and dominant negative Brg1(1.0 μg/μl) (25) were co-injected into the hind limb and electroporated with a CUY21EDIT square wave electroporator (Nepa Gene). Muscle tissue was analyzed 2 days after electroporation.

Immunohistochemistry—The dissected mouse hind limb muscle was fixed in 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde and 5% sucrose for 4 h at 4 °C. After fixation, the sample was cryoprotected with 25% sucrose in 0.1 M PB and embedded in OCT compound. Frontal sections (10 μm thick) were prepared. The following procedures were carried out at room temperature. The sections were pretreated with blocking solution (5% normal goat serum, 0.2% bovine serum albumin, 0.1% Triton X-100, 5 mM NaN_3, 0.1 M phosphate-buffered saline) for 1 h, incubated with rabbit polyclonal anti-myogenin antibody (1:100; Santa Cruz) for 16 h, and incubated with the Alexa Fluor 546 secondary antibody (1:500; Molecular Probes) in blocking solution. Fluorescence images were obtained using an IX-71 fluorescent microscope (Olympus) and processed with Adobe Photoshop software.

RESULTS

Our previous results indicated that at the time of expression in developing embryos, myogenic late genes that specify the skeletal muscle phenotype are predominantly bound by myogenin and Mef2D and that these factors cooperate with the Brg1 chromatin-remodeling enzyme to alter promoter chromatin structure and facilitate late gene expression (11). Though multiple studies have examined the initiation of myogenin expression by MyoD, we recently demonstrated that both MyoD and myogenin could bind to the myogenin promoter in MyoD-differentiated fibroblasts 24 h post-differentiation (22). Therefore, we asked whether myogenin was present at its own promoter in the developing embryo after the point at which MyoD had initiated gene expression.

ChIP analysis of tissue samples at embryonic (E) 10.5 (body with head and internal organs removed), E12.5 (limb buds), and E14.5 (limb muscle) revealed that myogenin binding to the myogenin promoter could be observed at E12.5, with increased binding at E14.5 (Fig. 1). The kinetics of myogenin binding to

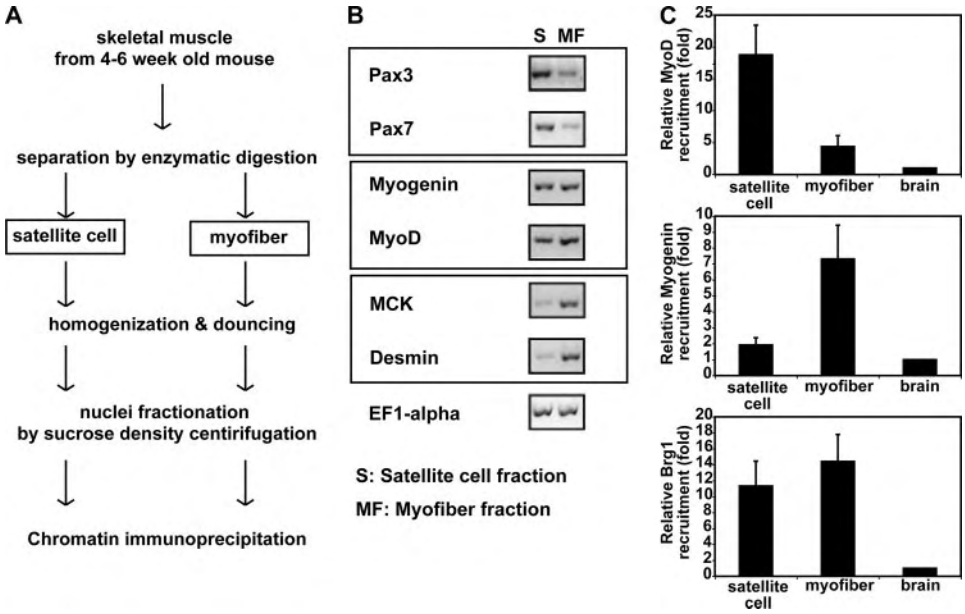

FIGURE 2. **Changes in recruitment of MRFs at the myogenin promoter in muscle satellite cells and myofibers.** *A*, flow chart of the separation and purification of nuclei from satellite cells and myofibers. *B*, transcript levels of muscle satellite cell and myofiber markers were analyzed by reverse transcriptase PCR. *C*, ChIP analysis using nuclei obtained from muscle satellite cells and myofibers. Binding of MyoD, myogenin, and Brg1 to the myogenin promoter in these tissues was quantified by quantitative PCR. The data reflect the mean ± S.D. from three independent experiments.

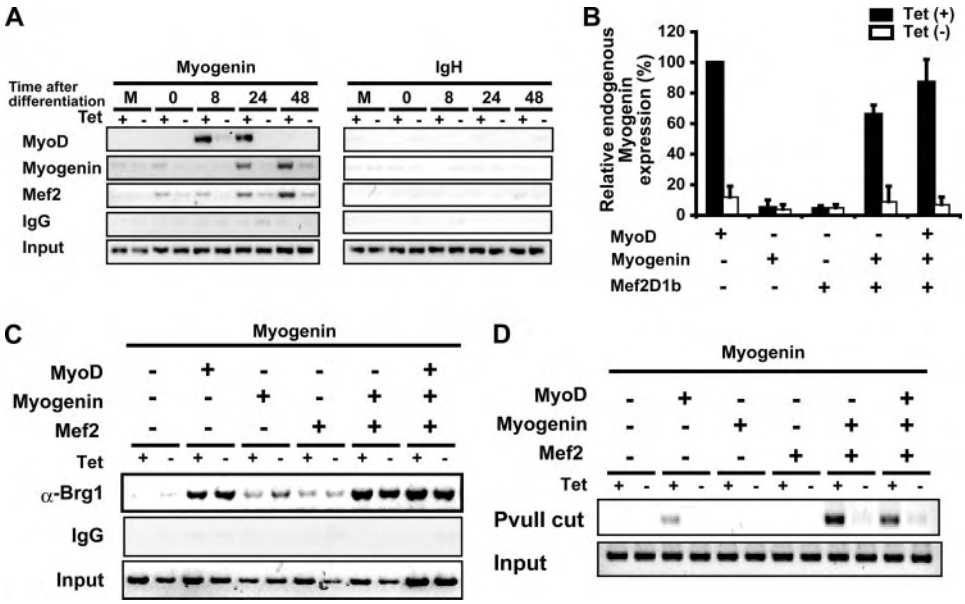

FIGURE 3. **Myogenin can activate expression from the endogenous myogenin locus.** *A*, ChIP analyses show changes in MRF binding to the endogenous myogenin locus during MyoD-mediated differentiation of B22 fibroblasts, which express dominant negative Brg1 in the absence of tetracycline (*Tet*). ChIPs were performed with antibodies against the indicated proteins or with nonspecific IgG on mock (*M*) or MyoD-differentiated samples at the indicated times. The IgH enhancer was amplified as a negative control. *B*, coexpression of myogenin and Mef2D1b induces endogenous myogenin expression. B22 cells expressing (−*Tet*) or not expressing (+*Tet*) dominant negative *Brg1* were infected with the indicated retroviruses and allowed to differentiate for 30 h. mRNA levels were examined by quantitative PCR. Myogenin levels present in the MyoD-infected +Tet sample were set at 100%. *C*, coexpression of myogenin and Mef2D1b recruits Brg1 to myogenic loci. ChIP assays were performed on B22 cells as described above. *D*, recruitment of functional Brg1 results in changes in restriction enzyme accessibility at the myogenin locus. Nuclei were harvested from B22 cells grown in the presence or absence of tetracycline and expressing the indicated factors were digested with PvuII. Cleavage at a PvuII recognition sequence at ~−370 relative to the start site of transcription was measured by a modified ligation-mediated-PCR protocol.

the myogenin promoter precisely matched those of myogenin binding to late genes (11), suggesting that at the time myogenin binds to genomic sequences in developing tissue, it does not discriminate between the previously inactive late gene loci and the transcriptionally active myogenin locus. Tissue culture studies indicate that Brg1 is essential for the initiation of myogenin transcription (20, 22, 26); as expected, Brg1 was present on the myogenin promoter at each of the embryonic stages examined (Fig. 1). We conclude that myogenin and Brg1 are present on the myogenin promoter at times when skeletal muscle tissue specification is occurring.

Mature skeletal muscle tissue is comprised of differentiated myofibers, which are the functional component of the tissue, and satellite cells, which associate with individual myofibers and are capable of dividing and either fusing with existing myofibers or creating new myofibers. The satellite cell population mediates the formation and repair of skeletal muscle tissue following the completion of development (reviewed in Refs. 27, 28). We separated satellite cells and mature myofibers from mouse hind limb muscles of 4- to 6-week-old mice and then isolated nuclei from the respective cell populations for molecular analyses of factor binding and chromatin accessibility. Nuclei were immediately utilized to isolate total RNA or were treated with cross-linking agents for ChIP analysis (Fig. 2*A*). Examination of marker gene expression permitted evaluation of the separation protocol. Proteins such as Pax3 and Pax7 are markers of satellite cells (29, 30), whereas the myogenic late gene products MCK and desmin are markers of differentiated skeletal muscle. The expression of these genes was enriched in the expected cell preparations (Fig. 2*B*). The satellite cell population is a pool containing both quiescent cells that are not expressing MyoD or muscle-specific markers and activated cells that express MyoD and have initiated differentiation but have not undergone cell fusion. Thus, both the satellite cells and the myofibers express mRNAs encoding the MyoD and myogenin transcriptional regulators (Fig. 2*B*).

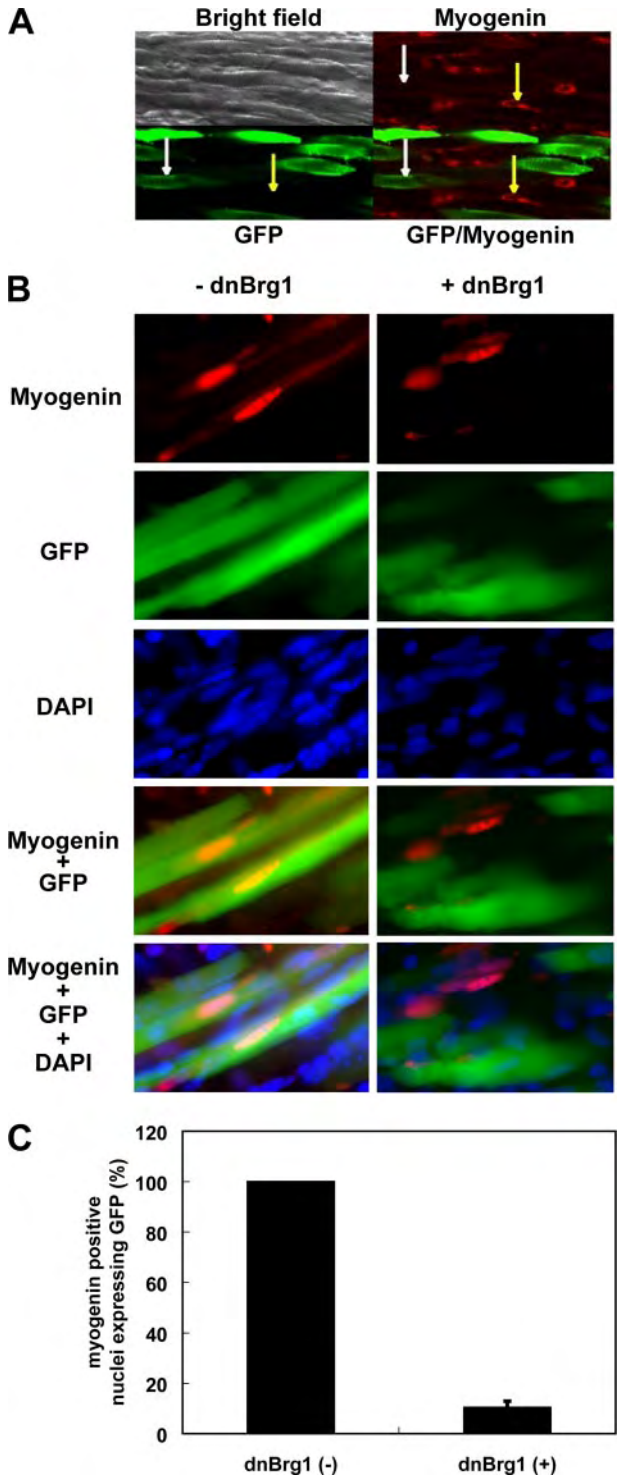

FIGURE 4. **SWI/SNF chromatin-remodeling activity is required for myogenin expression in differentiated skeletal muscle.** *A*, expression vectors encoding dominant negative Brg1 and GFP were introduced directly into mouse hind limb muscle of P2 mice by *in vivo* electroporation. Thin sections of hind limb skeletal muscle were examined in bright field, were immunostained with anti-myogenin antibody, and were monitored for GFP fluorescence 48 h later. *B*, control experiments show co-localization of GFP and myogenin in the absence of dominant negative Brg1 (−*dnBrg1*). Nuclei were visualized by 4′,6-diamidino-2-phenylindole. Tissue sections were permeable to 4′,6-diamidino-2-phenylindole, so all nuclei in the field are visible. Permeability issues limit the myogenin staining to nuclei at or near the surface of the section. *C*, quantification of the inhibition of myogenin expression. The number of myogenin-positive nuclei present in GFP fluorescent myofibers were counted in the control electroporation (GFP alone without dnBrg1) and in samples from dnBrg1-electroporated tissue. A total of 400 myogenin-stained nuclei from 16 different sections from both the GFP and GFP + dnBrg1 samples were counted for quantification.

Prior work revealed that MyoD targets Brg1 to the myogenin promoter during the initiation of myogenesis in tissue culture models for skeletal muscle differentiation (22, 26). ChIP analysis of the myogenin promoter showed that MyoD, not myogenin, bound along with Brg1 in satellite cells, suggesting that these two factors cooperate in the initiation of myogenin expression during adult myogenesis (Fig. 2*C*). In contrast, myogenin was preferentially associated with its own promoter in mature myofibers (Fig. 2*C*). These data indicate that myogenin binds to its own promoter in differentiated tissue, consistent with its appearance at times during embryogenesis when terminal differentiation is occurring or has already occurred (Fig. 1). Brg1 was also present on the myogenin promoter in myofibers, consistent with its presence on the promoter during the later stages of embryogenic myogenesis.

The data suggest the possibility that activation of the myogenin gene is mediated by MyoD whereas maintenance of myogenin expression may be mediated by myogenin itself. To mechanistically address this possibility, we utilized MyoD-differentiated B22 cells, which are NIH3T3-derived cells that express an ATPase-deficient, dominant negative Brg1 protein in a tetracycline-suppressible manner (17). Previous work using this system demonstrated that MyoD-driven differentiation faithfully recapitulated the events that occur during the activation of myogenic late genes during embryogenesis (11). We previously reported that MyoD binding to the myogenin promoter correlated with the changes in nuclease accessibility at the myogenin locus and activation of myogenin transcription (22). We also noted that both MyoD and myogenin could interact with the myogenin promoter in a Brg1-dependent manner 24 h after the initiation of differentiation (22). ChIP experiments over a time course of MyoD-mediated differentiation confirmed our earlier results and further revealed that as differentiation progressed beyond 24 h, MyoD binding to the promoter diminished whereas myogenin binding was enhanced (Fig. 3*A*). This apparent transition from MyoD binding to the promoter at the onset of myogenin expression to myogenin binding as the cells completed the differentiation process is similar to the observations made regarding MyoD and myogenin binding to the myogenin promoter in satellite cells and myofibers.

The ChIP data support the idea that myogenin, in combination with Mef2 and Brg1, is activating its own transcription at times subsequent to the initial activation of gene expression by MyoD and Brg1. To directly test this hypothesis, we ectopically expressed MyoD, myogenin, and Mef2D1b, the muscle-specific isoform of the Mef2D protein that is highly induced during myogenesis *in vivo* (11, 31), alone or in combination, in B22 cells to determine whether the endogenous myogenin gene could be activated. As expected, MyoD stimulated myogenin expression in a Brg1-dependent manner (Fig. 3*B*). Neither myogenin nor Mef2D1b alone could activate the myogenin gene; however, the combination of myogenin and Mef2D1b, in the presence of functional Brg1, could activate endogenous

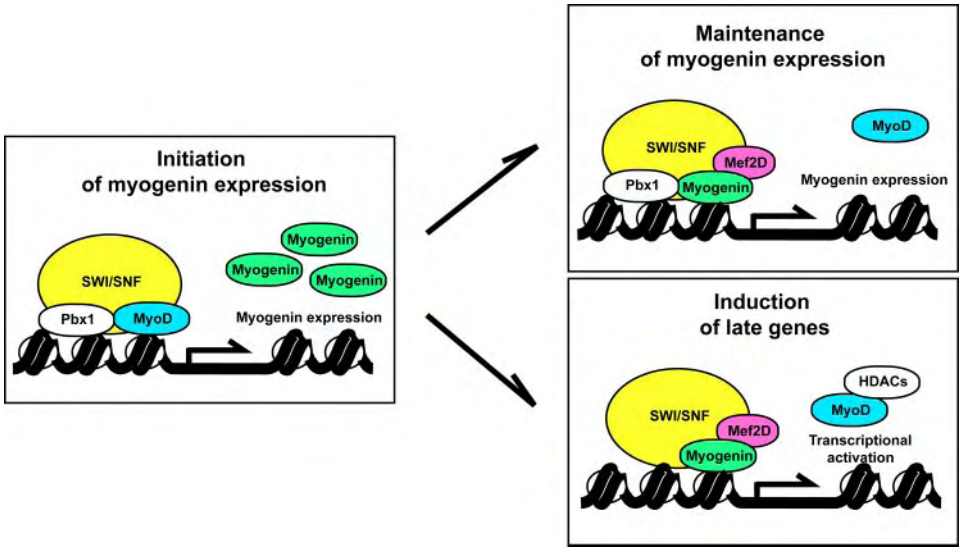

FIGURE 5. **Schematic representation of factor interactions at the myogenin promoter during myogenesis.** Genomic DNA is depicted as being nucleosomal. Initiation of myogenin transcription is dependent upon MyoD, the Pbx homeodomain factor, and SWI/SNF chromatin-remodeling enzymes (*left panel*) (reviewed in Ref. 2). Upon the expression of myogenin, myogenin, Mef2D, and Brg1 localize to the myogenin promoter (*right*, *top panel*) to maintain myogenin expression. At the same time, myogenin, Mef2D, and Brg1 also localize to and activate late gene loci, displacing the MyoD and HDAC2 present at these loci prior to late gene expression (11). The schematic representation of these molecules is for illustrative purposes; the relative size of each molecule is not to scale.

myogenin expression. Our previous data demonstrated that the combination of myogenin and Mef2D1b did not activate the endogenous *MyoD*, *Myf5*, or *Mrf4* loci in NIH3T3-based cell lines (Refs. 11, 19 and data not shown). Thus, expression from the endogenous myogenin locus is due solely to the activation potential of myogenin and Mef2D1b. ChIP experiments showed that the mechanism by which myogenin and Mef2D1b mediate myogenin expression is via recruitment of Brg1 to the myogenin promoter (Fig. 3*C*). The presence of Brg1 then mediated changes in chromatin structure at the myogenin locus, which are reflected as increases in restriction enzyme accessibility at a PvuII site upstream of the myogenin mRNA start site (Fig. 3*D*). Both MyoD and the combination of myogenin and Mef2D1b could recruit both the functional and ATPase-deficient, dominant negative forms of Brg1 to the promoter, but chromatin accessibility required functional Brg1. Thus, whereas myogenin is not believed to initiate its own expression *in vivo*, the data indicate that the myogenin protein, in conjunction with Mef2D1b, has the intrinsic ability to activate expression from the endogenous myogenin locus in the absence of MyoD. These data and the differential binding of MyoD and myogenin to the myogenin promoter in satellite cells and myofibers support the conclusion that myogenin and Mef2D1b are responsible for maintaining the expression of myogenin after the onset of myogenesis.

We further note that though the data indicate a role for myogenin in maintaining its own expression, a prior report has shown that the loss of myogenin in postnatal mice does not impact skeletal muscle formation (9). Thus, myogenin is not required to maintain expression from the myogenin locus nor is it required for any skeletal muscle-specific function. These data strongly suggest that there are redundant factors that can compensate for the loss of myogenin during adult myogenesis.

The involvement of the Brg1 SWI/SNF ATPase in the initiation of myogenesis in culture and during embryogenesis is well established (11, 19, 20, 22, 26). The data presented here suggest that Brg1 function is also required in differentiated tissue, but detailed information about the *in vivo* relevance of Brg1 function in myogenesis in the mouse has been difficult to obtain because knock out of the *Brg1* locus results in early embryonic lethality (32). To circumvent this problem, we adapted an *in vivo* electroporation protocol (24) to directly deliver plasmid DNAs encoding green fluorescent protein (GFP) and a *Brg1* allele containing a mutated ATP binding site to differentiated muscle tissue in the hind limb of a postnatal day 2 (P2) mouse. This ATPase-deficient protein has been characterized extensively and acts as a dominant negative by interfering with normal Brg1-dependent functions (17, 25). Tissue isolated 48 h post-electroporation revealed that nuclei showing GFP fluorescence (Fig. 4*A*, *white arrow*) did not express myogenin protein. Conversely, nuclei that did not take up plasmid DNA showed no GFP fluorescence but immunostained for myogenin (*yellow arrow*). Control experiments where GFP alone was electroporated showed that cells expressing GFP also expressed myogenin (Fig. 4*B*). Quantification of these results revealed that introduction of dominant negative Brg1 inhibited myogenin expression in ~90% of electroporated nuclei (Fig. 4*C*). The results demonstrate that functional Brg1 is required for expression of myogenin in fully differentiated skeletal muscle tissue. Thus, Brg1 is required for both the initiation and maintenance of myogenin gene expression *in vivo*.

DISCUSSION

Our earlier work demonstrated that although MyoD was present on myogenic late gene regulatory sequences during embryogenesis prior to activation of these genes, the predominant factors present at the time of late gene expression in the developing tissue were myogenin and Mef2D, along with the chromatin-remodeling enzyme Brg1 (11). We reasoned that if myogenin were being utilized to activate late gene expression during the time of terminal differentiation, it might also activate its own expression both at this time and following the completion of differentiation. Although there have been prior molecular studies examining the regulation of myogenin, we believe this is the first report to explore the relationship between specific MRF occupancy of promoters and the timing of differentiation in primary embryonic and adult tissue.

The data support our hypothesis. ChIP analysis in embryonic tissue shows that myogenin binds the myogenin promoter with the same kinetics it binds to late gene promoters. Further sup-

port was derived from ChIP studies of adult satellite cells and myofibers, where the myogenin promoter in differentiating satellite cells was occupied by MyoD and the promoter in differentiated myofibers was predominantly bound by myogenin. This implies that when myogenin becomes competent to activate gene expression in the developing embryo or in differentiating adult tissue, there are no structural features associated with the myogenin promoter that distinguish it from the late gene loci.

Based on these and earlier data, we propose that there is a transition in MRF binding during the terminal differentiation stages of embryonic and adult myogenesis. MyoD initially binds to both early and late myogenic genes, but activation of late genes is repressed due to the simultaneous presence of histone deacetylases and possibly other repressive chromatin-modifying enzymes at these loci (11). Following the induction of myogenin and Mef2 family members by MyoD, these factors mediate the transcriptional activation of late genes that specify muscle formation and the continued transcription of early myogenic genes such as myogenin. This model is presented schematically in Fig. 5. Such a model is both similar to and different from the conclusions of previous studies. Prior work comparing the activation domains of MyoD and myogenin in culture led to a model of sequential functions at some genes by MyoD and then myogenin (33). However, genome-wide factor binding studies, where analyzed regulatory sequences were identified as targets of MyoD, myogenin, or both (34, 35), were more recently used to conclude that the myogenic genes identified as targets of both MyoD and myogenin are cooperatively regulated by these MRFs. Although the concept of cooperative regulation could encompass either sequential or simultaneous binding by MyoD and myogenin, we note that the cell culture data shown here and previously (11) demonstrate that myogenin, in the presence of Mef2D, is capable of initiating and maintaining its own transcription and that of the rest of the myogenic genes without cooperative effects by MyoD or any other MRF. Further support for sequential function was recently provided by studies of the E box binding factor HEB, which can heterodimerize with MRFs. That study demonstrated that HEB acted synergistically with MyoD to activate transcription in cell culture at the onset of differentiation but synergized with myogenin following differentiation (36).

Although the data support the idea that myogenin is the relevant factor in maintaining its own expression, it is clearly not required for this function. Early studies demonstrated that a myogenin-lacZ transgene was expressed normally in myogenin null mice (37), and recent analysis of a conditional myogenin knock-out mouse indicated that loss of myogenin after myogenesis affected body size but no skeletal muscle deficiencies were observed (9). Thus, unlike during embryonic myogenesis, functional redundancy by other factors, presumably MRFs, can compensate for the lack of myogenin in adult tissues.

In contrast, the SWI/SNF chromatin-remodeling enzyme is required for continued expression of myogenin in differentiated tissue. Multiple reports show the requirement for SWI/SNF chromatin-remodeling function in culture, and the presence of SWI/SNF subunits on gene regulatory sequences in cultured cells and primary tissue (11, 19, 20, 22, 26) and genetic analysis of a non-enzymatic subunit, Baf60c, revealed a requirement for embryonic myogenesis (38). Here we provide the first *in vivo* evidence that the SWI/SNF enzymes are required for myogenic gene expression in differentiated muscle tissue. In combination with earlier cell culture studies, we suggest that Brg1 and SWI/SNF enzymes are required continuously throughout every stage of adult and embryonic myogenesis.

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Appendix D

MyoD Targets Chromatin Remodeling Complexes to the Myogenin Locus Prior to Forming a Stable DNA-Bound Complex

Transcription activation of muscle-specific genes requires the cooperation of muscle regulatory factors and chromatin remodeling enzymes. Gene expression analysis using microarray indicated that 30% of MyoD-induced genes required the function of SWI/SNF enzymes. Our contributions included the generation of mock and MyoD-differentiated samples and confirmation of targets identified in the microarray study. ChIPs were performed to analyze the molecular events occurring at the myogenin promoter and revealed that hyperacetylation of histone H4 preceded the binding of Brg1 in a MyoD-dependent manner. Binding of MyoD occurred before histone H4 modification and association of Brg1 with the promoter. Coimmunoprecipitations revealed that MyoD was able to interact with the constitutively bound homedomain factor Pbx1, prior to all chromatin remodeling events. This initial non-consensus binding permitted subsequent targeting of HATs and SWI/SNF. We performed coimmunoprecipitation in mock and MyoD-differentiated smaples and determined that Brg1 interacts with MyoD and Mef2. Thus a two-step mechanism exists where MyoD binds to constitutively bound Pbx1-Meis allowing binding of HAT and SWI/SNF, which facilitates chromatin remodeling at the myogenin locus, and ultimately permits direct binding by MyoD and other coactivators to initiate transcription.

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MyoD Targets Chromatin Remodeling Complexes to the Myogenin Locus Prior to Forming a Stable DNA-Bound Complex†

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The activation of muscle-specific gene expression requires the coordinated action of muscle regulatory proteins and chromatin-remodeling enzymes. Microarray analysis performed in the presence or absence of a dominant-negative BRG1 ATPase demonstrated that approximately one-third of MyoD-induced genes were highly dependent on SWI/SNF enzymes. To understand the mechanism of activation, we performed chromatin immunoprecipitations analyzing the myogenin promoter. We found that H4 hyperacetylation preceded Brg1 binding in a MyoD-dependent manner but that MyoD binding occurred subsequent to H4 modification and Brg1 interaction. In the absence of functional SWI/SNF enzymes, muscle regulatory proteins did not bind to the myogenin promoter, thereby providing evidence for SWI/SNF-dependent activator binding. We observed that the homeodomain factor Pbx1, which cooperates with MyoD to stimulate myogenin expression, is constitutively bound to the myogenin promoter in a SWI/SNF-independent manner, suggesting a two-step mechanism in which MyoD initially interacts indirectly with the myogenin promoter and attracts chromatin-remodeling enzymes, which then facilitate direct binding by MyoD and other regulatory proteins.

In eukaryotes, activation of gene expression involves the ordered assembly of transcriptional regulators, chromatin-modifying enzymes, RNA polymerase II, and associated general transcription factors onto *cis*-acting elements that are embedded in chromatin. Chromatin-remodeling enzymes play an integral role in gene activation by perturbing chromatin structure and making specific loci permissive for transcription. Molecular analysis of multiple gene activation events suggests that the temporal recruitment of transcription factors and chromatin-remodeling enzymes is gene specific and dictated by the interplay between specific activators and local chromatin structure (1, 52, 55).

Two classes of enzymes have been shown to remodel chromatin structure either by catalyzing covalent modifications of histones or by hydrolyzing ATP to mobilize nucleosomes. Among the latter class of enzymes are the SWI/SNF chromatin-remodeling complexes. A distinguishing feature of this family is the presence of a bromodomain in the ATPase subunit, which promotes interaction with acetylated histones and links the activities of the two classes of chromatin remodelers in the regulation of gene expression (22). SWI/SNF enzymes physically interact with histone acetyltransferases (HATs), histone deacetylases (HDACs), and methyltransferases, showing the potential for coordination of chromatin-remodeling activities (reviewed in reference 53).

Mammalian SWI/SNF chromatin-remodeling enzymes are multisubunit complexes that contain either the Brg1 or Brm ATPase subunits and can activate or repress expression of a subset of genes (39, 53). They function in cell cycle control, and some of the subunits are tumor suppressors (49). Diverse SWI/SNF complexes exist that are distinguished by the particular ATPase, the presence of unique subunits, and tissue-specific isoforms of common subunits (60, 61). The Brg1- and Brm-containing complexes are similar biochemically but display different physiological characteristics. In mice, disruption of Brg1 is early embryonic lethal while disruption of Brm has a mild effect on proliferation (6, 48). Moreover, the two ATPase subunits can be associated with different promoters (25, 38).

Mammalian SWI/SNF enzymes have been shown to facilitate the binding of TBP and other factors involved in polymerase II (Pol II) preinitiation complex formation and to promote transcriptional elongation both in vitro and in vivo (5, 11, 24, 33, 52, 55). Multiple models to explain targeting of SWI/SNF enzymes to specific regulatory sequences exist: interactions with RNA polymerase II holoenzyme (62), binding of bromodomains to acetylated histones (22), and recruitment by sequence-specific transcriptional activators (12). In yeast, interaction with activators is critical for SWI/SNF function (47), and in mammalian cells, SWI/SNF components interact with numerous activators, at least some of which likely target SWI/SNF to specific promoters (8, 11, 18, 26, 30, 32, 35, 46).

During the differentiation of skeletal muscle, the myogenic basic helix-loop-helix family of regulatory factors (MRFs) heterodimerize with ubiquitously present E proteins and bind to 6-bp elements called E boxes. MRFs interact with members of the myocyte enhancer family (MEF2) of proteins, which bind a conserved A/T-rich sequence in the regulatory regions of muscle-specific genes, to synergistically activate downstream muscle gene expression (42). Although each MRF can bind to the

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E box with similar affinities, myogenin promotes myogenesis less efficiently than Myf5 in mouse embryos and is less effective than MyoD in activating endogenous muscle-specific genes when introduced into fibroblasts (3, 19). MyoD-mediated gene activation is associated with chromatin remodeling in the regulatory regions of muscle-specific genes and depends on a cysteine-histidine-rich region and a carboxy-terminal region (16, 19). The carboxy-terminal alpha-helical region of MyoD that is distinct from that of myogenin specifies the ability to initiate muscle-specific gene expression (3).

During embryogenesis and skeletal muscle regeneration, it is the induction of MyoD and/or other MRF proteins that is critical for commitment to the skeletal muscle lineage, such as occurs in primary cell cultures and in activated satellite cells (44, 58). To model events controlling myogenic differentiation via the induction of MyoD, we have utilized the well-established model of MyoD-induced transdifferentiation of fibroblast cells, first used to identify MyoD as the regulator of myogenic differentiation (14).

We previously used this system to establish a role for SWI/SNF chromatin-remodeling enzymes in MyoD-mediated activation of two muscle-specific genes and correlated activation of myogenin transcription with changes in myogenin promoter chromatin structure (16). We later extended our results to show that several muscle-specific genes were also inhibited by dominant-negative SWI/SNF enzymes but that cell cycle control and expression of key cell cycle regulators, such as p21, cyclin D3, and Rb, were unaffected during muscle differentiation induced by the different MRFs (17, 50).

To more specifically describe the role that SWI/SNF chromatin-remodeling enzymes play in muscle differentiation, we performed a microarray analysis of cells differentiated by MyoD in the presence or absence of dominant-negative BRG1 and found that a subset of genes activated by MyoD require SWI/SNF enzymes. We demonstrate that MyoD induces histone H4 acetylation and localization of Brg1 at the myogenin promoter; however, stable MyoD binding to the promoter occurs only after chromatin modifications. Expression of dominant-negative BRG1 interferes with MyoD binding to its cognate E box on the myogenin promoter but does not affect acetylation of histone H4. This raises a paradox: interaction of SWI/SNF and acetylation of histones at the myogenin promoter require MyoD, but MyoD does not stably bind to the promoter in the absence of functional SWI/SNF enzyme. To address this, we demonstrate that the Pbx-1 homeodomain factor, which cooperates with MyoD to stimulate myogenin transcription, is constitutively bound to the myogenin promoter in a SWI/SNF-independent manner. This suggests a novel mechanism by which MyoD interacts with the promoter indirectly via Pbx-1 and recruits chromatin-remodeling enzymes, which then facilitate the binding of MyoD and other regulators. Demonstration of physical interactions between Brg1 and MyoD and Brg1 and Pbx support this conclusion. Models describing the role of SWI/SNF enzymes in the activation of the myogenin locus that address these and other recently published data (54) are discussed.

MATERIALS AND METHODS

Cell culture. The B22 cell line inducibly expressing dominant-negative BRG1 (15) was infected with retrovirus expressing MyoD- or MyoD-related regulators as previously described (14, 16, 17, 50). Briefly, this protocol involves culturing cells for 3 days in the presence of tetracycline (dominant-negative BRG1 repressed) or in the absence of tetracycline (dominant-negative BRG1 expressed) and passaging the cells so that 24 h later the cells are at about 50% confluence. The cells were infected with the retrovirus and incubated for 30 h. A low-serum differentiation medium was then added to induce myogenic differentiation. The time at which the differentiation medium was added is referred to as time zero. Samples were collected at the times indicated (hours) for analysis. Control samples were mock infected but still subjected to the differentiation protocol and are labeled "M" or "mock" for mock differentiated. Since the dominant-negative allele was derived from the human gene (28), capital letters are used throughout this report when describing the protein produced from this allele. Endogenous Brg1 in mouse-derived cell lines and the total amount of protein in mouse cells expressing the dominant-negative human allele are referred to as "Brg1."

Microarray analysis. For microarray analysis, RNA was prepared with the RNeasy kit from QIAGEN, and cDNA was generated as described previously (2). MyoD target genes were identified by comparing cells infected with MyoD-producing retrovirus ($n = 3$) to control cells ($n = 3$). Data points identified as unreliable by the scanner software were discarded. Data were normalized using the Lowess algorithm in the GeneSpring 6.0 analysis package (Silicon Genetics, Redwood City, CA). Normalized data were then transformed into log2 space. A heterocedastic *t* test was performed on each gene. The false discovery rate was estimated using Storey's *q* value (57). The resulting *q* values were used in conjunction with the magnitude severalfold change to identify significant genes at the thresholds described in the text. Brg1-dependent genes were identified in a similar fashion by comparing cells expressing a dominant-negative BRG1 ($n = 3$) to control cells ($n = 3$). Estimation of false-discovery rate (*q* value) for the Brg1 analysis was limited to the 94 genes identified as upregulated by MyoD.

RNA analysis. For reverse transcription (RT)-PCR, RNA was isolated and reverse transcribed as previously described (17). The cDNA was amplified with AmpliTaq Gold (Applied Biosystems) with 200 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 0.1 μg of each primer as described previously (17). MyoD and myogenin were amplified for 20 cycles with previously described primers (59). Hprt was amplified for 20 cycles with previously described primers (17). Amplification of p21 was for 20 cycles with (5′-ACACACAGAGAGAGG GCTAGG-3′) and (5′-AGATCCACAGCGATATCCAGAC-3′). Flag-BRG1 was amplified for 23 cycles with a primer to the BRG1 coding region (5′-GTA CAAGGACAGCAGCAGTGGA-3′) and primer to the Flag coding region (5′-TTTGTCATCGTCGTCCTTGTAGTC-3′). [^{32}P]dATP incorporation was detected with PhosphorImager (Molecular Dynamics), and quantification was performed using ImageQuant software.

Antibodies, protein extracts, Western analysis, and immunoprecipitations (IP). Commercial antibodies utilized were phosphatidylinositol 3-kinase (PI 3-kinase) (06-496; Upstate), Pbx1 (sc-889; Santa Cruz), Mef2 (sc-313; recognizes the Mef2A, -C, and -D isoforms; Santa Cruz), and MyoD (sc-304; Santa Cruz). MyoD chromatin immunoprecipitation (ChIP) experiments were confirmed using an affinity-purified rabbit antibody generated against a fusion protein between glutathione *S*-transferase (GST) and full-length MyoD. Polyclonal rabbit antisera raised against GST fused to a unique portion of BRG1 (15) was used for all experiments except for the coimmunoprecipitation studies in Fig. 8D and 9A, which utilized affinity-purified antibody isolated from rat antisera that was generated against the same GST-BRG1 fusion protein. Flag-tagged proteins were detected using rabbit antisera against a peptide encoding the Flag epitope or M2 Flag antibody (Sigma).

Isolation of protein and Western analyses were previously described (15). For coimmunoprecipitations in Fig. 9B, and C, cells were washed three times with phosphate-buffered saline and lysed with hypotonic buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 10 mM NaF, 2 mM sodium orthovanadate, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 3 μg/ml cytochalasin B, 5 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml aprotonin). Cell lysates were incubated at 4°C for 30 min, homogenized, and centrifuged at 3,000 × *g* for 10 min. Nuclei were then lysed with IP buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 200 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 2 mM sodium orthovanadate, 1 mM β-glycerophosphate, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin, and 2 μg/ml aprotonin). Nuclear extracts were incubated with 200 μg/ml DNase I and 10 μg/ml RNase A for 30 min at 26°C and then centrifuged at 15,000 × *g* for 15 min. The supernatant (250 μl) was rocked with 2 μg of antibody for 12 h at 4°C, followed by the addition of protein A Sepharose (Amersham) and an additional incubation for 6 h. The beads were washed three times in lysis buffer and eluted with 2% sodium dodecyl sulfate (SDS) sample buffer. The coimmunoprecipitation experiments in Fig. 8D and 9A utilized a previously published protocol (43). The coimmuno-

TABLE 1. Selected MyoD-induced genes affected twofold or more by dominant-negative BRG1

GenBank no.	UniGene no.	Name of gene product	Fold change	
			MyoD+/BRG1+[a]	Dom. Neg. BRG1[b]
NM_009394	Mm.1716	Troponin C2, fast	307.3	−10.1
NM_011620	Mm.14546	Troponin T3, skeletal, fast	225.7	−8.4
NM_011619	Mm.247470	Troponin T2, cardiac	219.3	−2.4
NM_016754	Mm.14526	Myosin light chain, phosphorylatable, fast skeletal muscle	209.9	−5.8
X15784	Mm.16528	Myogenin	186.3	−10.7
M38129	Mm.340090	Myosin, heavy polypeptide 3, skeletal muscle, embryonic	90.7	−19.1
X15784	Mm.16528	Myogenin	83.4	−7.3
NM_009405	Mm.39469	Troponin 1, skeletal, fast 2	71.2	−7.4
AL385643	Mm.269621	Myosin binding protein H	19.8	−10.4
Al324268	Mm.2375	Creatine kinase, muscle	16.6	−8.3
M19436	Mm.247636	Myosin, light polypeptide 4, alkali; atrial, embryonic	12.9	−4.1
Al324023	Mm.1000	Myosin, light polypeptide 1, alkali; atrial, embryonic	9.6	−3.6
NM_010518	Mm.309617	Insulin-like growth factor binding protein 5	9.1	−2.0
Al324248	Mm.35134	ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1	8.6	−4.1
Al414541	Mm.24059	Schwannomin-interacting protein 1	6	−2.2
Al661474	Mm.7342	PDZ and LIM domain 3	4.3	−2.2
Al325234	Mm.251322	Enolase 3, beta muscle	7.4	−2.5
NM_009668	Mm.4383	Bridging integrator 1	3.8	−2.3
Al604795	Mm.220982	Dysferlin	3.3	−2.1
Al326773	Mm.39968	Histidine-rich calcium binding protein	2.9	−2.5
Al447277	Mm.295105	(PTPRF), interacting protein (liprin), alpha 4	2.9	−2.1
M12866	Mm.214950	Actin, alpha 1, skeletal muscle	2.9	−2.2
Al430815	Mm.29475	CK2-interacting protein 1	2.8	−2
Al325457	Mm.22513	Kinesin family member C3	2.8	−2.3
Al385590	Mm.275654	Glycogen synthase 3, brain	2.3	−2.8
Al326236	Mm.31646	Actin-like 6	−2.2	−2.1

[a] Induction of gene expression after 24 h of differentiation with MyoD in the absence of dominant-negative BRG1.
[b] Fold change in induction after 24 h of differentiation with MyoD when dominant-negative BRG1 is expressed.

precipitation of Flag and MyoD (data not shown) utilized a different previously published protocol (15).

ChIPs. ChIPs were performed using the antibodies listed above as described previously (52), except that immune complexes were eluted with 0.1 M $NaHCO_3$ and 1% SDS, and following reversal of cross-links, the DNA was purified by proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. The purified DNA was dissolved in 50 μl Tris-EDTA, and 2 μl was used for PCR. For acetylated H4 ChIPs, the dissolved DNA was diluted 20-fold before PCR. Inputs were 0.5% to 1% of chromatin before immunoprecipitation. PCRs were performed with QIAGEN HotStart *Taq* master mix with 2 μCi [α-^{32}P]dATP for 32 cycles. PCR products were run on polyacrylamide gels and exposed to a PhosphorImager. Band intensities were quantified using the ImageQuant program. Primers to the myogenin regulatory region (34), the immunoglobulin H (IgH) enhancer (2), and the β-actin promoter (52) were described. Primers to the p21 promoter region were 5′-GTTGGTCTCCATCGGAATAG-3′ and 5′-GCCACATACATCTATGAACA-3′.

Restriction enzyme accessibility assay. Restriction enzyme accessibility experiments were performed as described previously (16) through purification of the digested genomic DNA. To visualize the cleaved DNA via PCR, a modified ligation-mediated PCR protocol was used. One microgram of digested DNA was ligated to 1 μl of 100 mM adaptor as described in reference 7 using Ligation kit version 2 (Takara). PCR amplification was performed with QIAGEN HotStart *Taq* master mix under the following conditions: 94°C for 15 min, followed by 26 cycles of 94°C for 30 s and then 65°C for 60 s, followed by 72°C for 60 s. PCR products were resolved in a 1.2% Tris-acetate-EDTA-agarose gel and stained with Sybr Green I. Primers used were the sense primer LM-PCR1, as described in reference 7, and the antisense primer used for myogenin ChIP (34). Quantification was performed by densitometry using NIH image 1.62 software.

RESULTS

A subset of MyoD-regulated genes are highly dependent on the activity of the SWI/SNF complex. Our previous studies demonstrated that SWI/SNF enzymes are necessary for MyoD to activate muscle gene transcription but not for MyoD to stimulate the expression of several cell cycle-regulated genes. To broadly assess the requirement of SWI/SNF enzymes for MyoD-mediated gene expression, we used spotted cDNA expression arrays with approximately 5,400 tiled features representing 4698 UniGene clusters (UniGene build no. 128, September 2003). We used NIH 3T3 murine fibroblasts that possess a tetracycline-suppressible, dominant-negative BRG1 allele (15, 28) and compared cells transduced with MyoD to control cells. Following 24 h in differentiation medium, MyoD increased the expression of 94 genes and decreased that of 70 genes ($q < 0.10$; change in expression greater than twofold) (Tables 1 and 2; see also Table S1 in the supplemental material). These 94 genes (represented by 96 array features) were analyzed for their dependence on a functional Brg1-based SWI/SNF complex. In the presence of dominant-negative BRG1, 29 genes did not achieve full activation by MyoD, as determined by statistical criteria ($q < 0.05$) and a twofold or more decrease in expression level (Table 1; see also Table S1 in the supplemental material). Some of the genes regulated by MyoD that were not dependent on SWI/SNF activity, such as pRb, cyclin D3, and p21 (17) (Table 2), are expressed prior to MyoD induction, whereas others, such as the beta and gamma subunits of the nicotinic acetylcholine receptor, are not expressed in fibroblasts and are induced by MyoD even in the presence of dominant-negative BRG1 (Table 2). We had previously documented that MyoD could activate cell cycle-regulated genes in the absence of functional SWI/SNF enzymes; however, the ability of MyoD to induce the expression of some previously silent loci in the absence of SWI/SNF function was not previously recognized. Of the 70 genes repressed by MyoD, only five were derepressed more than twofold in the presence

TABLE 2. Selected MyoD-induced genes affected twofold or less by dominant-negative BRG1

GenBank no.	UniGene no.	Name of gene product	Fold change	
			MyoD+/BRG1+[a]	Dom. Neg. BRG1[b]
NM_010866	Mm.1526	Myogenic differentiation 1[c]	42.7	1.1
M30514	Mm.2810	Cholinergic receptor, nicotinic, gamma polypeptide	19.6	−1
AI427434	Mm.256342	Kinesin family member 5C	12.3	1.8
AI385656	Mm.4583	Cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	10	−1.9
M14537	Mm.86425	Cholinergic receptor, nicotinic, beta polypeptide 1 (muscle)	8.6	−1.2
NM_013597.2	Mm.132788	Myocyte enhancer factor 2A	7.5	−1.7
AI323806	Mm.195663	Cyclin-dependent kinase inhibitor 1A (p21)	7.2	−1.5
AI894122	Mm.4261	Kangai 1 (suppression of tumorigenicity 6, prostate)	7.1	−1.2
AI326893	Mm.280029	Hairy and enhancer of split 6 (Drosophila)	5.5	1.0
AI323835	Mm.273862	Purinergic receptor (family A, group 5)	4.9	−1.0
NM_009029	Mm.273862	Purinergic receptor (family A, group 5)	4.8	−1.2
AI324186	Mm.297976	Glypican 1	4.7	−1.4
NM_011817	Mm.281298	Growth arrest and DNA-damage-inducible 45 gamma	4.2	1.1
AI451932	Mm.4081	Runt-related transcription factor 1	4.0	−1.8
AI450263	Mm.333762	Lectin, galactose binding, soluble 4	3.8	1.3
AI415710	Mm.25559	Serine/threonine kinase 17b (apoptosis-inducing)	3.5	−1.9
AI426448	Mm.28683	Transferrin receptor	3.4	−1.5
AI528676	Mm.347398	B-cell leukemia/lymphoma 6	3.1	−1.8
NM_011484	Mm.273174	Signal transducing adaptor molecule (SH3 domain and ITAM motif)	2.9	−1.2
AI428484	Mm.30841	Calcium channel, voltage-dependent, alpha2/delta subunit 1	2.9	−1.2
NM_007483	Mm.687	ras homolog gene family, member B	2.9	−1.6
NM_010722	Mm.7362	Lamin B2	2.8	1.2
AI326964	Mm.3862	Insulin-like growth factor 2	2.8	−1.9
AI450264	Mm.180750	Prion protein dublet	2.8	−1.7
AB025099	Mm.30262	Kruppel-like factor 5	2.8	−1.5
AI326148	Mm.289832	Proteasome (prosome, macropain) 26S subunit, ATPase 3	2.7	−1.3
AI429452	Mm.280805	Huntingtin-interacting protein 1	2.6	1.1
AI451071	Mm.25594	Protein kinase, cAMP dependent regulatory, type II beta	2.5	1.3
AI425917	Mm.29495	CUG triplet repeat, RNA binding protein 1	2.5	−1.0
AI326978	Mm.280103	ATPase, Na^+/K^+ transporting, alpha 1 polypeptide	2.5	−1.2
AI573376	Mm.22673	Fc receptor, IgE, high-effinity I, gamma polypeptide	2.5	−1.3
AI414367	Mm.65906	Pre-B-cell leukemia transcription factor-interacting protein 1	2.5	1.2
AI447937	Mm.12863	Heparan sulfate 2-*O*-sulfotransferase 1	2.5	−1.2
AI324262	Mm.294083	Annexin A11	2.5	−1.4
AI449015	Mm.206218	Histone deacetylase 11	2.4	1.0
AI324952	Mm.289131	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2	2.4	−1.1
AI323871	Mm.246520	Cyclin D3	2.3	−1.1
AI894225	Mm.19016	Drebrin 1	2.3	−1.1
AI449069	Mm.6529	Dystrophia myotonica kinase, B15	2.3	−1.3
U22445	Mm.177194	Thymoma viral proto-oncogene 2	2.2	−1.5
AI428800	Mm.206779	Cytoplasmic polyadenylation element binding protein 3	2.2	−1.3
AI325922	Mm.276826	Cofilin 2, muscle	2.2	1.3

[a] Induction of gene expression after 24 h of differentiation with MyoD in the absence of dominant-negative BRG1.
[b] Fold change in induction after 24 h of differentiation with MyoD when dominant-negative BRG1 is expressed.
[c] Exogenously expressed from retroviral vector.

of dominant-negative BRG1, suggesting that SWI/SNF enzymes play a limited role in MyoD-mediated gene repression (see Table S1 in the supplemental material).

Despite the fact that only a subset of MyoD-regulated genes are highly dependent on SWI/SNF enzymes based on a severalfold change and statistical criteria, the expression of most of the MyoD-regulated genes was reduced in the absence of an active SWI/SNF complex. Graphing the log of the ratio of gene expression in the presence or absence of dominant-negative BRG1 revealed a distribution centered on zero for all the genes in the array (median ratio in log2 space is −0.02) (Fig. 1A), indicating that SWI/SNF activity does not globally alter gene expression. In contrast, limiting the analysis to the 94 genes regulated by MyoD shifted the center of the distribution below zero (median ratio in log2 space is −0.56), indicating that MyoD-regulated genes require SWI/SNF activity to achieve their full level of expression more than the typical gene spotted on the array (Fig. 1B). This is not solely due to the contribution of the highly SWI/SNF-dependent MyoD targets, because limiting the analysis to the MyoD target genes that were not identified as Brg1 dependent by the statistical criteria also provides a skewed histogram, with a median ratio of −0.23, as opposed to the median near 0 for all genes (Fig. 1B, double-headed arrow). The subset of genes that is highly dependent on SWI/SNF for MyoD activation is represented in the asymmetric tail of genes with negative ratios (29 genes demonstrate more than a twofold decrease in expression in the presence of dominant-negative BRG1; see Fig. 1B, unfilled bars). Therefore, the array data reveal a modest global dependence of MyoD-regulated genes on SWI/SNF enzymes and a more profound dependence for approximately one-third of the MyoD-regulated genes tested.

To better document the role of SWI/SNF enzymes during the induction of the subset of muscle marker genes that are highly dependent on these chromatin remodelers for MyoD-mediated gene activation, we elected to focus on the myogenin gene. Previously, we demonstrated that myogenin activation by MyoD or other members of the MyoD family of muscle regu-

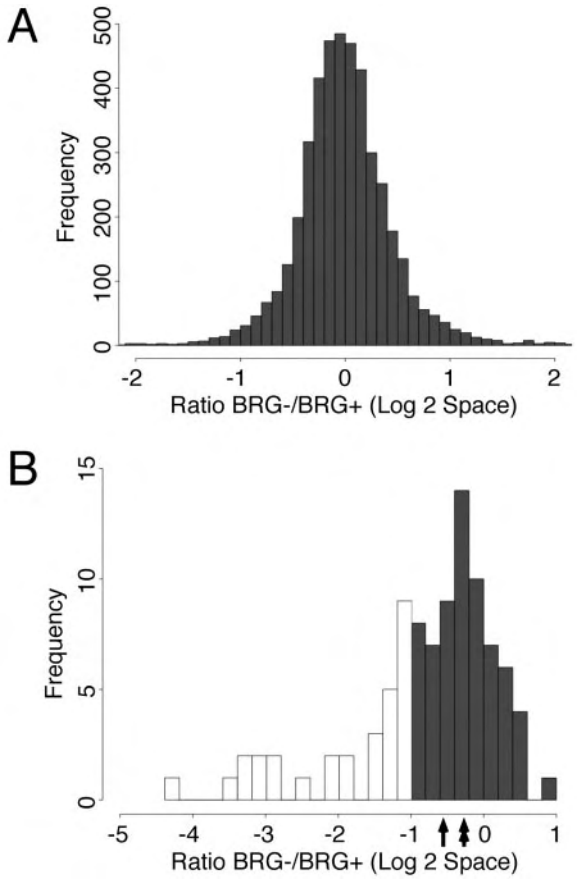

FIG. 1. (A) Histogram of the expression ratio in cells expressing dominant-negative BRG1 (BRG1−) to nonexpressing cells (BRG1$^+$) for the 4,282 array features that reported reliable data. The median value in log2 space is −0.02. (B) Histogram of the BRG1−/BRG1+ expression ratio for the 94 genes upregulated by MyoD. Hollow boxes indicate the 29 genes identified as strongly BRG1 dependent at the twofold change ($q < 0.05$) threshold. The single-headed arrow indicates the median ratio for all 94 MyoD-dependent genes (−0.56). The double-headed arrow indicates the median for the 65 genes not identified as strongly BRG1 dependent (−0.23).

latory proteins failed in the presence of dominant-negative SWI/SNF enzymes and showed that SWI/SNF-dependent myogenin activation correlated with a SWI/SNF-dependent increase in promoter accessibility at the endogenous myogenin locus (16, 17, 50). We therefore sought to build on this base of knowledge by temporally examining the interplay of SWI/SNF enzymes and myogenic transcription factors during myogenin activation.

Kinetics of myogenin activation during MyoD-mediated differentiation. We first performed a temporal analysis of gene expression. We infected cells with a MyoD-expressing retrovirus for 30 h, induced differentiation by adding a low-serum differentiation medium at time zero, and took samples for analysis at the indicated time points. RT-PCR analysis showed that MyoD was expressed 8 h before the addition of differentiation medium and remained constant throughout differentiation in the presence and absence of tetracycline (Fig. 2A). A slight increase in the amount of myogenin mRNA was apparent in the hours following addition of the differentiation medium, with a significant increase in mRNA levels after 8 h of differentiation. Myogenin expression was inhibited by dominant-negative BRG1 at all time points (Fig. 2A and B). Induction of p21 cyclin-dependent kinase inhibitor occurred 4 h after differentiation and continued to increase but was less than twofold affected by dominant-negative BRG1, in agreement with previous work (17, 50) and with the microarray results (Table 2).

The levels of Brg1 and Brm as well as the levels of Flag-tagged dominant-negative BRG1 remained constant during MyoD-mediated differentiation (Fig. 2C). Interestingly, overall expression of Brg1 did not change when dominant-negative BRG1 was present, demonstrating that induction of dominant-negative BRG1 did not result in overexpression of total Brg1.

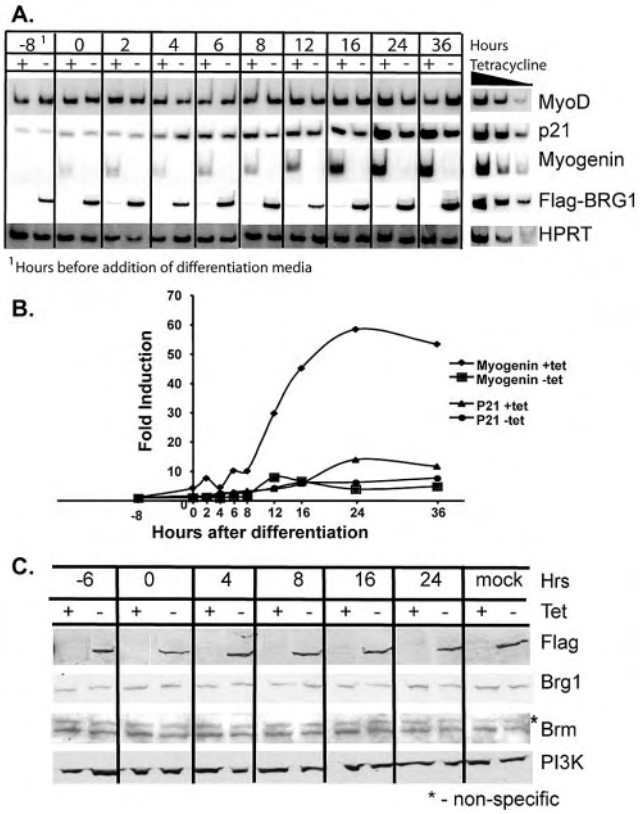

FIG. 2. (A) Time course of myogenin, p21, and ectopic MyoD expression during differentiation. Cells expressing or not expressing dominant-negative flag-tagged BRG1 were infected with retrovirus containing MyoD. Thirty hours later, differentiation was initiated by replacement of the medium with a low-serum differentiation medium (time zero). mRNA levels of each gene were examined at the indicated time points by RT-PCR. The −8 time point is 8 h prior to addition of differentiation medium. A titration of twofold dilutions of cDNA shows the linearity of the PCRs. The 36-h time point, plus-tetracycline (Tet) sample, was used for all titrations except for Flag-tagged dominant-negative BRG1, which was amplified with the 36-h, minus-tetracycline sample. (B) Quantification of mRNA levels observed in panel A. Fold induction was defined as the ratio of myogenin or p21 levels in a given sample relative to the levels of Hprt in the same sample and standardized to the −8-h time point. (C) Time course of dominant-negative BRG1, total Brg1, and Brm protein levels during differentiation. A Western blot was performed with protein extracts isolated at the indicated time points. "Mock" refers to the samples that were not infected with the MyoD-encoding retrovirus but were instead mock infected, subjected to the same differentiation protocol, and harvested 24 h after the addition of differentiation medium.

This effectively eliminates the possibility that expression of dominant-negative BRG1 results in nonspecific effects on transcription and promoter localization due to overexpression. It also suggests that a compensating mechanism exists for regulating Brg1 levels in cells. Tight regulation and compensation for levels of the SWI/SNF enzyme subunits Brm, Baf57, and Ini1 have previously been demonstrated (9, 20, 48).

We then examined changes in nuclease accessibility at the endogenous myogenin promoter. Previously, we reported a differentiation-dependent increase in restriction enzyme accessibility at a PvuII site 370 bp upstream of the transcription initiation site and showed that the change in accessibility required SWI/SNF chromatin remodeling activity (16). We monitored the change in accessibility at this site as a function of time of differentiation, using a modified protocol in which a linker DNA was ligated to the purified, digested genomic DNA fragments followed by PCR amplification to permit visualization of the cleaved DNA (see Materials and Methods). We observed a small but noticeable increase in accessibility at 4 h postdifferentiation and a continued increase in accessibility as differentiation proceeded (Fig. 3). Thus, the change in promoter accessibility precedes the onset of myogenin mRNA accumulation.

Histone acetylation and recruitment of SWI/SNF to muscle-specific promoters occurs in response to MyoD-mediated differentiation. We previously showed that chromatin remodeling is inhibited by dominant-negative BRG1 in MyoD-differentiated cells (16). Upon differentiation, hyperacetylation of histones surrounding the MyoD and Mef2 binding sites of several muscle-specific genes has been reported (34, 37). To determine whether dominant-negative BRG1 inhibits acetylation of histone H4, we differentiated cells with MyoD in the presence or absence of tetracycline, cultured the cells in differentiation media for 36 h, and then performed ChIP analysis. Figure 4 shows that muscle differentiation resulted in histone H4 hyperacetylation at the myogenin promoter and that H4 hyperacetylation was not inhibited by dominant-negative BRG1 in cells differentiated in the absence of tetracycline. This indicates that hyperacetylation of histone H4 occurs independently of SWI/SNF chromatin remodeling. We also looked at the acetylation status of the p21 promoter, because p21 expression is up-regulated during muscle differentiation but is not dependent on functional SWI/SNF chromatin-remodeling enzymes (17). We found a high level of histone acetylation at the p21 promoter in both mock-differentiated and differentiated cells compared to results with the silent IgH enhancer (Fig. 4B and C). This substantiates our previous conjecture that in fibroblasts, the promoters of genes that are constitutively expressed at low levels or that are regulated in a cell cycle-dependent manner may not require extensive chromatin remodeling.

To show that Brg1 is directly acting at these regulatory regions, we performed ChIP analysis with a BRG1 antibody and an antibody to the Flag epitope to detect epitope-tagged dominant-negative BRG1. We found that Brg1 was recruited to the myogenin promoter upon differentiation in the presence and absence of tetracycline and that Flag-tagged dominant-negative BRG1 could also be localized to these regions in differentiated cells (Fig. 4B and C). Surprisingly, although p21 up-regulation during muscle differentiation is not appreciably affected by dominant-negative BRG1 (17, 50) (Fig. 2A and B and Table 2), Brg1 was localized to the promoter region both

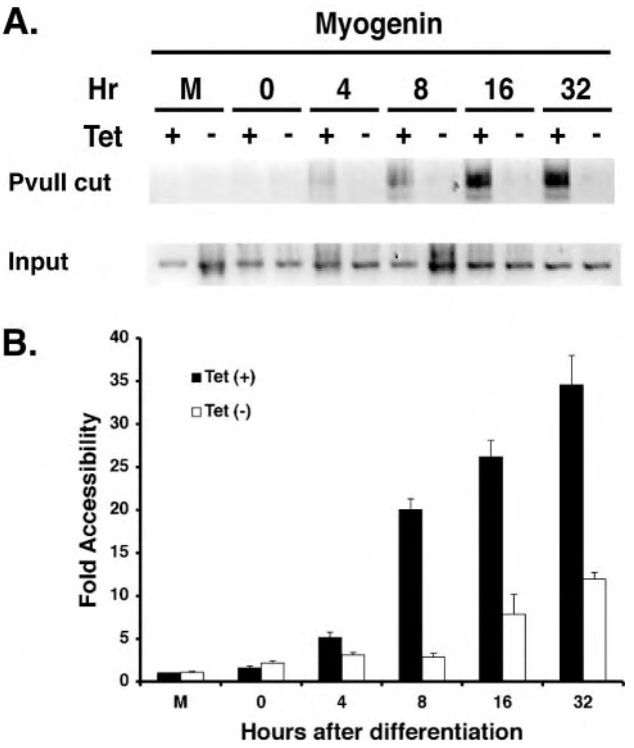

FIG. 3. Restriction enzyme accessibility increases at the endogenous myogenin promoter as a function of MyoD-induced differentiation and requires functional Brg1 based-SWI/SNF enzymes. Nuclei were isolated from cells that were differentiated in the presence or absence of tetracycline at the indicated time points or from cells that were mock differentiated (M) in the presence or absence of tetracycline. The mock-differentiated cells were not infected with the MyoD-encoding retrovirus but were subjected to the differentiation protocol for 32 h. (A) A modified LM-PCR protocol (see Materials and Methods) was utilized to visualize cleaved genomic DNA isolated from nuclei digested with PvuII, which cleaves the myogenin promoter at −370 relative to the start site of transcription. The PCR product was visualized by Sybr Green I staining, and an inverse image is shown. To monitor the input DNA, 10% of the amount of purified, cleaved DNA that was used for ligation-mediated PCR was used to amplify the sequences between −143 and −5 of the myogenin promoter, which contain no PvuII site. (B) Quantification of the change in nuclease accessibility at the myogenin promoter. The relative values for each time point were normalized to input and graphed relative to the value obtained for cleavage in the mock-differentiated, plus-tetracycline [Tet(+)] sample, which was arbitrarily set at 1.0. Each value is the mean ± standard deviation from three independent experiments.

in mock-differentiated and differentiated cells (Fig. 4B). SWI/SNF enzymes have been reported to play a direct role in the regulation of p21 transcription in other cell types that are actively proliferating; therefore, the requirement for SWI/SNF enzymes may be cell type or cell cycle stage specific (23, 27). In fibroblasts, the local chromatin structure on the p21 promoter may not require extensive chromatin remodeling by SWI/SNF enzymes during muscle differentiation and/or there may be redundant mechanisms for achieving remodeling. Thus, SWI/SNF enzymes likely contribute to p21 expression but are not required as they are for many of the muscle-specific genes.

MRF and Mef2 association with muscle-specific promoters at an endpoint of muscle differentiation is inhibited by dominant-negative BRG1. It is generally thought that transcrip-

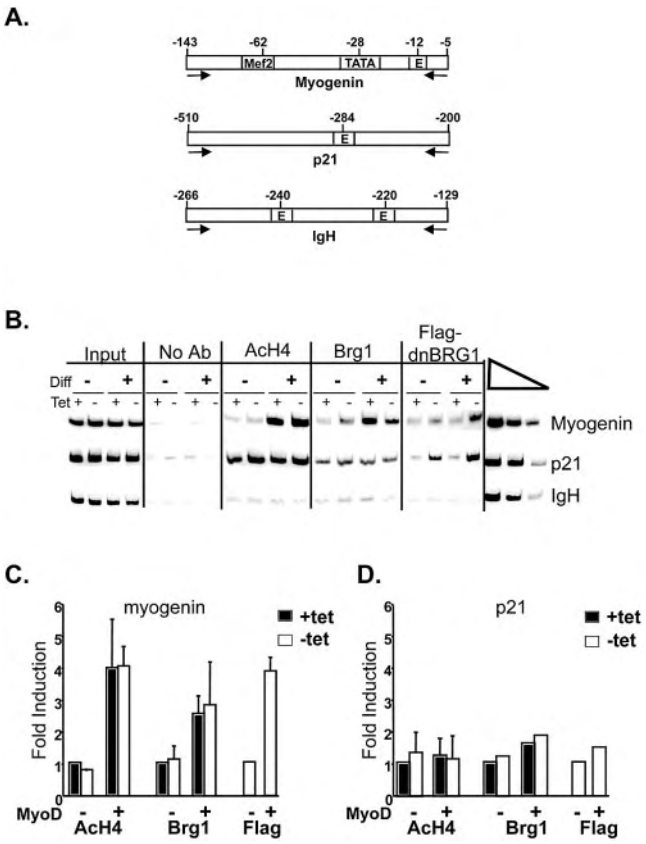

FIG. 4. Brg1 and hyperacetylated H4 are associated with the myogenin promoter. (A) A schematic diagram indicating the regions of the myogenin and p21 promoters and the IgH enhancer that were amplified. Arrows indicate the location and direction of primers used for amplification. The approximate locations of transcription factor binding sites are indicated. (B) Chromatin immunoprecipitations were performed with antisera against BRG1, Flag, or tetra-acetylated histone H4 (AcH4) or with no antibody (No Ab) on mock-differentiated (−) or MyoD-differentiated (+) samples that had been cultured in the presence or absence of tetracycline (tet) and that were harvested for analysis 36 h after the onset of differentiation. PCR amplification of 1% of the input DNA is shown on the left. A twofold titration of input DNA using the undifferentiated, plus-tetracycline sample was performed (far right) to show that the PCR was in the linear range. (C and D) Quantification of the levels of hyperacetylated H4, Brg1, and Flag-tagged dominant-negative BRG1 present on the myogenin and p21 promoters by ChIP analysis. Band intensities in each lane were normalized to input. Induction relative to the plus-tetracycline, mock-differentiated sample is shown for hyperacetylated H4 and Brg1. Induction relative to the minus-tetracycline, mock-differentiated sample is shown for Flag-tagged dominant-negative BRG1. The data reflect the means ± standard deviations from three to four independent experiments, except for the levels of Brg1 and Flag-tagged dominant-negative Brg1 on the p21 promoter, which reflect the average values from two independent experiments.

tional regulators play a critical role in targeting of chromatin-remodeling complexes to specific promoters. Both MyoD and Mef2 have been shown to interact with HATs and/or HDACs (41). We therefore conducted ChIP analysis of cells following differentiation in the presence or absence of dominant-negative BRG1 using antibodies to MyoD, myogenin, and Mef2 to localize these proteins on the myogenin promoter. Figure 5A and B shows that MyoD, myogenin, and Mef2 were associated with the myogenin promoter in differentiated cells with wild-type Brg1 activity; however, there was marked inhibition of binding by all three proteins when dominant-negative BRG1 was expressed. No binding of MyoD, myogenin, or Mef2 was observed on the β-actin promoter or the inactive IgH enhancer, which contains a consensus E box. Since MyoD and myogenin are structurally similar, we infected separate NIH 3T3 cell cultures with retroviruses encoding one of each of the MyoD family of myogenic regulators (50) to demonstrate that the MyoD and myogenin antibodies do not cross-react (Fig. 5C). This indicates that both MyoD and myogenin can occupy the myogenin promoter.

We have previously shown that ectopic expression of MyoD is not affected by dominant-negative BRG1 (16) (Fig. 1; see also Fig. 8A), while endogenous myogenin induction is profoundly inhibited and Mef2 induction is inhibited to a lesser extent (16, 17) (Fig. 2A and B; see also Fig. 9). Thus, the observed inhibition of myogenin and Mef2 binding in the presence of dominant-negative BRG1 is at least in part due to reduced levels of these proteins. However, this is not the case

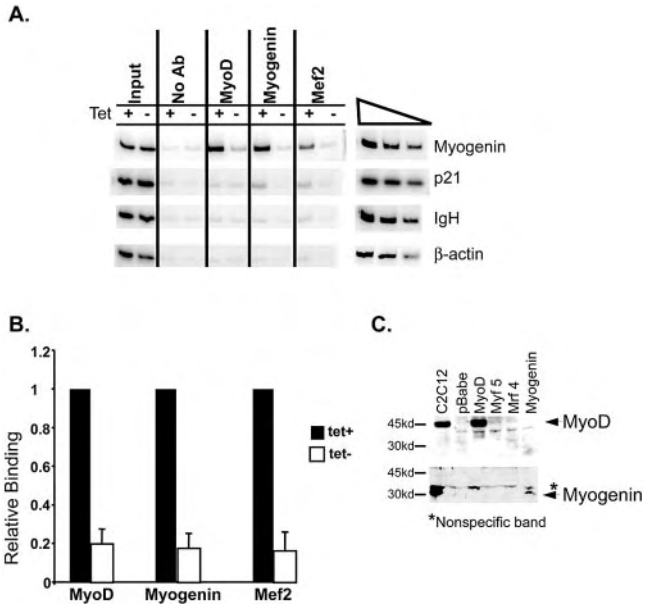

FIG. 5. Binding of muscle regulatory proteins to the myogenin promoter is inhibited by dominant-negative BRG1. (A) Chromatin immunoprecipitations were performed with antisera against MyoD, myogenin, or Mef2 or with no antibody (No Ab) on MyoD-differentiated samples that had been cultured in the presence or absence of tetracycline (tet) and that were harvested for analysis 36 h after the onset of differentiation. PCR amplification of 0.5% of the input DNA is shown on the left. A twofold titration of input DNA using the undifferentiated, plus-tetracycline sample was performed (far right) to show that the PCR was in the linear range. (B) Quantification of the level of MyoD, myogenin, or Mef2 present on the myogenin promoter by ChIP analysis. Band intensities in each lane were normalized to input. The decrease in promoter association due to the expression of dominant-negative BRG1 is expressed relative to the differentiated, plus-tetracycline sample, which was set at 1.0. The data reflect the mean ± standard deviation from three independent experiments. (C) MyoD and myogenin antisera do not cross-react. Protein extracts from C2C12 myotubes or NIH 3T3 cells infected with MyoD, myogenin-, MRF4-, or Myf5-containing retrovirus or the empty retroviral vector (pBABE) were run on an SDS-polyacrylamide gel, blotted, and probed with either anti-MyoD or antimyogenin antiserum.

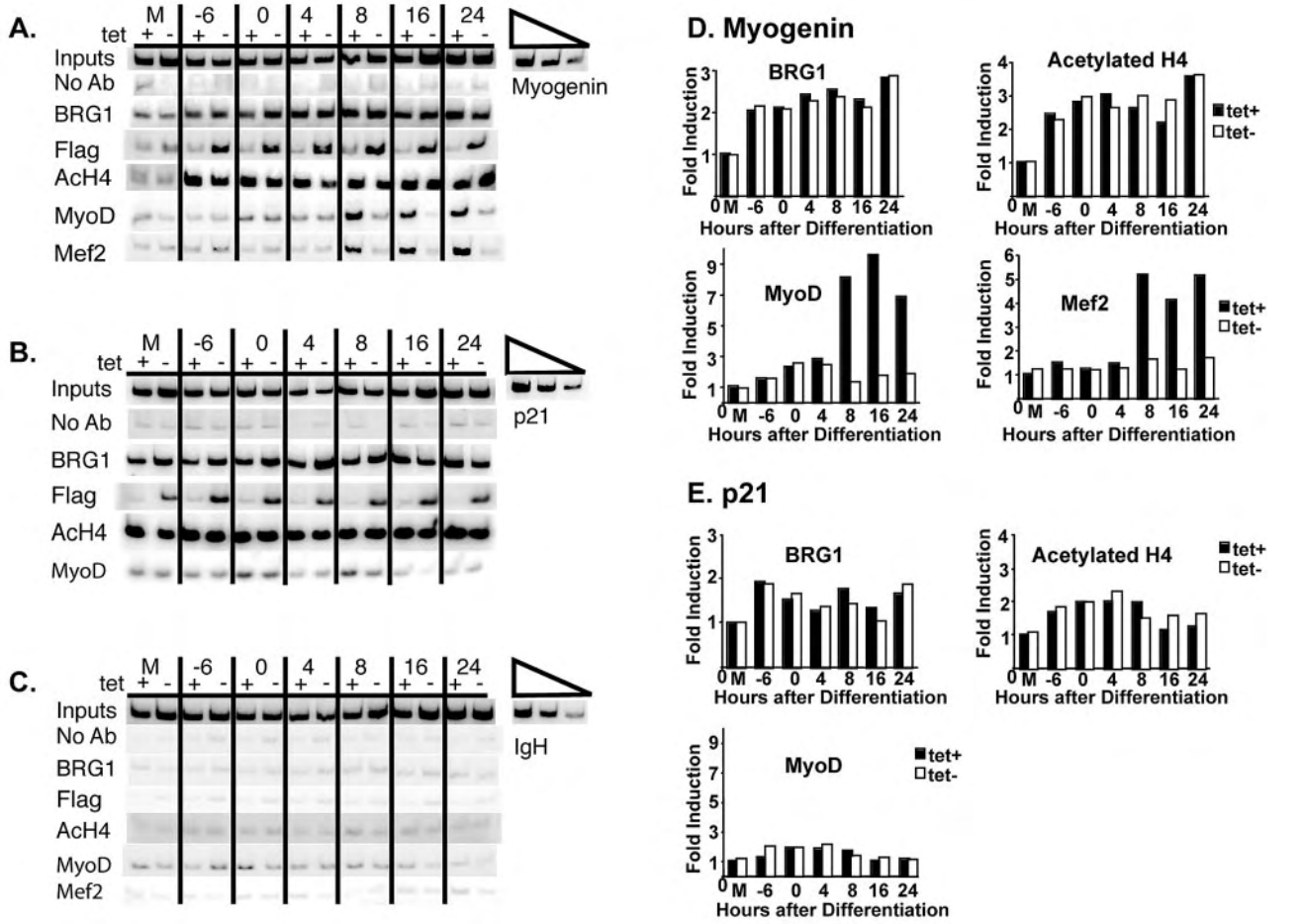

FIG. 6. Brg1 and acetylated H4 associate with the myogenin promoter prior to stable binding of MyoD and Mef2. Shown is a time course of histone H4 acetylation, total Brg1, dominant-negative BRG1, MyoD, and Mef2 association with (A) the myogenin promoter, (B) the p21 promoter, and (C) the IgH enhancer as measured by ChIP during a time course of differentiation induced by MyoD in the presence or absence of tetracycline (tet). M indicates samples that were mock differentiated for 24 h. "No Ab" indicates ChIP reactions performed in the absence of antibody. Linearity of the PCRs was demonstrated by twofold titrations of input DNA using the mock-differentiated, plus-tetracycline sample. PCR amplification of 1% of the input DNA is shown. (D and E) Quantification of the levels of acetylated H4, Brg1, MyoD, or Mef2 present on the myogenin promoter (D) or on the p21 promoter (E) by ChIP analysis. Band intensities in each lane were normalized to input. Induction relative to the plus-tetracycline, mock-differentiated sample is shown. The data reflect the average value from two independent experiments.

for the inhibition of MyoD binding caused by dominant-negative BRG1. The inhibition of MyoD association with the myogenin promoter suggests that SWI/SNF enzyme function is required to form a stable DNA binding complex within chromatin and does not support the idea that MyoD stably bound to chromatin directly targets Brg1-containing SWI/SNF enzymes to muscle-specific promoters.

Activation of p21 expression is critical for muscle differentiation and is promoted by MyoD (21, 45, 63). Although there are potential E boxes in the upstream region of p21, we did not detect significant levels of MyoD, myogenin, or Mef2 binding on the endogenous p21 upstream region at the end of the differentiation protocol by ChIP analysis (Fig. 5A), suggesting that MyoD activates p21 expression by an indirect mechanism and/or that our ChIP experiments do not detect an indirect association of MyoD with the p21 promoter through protein-protein interactions as previously was demonstrated for MyoD and CREB on the Rb promoter (36).

Kinetics of myogenin promoter interactions during MyoD-mediated differentiation. To determine how promoter interactions might influence the timing of myogenin expression, we performed ChIPs over the time course of differentiation (Fig. 6A). We found that Brg1 was recruited to the promoter 6 h before addition of differentiation medium and remained present throughout differentiation. Dominant-negative BRG1 was also present on the myogenin promoter, as seen by the ChIPs of Flag-tagged dominant-negative BRG1. Likewise, histone H4 on the myogenin promoter was hyperacetylated by 6 h prior to differentiation and was unaffected by dominant-negative BRG1. This indicates that chromatin-remodeling enzymes are associated with the myogenin promoter prior to significant gene expression and suggests that additional chromatin modifications may occur before activation of transcription. On the p21 promoter, recruitment of Brg1 and acetylation of histone H4 did not change significantly as a function of muscle differentiation, and neither was affected by expression of dominant-

negative BRG1 (Fig. 6B). Neither Brg1 nor hyperacetylated H4 was present on the inactive IgH enhancer (Fig. 6C).

ChIPs with the MyoD antibody showed that MyoD was bound at 8, 12, and 24 h after the addition of differentiation medium but not if the cells were differentiated in the presence of dominant-negative BRG1. Stable association of MyoD with the myogenin promoter was dependent on functional SWI/SNF enzymes and occurred just prior to the significant increase in myogenin gene expression after 8 h postdifferentiation (Fig. 2A and B and 6A). These results were confirmed with a different antibody against MyoD (data not shown). ChIP experiments to detect Mef2 binding on the promoter generated similar results (Fig. 6A). In contrast, amplification of the p21 promoter or IgH enhancer showed no or minimal interaction of MyoD with these sequences (Fig. 6B and C).

These results indicate that histone hyperacetylation and recruitment of Brg1 occur early during the differentiation process and well prior to stable binding of MyoD to the myogenin promoter. Bromodomains of chromatin-remodeling enzymes display high affinity for acetylated histones, and histone acetylation can facilitate the recruitment of SWI/SNF enzymes (22). To determine whether histone acetylation occurs prior to the recruitment of Brg1 to the myogenin promoter, we conducted ChIPs at earlier time points. Figure 7A shows that histone H4 hyperacetylation could first be detected 12 h after infection with the MyoD retrovirus or 18 h before addition of the differentiation medium at time zero, whereas recruitment of Brg1 occurred 19 h after retroviral infection or 11 h before addition of differentiation media. These results demonstrate that histone H4 acetylation coincides with the appearance of detectable levels of MyoD protein (Fig. 7B and C) and precedes the recruitment of Brg1, suggesting that histone acetylation facilitates the interaction of SWI/SNF complexes with the myogenin promoter. Thus, during MyoD-directed differentiation, the myogenin promoter becomes hyperacetylated on H4 prior to Brg1 recruitment. Although these initial events depend on MyoD, they precede stable binding of MyoD to the promoter in a SWI/SNF-dependent manner, raising the question of how the chromatin-remodeling enzymes become specifically localized to the promoter.

Pbx1 is constitutively associated with the myogenin promoter and participates in the recruitment of chromatin-remodeling complexes. Recent studies suggest that MyoD is recruited to the myogenin promoter through interaction of its cysteine/histidine and helix 3 regions with a homeodomain protein complex containing Pbx and Meis that is constitutively bound to the myogenin promoter (4). If targeting of chromatin-remodeling enzymes occurs via the Pbx-Meis site, one would predict that factor binding to this site would be independent of chromatin-remodeling activities. To address this question, we performed ChIPs with an antibody against Pbx1 during a time course of differentiation in the presence or absence of dominant-negative BRG1. Western analyses showed that Pbx1 levels did not change during MyoD-mediated muscle differentiation and were not affected by dominant-negative BRG1 (Fig. 8A). Figure 8B and C shows that, as previously reported, Pbx1 was constitutively associated with the myogenin promoter (4). Significantly, association of Pbx1 was not affected by dominant-negative BRG1; thus, Pbx1 potentially plays a role in the recruitment of Brg1 and other chromatin-remodeling activities to the myogenin promoter, perhaps via a direct interaction or via indirect recruitment through MyoD bound to the Pbx/Meis complex.

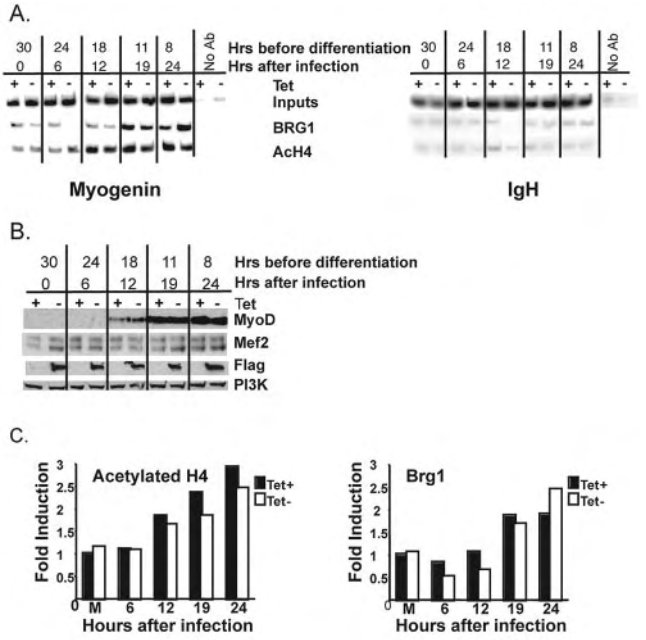

FIG. 7. (A) Histone H4 hyperacetylation precedes the binding of Brg1 at the myogenin promoter. Shown is a time course of histone H4 hyperacetylation and Brg1 association with the myogenin promoter. Cells were grown in the presence or absence of tetracycline (Tet), infected or not with MyoD-encoding retrovirus, and harvested for ChIP at the indicated times prior to the addition of differentiation medium. Time points are also indicated as hours following retroviral infection. Association with the IgH enhancer is presented as a control. One percent of the input is shown. (B) Western analysis of MyoD and Mef2 protein present at the indicated times prior to addition of differentiation medium. Anti-Flag antiserum was used to document the presence of the Flag-tagged dominant-negative BRG1. PI 3-kinase (PI3K) is shown as a loading control. (C) Quantification of the levels of acetylated H4 and Brg1 present on the myogenin promoter by ChIP analysis. Band intensities in each lane were normalized to input. Induction of levels of association is presented relative to the plus-tetracycline, mock-differentiated sample. The data reflect the average value from two independent experiments.

The previously published studies indicate that MyoD and Pbx1 interact at the myogenin promoter both in vivo and in vitro (4, 29). We therefore tested whether endogenous Brg1 and endogenous Pbx1 can physically interact before the onset of stable MyoD binding and the initiation of myogenin transcription. Figure 8D demonstrates that Brg1 from nuclear extracts prepared from differentiated cells coimmunoprecipitated with Pbx1 at the onset of differentiation and at 4 h postdifferentiation. The interaction was not observed in mock-differentiated cells and was not appreciably affected by the expression of dominant-negative BRG1 in the cells, indicating that the mutant BRG1 molecule also likely interacts with Pbx1. The results reveal that a specific Brg1-Pbx1 interaction occurs in the presence of MyoD, thereby supporting the idea that SWI/SNF enzymes are targeted by a MyoD-Pbx1 complex that is present on the promoter prior to the stable interaction of MyoD with the chromatin.

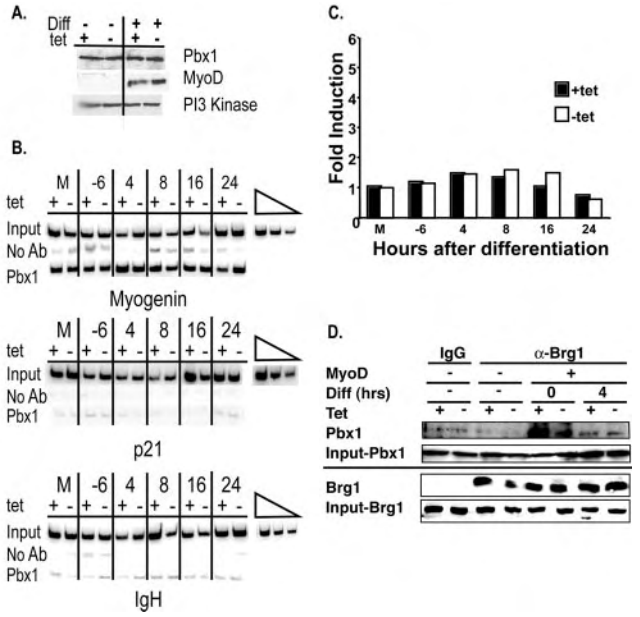

FIG. 8. Pbx1 mediates targeting of Brg1 to the myogenin promoter. (A) Pbx1 protein levels are unaffected by differentiation (Diff.) or by the expression of dominant-negative BRG1. Protein extracts from mock-differentiated cells (−) or cells differentiated with MyoD (+) in the presence or absence of tetracycline were run on an SDS-polyacrylamide gel and probed with Pbx1, MyoD, or PI 3-kinase antibodies. (B) Pbx1 association with the myogenin promoter, the p21 promoter, and the IgH enhancer as measured by ChIP during a time course of differentiation induced by MyoD. M indicates samples that were mock differentiated for 24 h. The linearity of the PCRs was demonstrated by a twofold titration of input DNAs using the mock-differentiated, plus-tetracycline (+tet) sample. PCR amplification of 1% of the input DNA is shown. (C) Quantification of Pbx1 association with the myogenin promoter. Band intensities in each lane were normalized to input. Induction relative to the plus-tetracycline, mock-differentiated sample is shown. The data reflect the average of values from two independent experiments. (D) Endogenous Pbx1 and Brg1 coimmunoprecipitate from MyoD-differentiated but not mock-differentiated cells. Nuclear extracts from mock (−) or MyoD-differentiated (+) cells were immunoprecipitated with Brg1 antibody or purified IgG as indicated, and the immunoprecipitated material was run on an SDS-polyacrylamide gel, transferred to a membrane, and probed for the presence of Pbx1 and Brg1. The levels of Pbx1 and Brg1 present in 10% of the input for each sample are shown.

Brg1 interacts with MyoD and Mef2 in differentiated cells. To further examine mechanisms for recruitment of SWI/SNF enzymes to the myogenin promoter, we investigated whether endogenous Brg1 could stably associate with MyoD and/or Mef2 in differentiating cells. Pulldown of Brg1 from differentiated cell nuclear extracts demonstrated that MyoD and Brg1 could be coimmunoprecipitated at the onset of differentiated as well as at later times and that MyoD was capable of interacting with the dominant-negative Brg1 protein as well (Fig. 9A). As an independent confirmation of this interaction, an antibody against the Flag epitope that marks the dominant-negative BRG1 was used for immunoprecipitation to show that the mutant BRG1 interacted with MyoD (data not shown). Others have also demonstrated that MyoD and Brg1 can be coimmunoprecipitated from extracts of differentiating cells (54).

Finally, we demonstrate that an antibody against BRG1 can coimmunoprecipitate Mef2 from MyoD-differentiated cells (Fig. 9B), while the converse experiment showed that an antibody against Mef2 could coimmunoprecipitate Brg1 (Fig. 9C). An interaction between Mef2 and Brg1 was also detected in the absence of tetracycline when dominant-negative BRG1 was expressed. The lighter band likely results from the inhibitory effect that dominant-negative BRG1 has on Mef2 expression (see input in Mef2 Western, Fig. 9B). Previously, we reported that expression of dominant-negative SWI/SNF complexes inhibited the expression of Mef2C RNA (17). These results demonstrate that endogenous SWI/SNF enzymes can associate with endogenous Mef2 in MyoD-differentiated cells.

Taken together, the results indicate that endogenous Brg1 interacts with both Pbx1 and MyoD at the onset of differentiation and with MyoD and Mef2 later during differentiation. The physical interactions between Brg1 and Pbx1 and between Brg1 and MyoD support the idea that MyoD is initially targeted to the myogenin promoter via the constitutively bound Pbx1. Because MyoD and Mef2 have been shown to physically and functionally interact (42), these data also suggest that

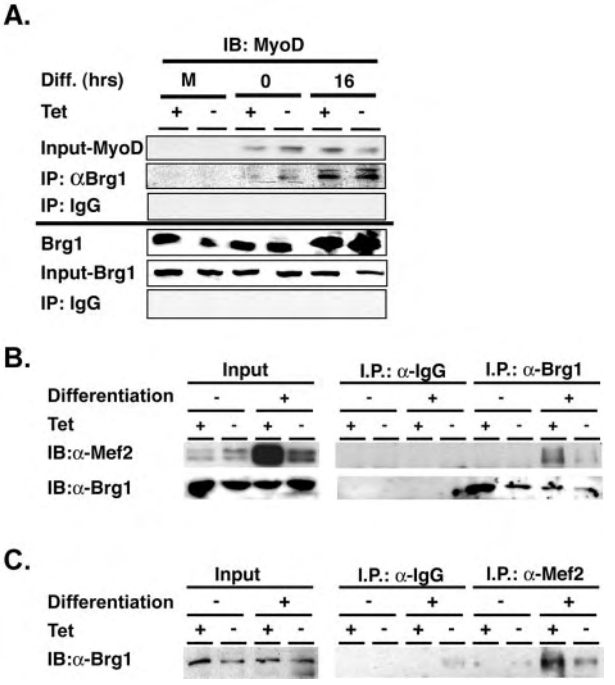

FIG. 9. Brg1 interacts with MyoD and Mef2 in differentiating cells. Extracts were prepared from mock-differentiated cells or cells differentiated with MyoD in the presence or absence of tetracycline. (A) Immunoprecipitation from nuclear extracts from cells that were mock differentiated (M) or MyoD differentiated was performed using purified IgG or antibody against Brg1 at the times indicated. Samples were run on an SDS-polyacrylamide gel and transferred to a membrane for Western blotting with MyoD and Brg1 antibodies. Ten percent of the input for each sample is shown. The samples shown in Fig. 8D and 9A were from the same time course; the Brg1 input and Brg1 IP bands for the mock and 0-h time point are the same data that were presented in Fig. 8D. (B and C) Nuclear extracts were immunoprecipitated from cells mock (−) or MyoD (+) differentiated in the presence or absence of tetracycline for 36 h using purified IgG or antibody against Brg1 or Mef2 and probed for the presence of Mef2 or Brg1. Confirmation of Mef2 immunoprecipitation by the Mef2 antibody could not be obtained because the Mef2 band was obscured by the antibody heavy chains.

Brg1-based SWI/SNF enzymes may be part of a higher-order complex containing both MyoD and Mef2 during the activation of muscle-specific genes. The data suggest that Brg1-based SWI/SNF enzymes are associated with the myogenin promoter throughout the differentiation process via protein-protein interactions with several regulatory factors.

DISCUSSION

Stable recruitment of MyoD and Mef2 transcriptional activators requires functional SWI/SNF enzymes. We found that Brg1 was localized to the myogenin promoter during differentiation. Stable association of muscle-specific activators with the myogenin promoter was inhibited by dominant-negative BRG1, indicating that functional SWI/SNF enzymes are necessary for activator binding. Although in vitro studies using reconstituted templates and purified factors long ago demonstrated that SWI/SNF enzymes have the potential to facilitate activator binding to chromatin (13, 31, 60), there is only one other direct example of this occurring in mammalian cells. Ma et al. recently demonstrated that ectopic expression of BRG1 in BRG1-deficient cells stimulated MMP2 transcription and increased the binding of Sp1 and AP2 to the MMP2 promoter (35). Temporal analysis of protein binding events at other mammalian promoters and enhancers has revealed that the order is gene specific and that SWI/SNF chromatin-remodeling enzymes are generally recruited during the later stages of the activation process (1, 40, 52, 55, 56). For example, induction of the beta interferon gene by viral infection results in ordered binding of an enhancesome complex to a nucleosome-free region, recruitment of the GCN5 histone acetyltransferase, association of the Pol II/CBP complex, and subsequent recruitment of SWI/SNF enzymes. Chromatin remodeling by SWI/SNF enzymes promotes association of TBP with the TATA box and transcription initiation (1, 33). During enterocyte differentiation, activation of α_1 antitrypsin transcription starts with association of the activator, HNF-1, TBP, and TFIIB with the promoter followed by Pol II, TFIID components, TFIIH, and mediator and then by recruitment of the activator, HNF-4α, CBP/PCAF, and BRM containing SWI/SNF enzymes (55). We recently demonstrated that during adipocyte differentiation, activation of the PPARγ nuclear hormone receptor promoter involves recruitment of SWI/SNF enzymes to the promoter after binding of the C/EBPβ activator and that SWI/SNF-promoter interactions facilitated or stabilized the binding of Pol II-associated general transcription factors (52). In these and other cases, localization of SWI/SNF enzymes and chromatin remodeling at the promoter occurred after activator binding, implying in most of these cases that SWI/SNF enzymes are needed to complete PIC formation and/or function. Indeed, early in vitro experiments indicated that SWI/SNF-mediated remodeling of nucleosomes could permit TBP/TFIIA binding to nucleosome particles (24). SWI/SNF enzymes are also required, both in vitro and in vivo, to promote transcriptional elongation of the *hsp70* gene (5, 11). Thus, our data reveal an additional role for SWI/SNF chromatin-remodeling enzymes in cells and show that SWI/SNF enzymes are needed at different steps during activation of different genes.

Requirement for MyoD to initiate chromatin remodeling at the myogenin promoter: a model for SWI/SNF recruitment to the myogenin promoter. The simplest mechanism for recruitment of chromatin-remodeling enzymes to muscle-specific promoters is recruitment by DNA-bound MyoD. In our study, there is an apparent paradox: expression of MyoD was necessary for early histone acetylation and SWI/SNF recruitment, yet ChIP assays showed that MyoD formed a stable, DNA-bound complex only after these changes occurred. A model to explain this apparent paradox is that MyoD initially associates with the myogenin promoter indirectly via interactions with Pbx1/Meis proteins at a Pbx binding site next to a noncanonical E box previously identified at −123/97 of the myogenin promoter. The initiation of myogenin expression in differentiating cells was recently shown to require the interaction of MyoD with a DNA-bound complex containing the Pbx homeodomain protein in the absence of a canonical MyoD binding site, and protein interactions between MyoD and the Pbx complex were shown to be necessary for the initial association of MyoD with the myogenin promoter (4). The interaction of MyoD with these factors might induce a conformational change in the Pbx/Meis proteins that permits targeting of Brg1 in differentiating cells. Alternatively, others have demonstrated that Pbx proteins can interact with HDACs and act to repress transcription, and then, upon specific cell signaling, Pbx can become associated with HATs and promote transcription (51). A similar switch potentially could occur upon muscle differentiation, with the Pbx protein facilitating transcription via interaction with HATs and/or SWI/SNF enzymes. Indeed, coimmunoprecipitation of endogenous Brg1 and endogenous Pbx1 (Fig. 8D) provides support for models involving targeting of SWI/SNF components by Pbx/Meis proteins in differentiating cells.

However, given the existing data indicating that both SWI/SNF and HAT enzymes can also interact with MyoD, we propose that recruitment of chromatin-remodeling enzymes occurs early during differentiation via interaction with MyoD bound to the promoter indirectly through the Pbx/Meis proteins. Because the interaction of MyoD is indirect in this scenario, it would not be easily cross-linked at early time points in our ChIP assays. MyoD is known to interact with p300 and P/CAF (reviewed in reference 41); targeting these HATs to the myogenin promoter would result in acetylation of histone tails, which would help promote the association of SWI/SNF complexes through interactions with the bromodomain present on the ATPase subunit. The ability of MyoD and Brg1 to stably associate (Fig. 9) (54), combined with the ability of Brg1 to interact with Pbx (Fig. 8D), would further promote the association of Brg1-based SWI/SNF enzymes with the myogenin promoter. Thus, SWI/SNF association with the promoter can be facilitated by its interactions with acetylated chromatin, with MyoD, and with Pbx. Subsequent chromatin remodeling by SWI/SNF would then open the canonical E boxes and the Mef2 site for factor binding, resulting in stably bound activator complex at the myogenin promoter that can be detected by ChIP assay. A schematized version of these events is presented in Fig. 10.

This model is generally consistent with the recent findings of Simone et al. (54). Using differentiating C2C12 myoblasts, they demonstrated that MyoD is recruited to the myogenin promoter and induces histone acetylation. Chromatin remodeling at the promoter required the subsequent recruitment and activity of SWI/SNF enzymes, which were dependent on an active p38 kinase. In that study, MyoD was cross-linked to the myogenin promoter prior to chromatin remodeling, which could

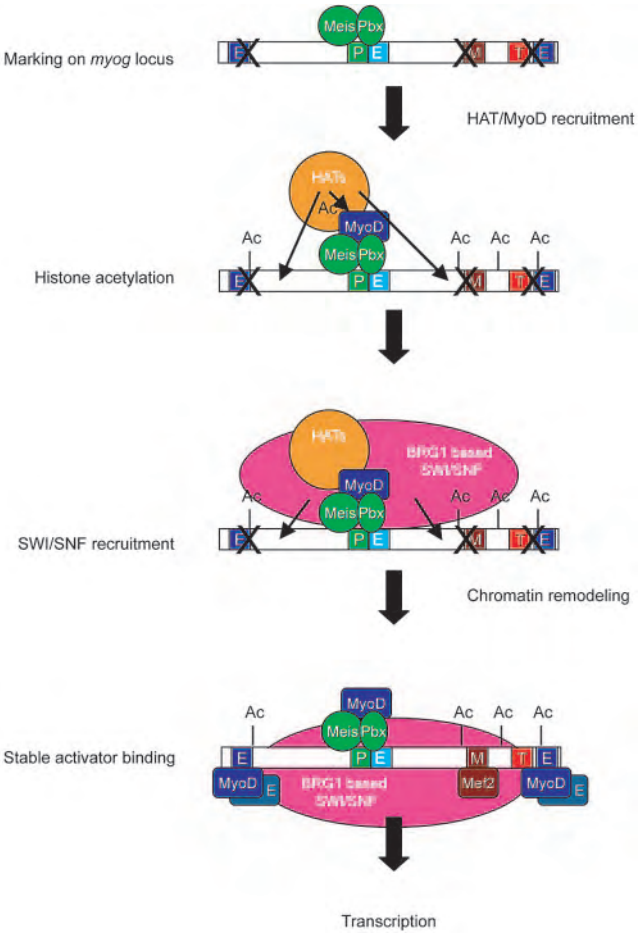

FIG. 10. A cartoon model of the order of events occurring during activation of the endogenous myogenin locus in MyoD-differentiated cells. Bold "X" marks placed over transcription factor binding sites indicate that the sites are inaccessible to factor binding. "P" indicates a Pbx binding site, "M" indicates a Mef2 binding site, "T" indicates the TATA box, and "E" indicates E boxes, which bind MyoD. The nonconsensus E box adjacent to the Pbx1 binding site is indicated in light blue to distinguish it from consensus binding sites elsewhere.

suggest that MyoD is directly bound to the promoter but alternatively could indicate that a tighter association exists between MyoD and the Pbx complex in C2C12 myoblasts than exists in the MyoD–differentiated fibroblasts, thereby permitting MyoD to be visualized at the promoter by ChIP at the beginning of the differentiation process. Both possibilities support a model of early recruitment of MyoD to the myogenin promoter prior to acetylation and SWI/SNF recruitment, though the loss of MyoD binding in the presence of dominant-negative BRG1 (Fig. 5 and 6) and the contribution of Pbx complexes during myogenin activation in C2C12 cells (4) argue for the latter possibility. Further experiments will be needed to evaluate the role of each of the potential interactions occurring at the myogenin promoter during the activation of gene expression.

MyoD-induced genes show differential requirements for SWI/SNF enzymes. The expression of the vast majority of genes measured by the expression array was not altered by the inhibition of SWI/SNF activity, whereas most genes induced by MyoD were modestly affected by SWI/SNF inhibition and a subset of MyoD-regulated genes were highly dependent on SWI/SNF (see Fig. 1). Our data demonstrate that chromatin modification and SWI/SNF activity are necessary for MyoD to form a stable interaction with DNA at the myogenin promoter, suggesting that chromatin remodeling may be necessary for MyoD binding at the subset of genes that are highly dependent on SWI/SNF activity. We suggest that the interaction between MyoD and Pbx is necessary to initially target MyoD to the myogenin promoter. It is interesting that there is a significant overlap between the MyoD-regulated genes that are highly dependent on SWI/SNF and the subset of genes that require the domains of MyoD that interact with Pbx for full activation (unpublished data), suggesting that these domains of MyoD might be necessary for targeting the protein to multiple different promoters as an initial step in gene activation.

Summary. During the establishment of new cell lineages, changes in chromatin structure become apparent as previously silent genes are activated. How activators initially gain access to their binding sites within condensed chromatin structure has been a topic of intense investigation. In some cases, such as during differentiation of liver cells, the HNF3 homeodomain protein can bind to nucleosomal DNA and disrupt nucleosomal structure in the absence of ATP-dependent chromatin-remodeling enzymes (10). In other cases, gene-specific activators recruit chromatin-remodeling enzymes to specific promoters (12). Our results suggest that during muscle differentiation, muscle-specific activators may both recruit and require chromatin-remodeling activities for stable binding to the regulatory regions of muscle-specific genes.

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