

CHROMATIN REMODELING AND MYOGENESIS

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By

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List of Abbreviations

Ac	Acetylated
ACF	ATP-utilizing chromatin assembly and remodeling factor
ATP	adenosine triphosphate
Avg	average
bHLH	basis helix loop helix
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
CBP	CREB-binding protein
CaMK	calcium/calmodulin-dependent kinase
cDNA	complementary DNA
ChIP	Chromatin Immunoprecipitation
CHRAC	chromatin remodeling and assembly complex
CREB	cAMP response element binding protein
Da	Dalton
DiMe	di-methylated
DMEM	Dulbecco's Modified Eagle's Media
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FAC	fluorescence activated cell sorting
5-FOA	5-fluoroorotic acid
GST	glutathione S transferase
HAT	histone acetyltransferase
HDAC	histone deacetylases
hEGF	human epidermal growth factor
HMTase	histone methyltransferase
Hrs	hours
Hu	human
IGF	Insulin-like growth factor
IP	immunoprecipitation
K9	lysine 9
Kb	kilo bases
kDa	kilo Dalton
MAP	mitogen-activated protein
MAPK	MAP kinase
MEK1	MAPK / ERK kinase 1
Min	minute
mM	millimoles
MPC	myoblast precursor cell
MRF	myogenic regulator factor

mRNA	message ribonucleic acid
NLS	nuclear localization signal
NoRC	Nucleolar Remodeling Complex
NURF	nucleosome remodeling factor
PBS	phosphate buffered saline
PCAF	p300/CBP associated factor
PCR	polymerase chain reaction
RB	retinoblastoma
RNA	ribonucleic acid
RPM	revolutions per minute
rRNA	ribosomal RNA
RSC	remodels the structure of chromatin
RSF	remodeling and spacing factor
RTA	repressed transactivator
RT-PCR	reversed transcribed PCR
P.C.	postcoitum
SC	synthetic complete
SDS	sodium dodecyl sulfate laury
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
Tris Base	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride
TSA	Trichostatin A
WGA	whole genome assay
WICH	WSTF-ISWI chromatin remodeling complex
WT	wildtype
µg	micrograms
µM	micromoles

Abstract

The lineage identity of a particular cell within a multi-cellular organism is defined by the repertoire of mRNA it expresses. Changes to the transcript repertoire can affect cell identity and stage of differentiation. Chromatin as the biologically relevant target of transcription factors has a direct impact on cell identity and differentiation, with changes to its structure central to lineage choice, cellular identity and differentiation. The requirement of chromatin remodeling in these developmental processes is evident throughout myogenesis, with changes to the cell's chromatin structure occurring during both the specification of myoblasts and the differentiation of specified myoblasts to myotubes.

This thesis examines three distinct aspects of chromatin remodeling in relation to myogenesis. Firstly, the role of Snf2h in MyoD-mediated repression of target genes during myoblast proliferation was investigated. Snf2h was identified as a MyoD-interacting protein in a repressed transactivator yeast two-hybrid screen. Overexpression of either an active or inactive form of human SNF2H in myoblasts accelerated the differentiation process without affecting either growth rate or cell cycle kinetics. Furthermore, the chromatin structure surrounding the myogenin E boxes was more open in myoblasts expressing inactive human SNF2H compared to control myoblasts suggesting functional Snf2h complexes are required to repress differentiation specific genes during growth and subsequently maintain the myoblast proliferative state. From these and published results, a model describing the role of Snf2h in myogenesis is suggested. This model proposes that Snf2h activity facilitates the repression of MyoD target genes by histone deacetylases.

In addition, genome-wide changes to histone H4 acetylation and histone H3-K9 dimethylation during myoblast differentiation were assessed. The patterns of genome-wide histone modifications in proliferating myoblasts were distinct from the patterns observed in myotubes, suggesting distinct mechanisms are targeting histone-modifying activity in myoblasts and myotubes. Furthermore, in our study, hyperacetylation of histone H4 and hypomethylation of histone H3 did not correlate strongly with transcriptional status. Our results support the hypothesis of the Histone Code Model, which theorizes that a combination of distinct histone modifications and not individual histone modifications defines the transcriptional competence of a particular gene.

The relationship between signal transduction pathways and chromatin remodeling in myoblast differentiation was also examined. U0126, a specific chemical inhibitor of the MAP kinase MEK1, accelerates myoblast differentiation. The inhibition of MEK1 resulted in an increase in the MyoD-associated histone acetyltransferase activity. Furthermore, U0126 promoted the acetylation of histone H4 at muscle specific E boxes. Together these results suggest a model in which MEK1 modulates MyoD activity by affecting the recruitment by MyoD of histone modifying enzymes. All three projects outlined in this thesis illustrate the integral role chromatin remodeling plays during myoblast differentiation.

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CHAPTER 1: INTRODUCTION

Forward

Sections of this introduction (excluding Chromatin and Chromatin Remodeling Enzymes) have previously been published in a book chapter entitled “The Myogenic Regulatory Factors” in *Advances in Developmental Biology and Biochemistry: Stem Cells and Cell Signalling in Skeletal Myogenesis*, D.A. Sassoon (Ed); Elsevier (London). C. Palmer wrote the review and drew all the figures. M.A. Rudnicki edited review.

Overview: Myoblasts – Recapitulating Muscle Differentiation In Tissue Culture

Muscle derived cells or myoblasts are a well characterized and an extensively used *in vitro* model of cellular specification and differentiation. The molecular events necessary for the formation of skeletal muscle from multipotent mesodermal precursor cells can, in part, be recapitulated in the laboratory. In culture, growing myoblasts can be induced to exit the cell cycle and terminally differentiate into elongated multinucleated myotubes by the removal of mitogens (McKinsey et al., 2001a). A single master regulator (myogenic regulatory factor) can initiate the cascade of molecular events necessary to form skeletal muscle from non-muscle cells. The reprogramming of non-muscle cells to the myogenic lineage and the differentiation of specified myoblasts to myotubes require changes to the cell's chromatin structure (McKinsey et al., 2001a). By modulating, in part, the activity of chromatin remodeling enzymes, signaling pathways translate changes in the extracellular milieu into changes in gene expression. Chromatin structure and its modification are central to lineage choice and cellular identity during mammalian development (Li, 2002).

Myogenic Regulatory Factors

The first hints to the existence of a dominant-acting myogenic transcription factor come from studies in which non-muscle cells were fused to myoblasts and muscle specific gene expression was activated in the non-muscle nuclei of the heterocaryons (Blau et al., 1983; Wright, 1984). This transcription factor was later identified by a subtractive cDNA approach and called MyoD ((Davis et al., 1987); for review (Weintraub et al., 1991a)). MyoD is a basic helix loop helix transcription factor capable

of inducing myogenic gene expression in non-muscle cells ((Davis et al., 1987); for review (Weintraub et al., 1991a)). Myf5, Myogenin and MRF4 were later identified as members of the MyoD family of transcription factors, also called the myogenic regulator factors (MRFs). A similar capacity to induce muscle specific gene expression in non-muscle cells was demonstrated for these MRFs. Members of this family have subsequently been identified in a diverse array of organisms including: *Drosophila* (reviewed in (Abmayr and Keller, 1998)); *Xenopus* (Harvey, 1990; Hopwood et al., 1991; Jennings, 1992); Chicken (Fujisawa-Sehara et al., 1992), mouse (Davis et al., 1987; Wright et al., 1989) and human (Pearson-White, 1991).

The MRFs are basic helix loop helix transcription factors

The superfamily of basic helix loop helix (bHLH) transcriptional regulators has over 400 members that are active in a diverse array of developmental processes including myogenesis and neurogenesis (for review see (Atchley and Fitch, 1997; Atchley et al., 1999; Atchley et al., 2000; Morgenstern and Atchley, 1999; Wollenberg and Atchley, 2000)). Based on homology within the bHLH domain, DNA binding affinity and the presence or absence of a leucine zipper motif, these proteins are classified into five subfamilies (Atchley and Fitch, 1997). The MRFs are members of subfamily A; other members of this subfamily include Twist, dHand and E12 (Atchley and Fitch, 1997). Subfamily A members bind the E Box sequence 5'- CAGCTG -3' with high affinity, lack a leucine zipper motif and have a conserved configuration of amino acids at residues 5, 8, and 13 of the bHLH domain (Atchley and Fitch, 1997).

The four vertebrate MRFs share significant amino acid sequence identity (over 80%) throughout the bHLH domain (Dias et al., 1994; Murre et al., 1989b). The individual MRF bHLH domains appear to be functionally equivalent, however, regions outside this domain give specific activities to individual MRFs (Asakura et al., 1993). Through the amphipathic helices of the bHLH domain, the MRFs can both homo and heterodimerize (Ferre-D'Amare et al., 1993; Ma et al., 1994). The MRFs form transcriptionally active heterodimers with the widely expressed E proteins, a distinct group of bHLH proteins including E12/E47, ITF-2 and HEB (Lassar et al., 1991; Murre et al., 1989b). There is evidence to suggest that DNA by acting as an allosteric ligand facilitates the dimerization of MyoD with E protein partners (Czernik et al., 1996; Maleki et al., 1997; Maleki et al., 2002; Weintraub et al., 1990; Wendt et al., 1998). However, under physiological conditions heterodimerization appears to precede DNA binding (Spinner et al., 2002).

MRF activity is regulated at the level of dimer formation.

Several proteins, for example Id, Mist1 and MyoR, have been identified which either sequester E proteins from MyoD or prevent MyoD/E protein heterodimerization by binding the bHLH domain of MyoD (Benezra et al., 1990; Lemercier et al., 1998; Lu et al., 1999). Id (inhibitors of DNA binding) proteins are helix loop helix proteins that lack a DNA binding domain (Benezra et al., 1990; Biggs et al., 1992; Christy et al., 1991; Deed et al., 1993; Ellmeier et al., 1992; Riechmann et al., 1994). Ids have been suggested to avidly bind E proteins sequestering them from bHLH transcription factors (Benezra et al., 1990; Langlands et al., 1997). In addition, Id1 and Id2 bind to MyoD and Myf5 strongly preventing dimer formation (Langlands et al., 1997). Ectopic expression of Id

in differentiating cultures of C2C12 myoblasts inhibits terminal differentiation (Jen et al., 1992). Together this data suggests Ids may function to prevent MRF dependent entry into the differentiation program during expansion of myogenic cells and that the downregulation of Id is a prerequisite to initiating the differentiation program (Langlands et al., 1997). The down regulation of Id during myoblast differentiation is dependent on calcineurin signalling (Benezra et al., 1990; Friday et al., 2003). However, mice null for Id1, Id2, Id3, or Id1 and Id3 do not have any overt muscle defects (Lyden et al., 1999; Rivera et al., 2000; Yokota et al., 1999; Yokota et al., 2000). The lack of a muscle phenotype may in part be due to functional redundancy between Id proteins; analysis of additional Id compound mutant mice may address this question.

MyoR is a muscle restricted basic helix-loop-helix protein that antagonises MyoD (Lu et al., 1999). In the developing mouse, MyoR is expressed in a subset of muscle precursor cells predominantly during primary myogenesis between 10.5 and 16.5 days postcoitum (p.c.) and appears to have a specific role in the development of facial muscles (Lu et al., 1999; Lu et al., 2002). In cell culture, the expression of MyoR is down regulated during differentiation by HES6, a mammalian homolog of enhancer of split (Gao et al., 2001; Lu et al., 1999). In addition to sequestering E proteins, MyoR/E12 heterodimers bind E boxes within the promoters of muscle specific genes competing with MRF/E protein heterodimer for access (Lu et al., 1999). MyoR may function to modulate the timing of muscle-specific gene expression and delay muscle fibre maturation (Lu et al., 1999).

Activators of myogenesis include proteins that stimulate the heterodimerization of MyoD and E proteins. In solution the formation of MyoD/E12 heterodimers is not favoured over the formation of MyoD homodimers (Maleki et al., 1997). However, Mos-dependent phosphorylation of MyoD promotes MyoD-E12 heterodimerization while inhibiting the MyoD homodimer DNA binding activity (Lenormand et al., 1997). Mos is a serine/threonine kinase expressed in adult skeletal muscle (Leibovitch et al., 1991). Mos directly phosphorylates serine 237 of MyoD (Pospel K et al., 2000). Mutation of this serine residue to alanine abolishes the Mos positive regulation of MyoD (Pospel K et al., 2000). Although *in vitro* data supports a role for Mos in myogenic differentiation, homozygous Mos mutant mice do not have obvious skeletal muscle defects (Colledge et al., 1994; Hashimoto et al., 1994; Lenormand et al., 1997). Since the phosphorylation status of serine 237 was not determined in the *Mos*^{-/-} mice, phosphorylation of this residue by yet-to-be identified kinases may compensate for the loss of Mos activity in muscle. In addition, the skeletal muscle defects in post-natal MyoD deficient mice are not overtly apparent except during muscle regeneration (Megeney et al., 1996; Rudnicki et al., 1992). If Mos is essential for MyoD activity *in vivo* *Mos*^{-/-} mice may exhibit a muscle regeneration defect similar to that of *MyoD* mutant animals.

MRFs bind E boxes in the promoters and enhancers of muscle specific genes.

Dimerization of bHLH proteins juxtaposes their basic domains forming a functional DNA binding domain (Ferre-D'Amare et al., 1993; Ma et al., 1994). In general, bHLH protein dimers bind to the consensus sequence 5'-CANNTG-3' (each basic region binds ½ of the consensus site), called an E box (Murre et al., 1989b).

MyoD/E protein heterodimers preferentially bind the E box sequence

5'CA(G/C)(G/C)TG-3' (Blackwell and Weintraub, 1990). Highly conserved residues within the basic domain are responsible for consensus binding. In particular, a glutamic acid residue conserved in the majority of bHLH proteins is in direct contact with the 'CA' nucleotides of the E box (reviewed in (Robinson and Lopes, 2000)). Residues within the basic domain, in part, dictate the consensus sequence specificity of different bHLH protein dimers. For example, Arg-111 of MyoD makes contact with the 3'G of the E box. A similar contact does not occur when non-myogenic bHLH proteins bind DNA (reviewed in (Robinson and Lopes, 2000)). The unique conformation of the MRF bHLH is likely due to the small size of Ala-114 (a residue required for myogenic activity). In non-myogenic bHLH proteins this position is occupied by a bulkier amino acid, it is this amino acid that is in contact with the G of CANNTG (Ellenberger et al., 1994; Ferre-D'Amare et al., 1993; Huang et al., 1998; Ma et al., 1994).

The Myogenic Code

Mutagenesis studies have identified conserved residues within the basic region of the MRFs essential for their myogenic activity, residues Ala-114 and Thr-115 of MyoD and residues Ala-86 and Thr-87 of Myogenin (Brennan et al., 1991; Davis et al., 1990). These residues are referred to as the myogenic code. Replacement of Ala-114 of MyoD with asparagine or histidine, the residue normally found at that position in E proteins and Max respectively, alters the conformation of the basic domain such that Arg-111 does not make direct contact with the E box DNA (Huang et al., 1998). Furthermore this local conformational change prevents the unmasking of the N-terminal activation domain of

MyoD upon DNA binding (Huang et al., 1998). Interestingly, other bHLH proteins can acquire myogenic specificity by the introduction of the myogenic code into the appropriate position (Davis and Weintraub, 1992; Weintraub et al., 1991b). Similarly, the neurogenic bHLH protein MASH-1 acquires myogenic specificity by replacement of leucine-130 with lysine and the addition of one alpha helical turn without significantly altering cell free DNA binding properties (Dezan et al., 1999).

Although the bHLH domains of the four MRFs are highly conserved, there is little sequence conservation outside this region, a notable exception include a cysteine/histidine rich region (Tapscott et al., 1988; Weintraub et al., 1991a). The transactivation domain is not conserved between individual MRFs. The transactivation domain of Myf5 is located in the C terminus while that of MyoD and MRF4 is in the N terminus (Braun et al., 1990; Mak et al., 1992; Weintraub et al., 1991b). Strong transcriptional activation domains have been identified in both the N- and C-termini of myogenin (Olson, 1992). Additional functional studies are required to define more precisely the MRF transactivation domains.

Embryonic Myogenesis

Embryonic Expression patterns of the MRFs

In vertebrates, skeletal muscles with the exception of the anterior head, and extraocular muscles originate from mesodermal precursor cells in the somites (for review see (Borycki and Emerson, 2000)). The paraxial head mesoderm gives rise to the anterior head and extraocular muscles (Christ and Ordahl, 1995; Wachtler and Christ, 1992). Somites are epithelial spheres of paraxial mesoderm that flanks the neural tube and form in a rostrocaudal progression (for review see (Christ and Ordahl, 1995)). As somites

mature, they become subdivided into the ventral sclerotome, which contributes to the vertebra and ribs, and the dorsal dermomyotome (skeletal muscle, distal ribs and dorsal dermis) (for review see (Tajbakhsh and Buckingham, 2000)). The dorsomedial region of the dermamyotome gives rise to epaxial muscles (axial muscles of the back). The limb or hypaxial muscles arise from cells that migrate into the limb bud from the ventrolateral dermamyotome (for review see (Molkentin and Olson, 1996)). Signals from the neural tube, notochord and paraxial mesoderm are involved in regulating somitic myogenesis (Cossu et al., 1996).

The four vertebrate MRFs have distinct spatiotemporal expression patterns. MRF expression occurs initially in rostral somites and progresses caudal with somite maturation. Myf5 is the first MRF expressed in the developing embryo. Using *in situ* hybridization, Myf5 is first detected in the dorsomedial quadrant of the most rostral somite at day 8 postcoitum (p.c.) (Ott et al., 1991; Tajbakhsh et al., 1996). As development progresses, Myf5 expression spreads to the whole myotome and is subsequently down regulated as the myotome matures. Myf5 expression in the developing limb bud is detected between day 10 p.c. and 12 p.c. MyoD mRNA first appears in the hypaxial somitic domain at 9.75 days p.c. (Faerman et al., 1995; Sassoon et al., 1989). MyoD expression is detected throughout the myotome by day 11.0 and is maintained throughout development. MyoD expression in the limb bud occurs together with Myf5 expression. Myogenin mRNA is detected approximately 12 hrs after Myf5 mRNA in the rostral somites. Myogenin expression spreads caudally as development progresses and is maintained throughout fetal life (Ott et al., 1991; Sassoon et al., 1989). Its expression is

down regulated postnatally. Myogenin mRNA is detected in the developing limb bud after 10.5 days p.c. MRF4 is expressed transiently between 9.0 and 11.5 days p.c. in the myotome (rostral to caudal progression). It is subsequently re-expressed in the muscles of the embryo at 16 days p.c. becoming the most abundant MRF mRNA postnatally (Bober et al., 1991; Hannon et al., 1992; Hinterberger et al., 1991). Successive waves of myogenic determination and differentiation likely contribute to the complex developmental expression pattern of the MRFs. (See figure 1)

Gene targeting experiments reveal a hierarchical relationship amongst the MRFs.

The four MRFs have similar capacities to convert 10T1/2 fibroblasts into myoblasts. In addition, the DNA binding and dimerization properties are similar amongst the MRFs (Braun and Arnold, 1991). Cross-activation of the endogenous MRF genes by ectopically expressed MRFs complicate analysis of individual MRF function (Braun et al., 1989; Thayer et al., 1989). Targeted disruption of the MRFs was essential to define the individual roles of these proteins in myogenesis.

MyoD

Mice deficient in MyoD are viable, fertile and do not display any overt skeletal muscle abnormalities (Rudnicki et al., 1992). MyoD deficiency does not affect sarcomere ultrastructure, fast/slow fiber ratio or muscle specific gene expression. Myogenin and MRF4 expression in MyoD mutant mice is unaffected. Both heterozygous and homozygous MyoD mutant mice however show significant postnatal over expression of Myf5; 1.8 and 3.5 fold respectively (Rudnicki et al., 1992). While the epaxial muscles in a MyoD^{-/-} embryo develop normally, development of the hypaxial musculature or

migrating muscle lineage is delayed by about 3.5 days (Kablar et al., 1998; Kablar et al., 1997; Kablar et al., 1999; Kaul et al., 2000). In addition, adult MyoD-deficient muscle have a severe regenerative defect (Megeney et al., 1996). The effect of MyoD deficiency on regeneration will be discussed later.

Myf5

Similar to MyoD mutant mice, newborn *Myf5*^{-/-} mice have no overt morphological defects in their skeletal muscle. Expression of the three remaining MRFs is not up regulated in *Myf5* mutant mice (Braun et al., 1992). Using a *Myf5* targeted nls-lacZ reporter gene, Tajbakhsh and colleagues tracked the migratory routes of *Myf5* expressing muscle progenitor cells (β -gal positive cells) in both heterozygous and homozygous mutant embryos. Interestingly in *Myf5* mutant embryos, muscle progenitor cells that have activated *Myf5* expression (β -gal positive) migrate aberrantly and fail to respond to positional cues. These cells ultimately adopt a non-myogenic fate (Tajbakhsh et al., 1996).

Epaxial myogenesis in *Myf5* mutant embryos is markedly delayed and is rescued by the induction of MyoD expression at 13.5 days p.c. (Braun et al., 1992; Kablar et al., 1998; Kablar et al., 1997; Kablar et al., 1999; Kaul et al., 2000). Hypaxial myogenesis is normal in *Myf5*^{-/-} embryos. Taken together the delay in epaxial and hypaxial myogenesis observed in *Myf5* mutant and MyoD mutant mice respectively supports a multiple lineage hypothesis in which *Myf5* regulates epaxial muscle formation and MyoD regulates hypaxial muscle formation (Kablar et al., 1998; Kablar et al., 1997; Kablar et al., 1999). Nevertheless, the lack of obvious skeletal muscle defect in *Myf5* and MyoD

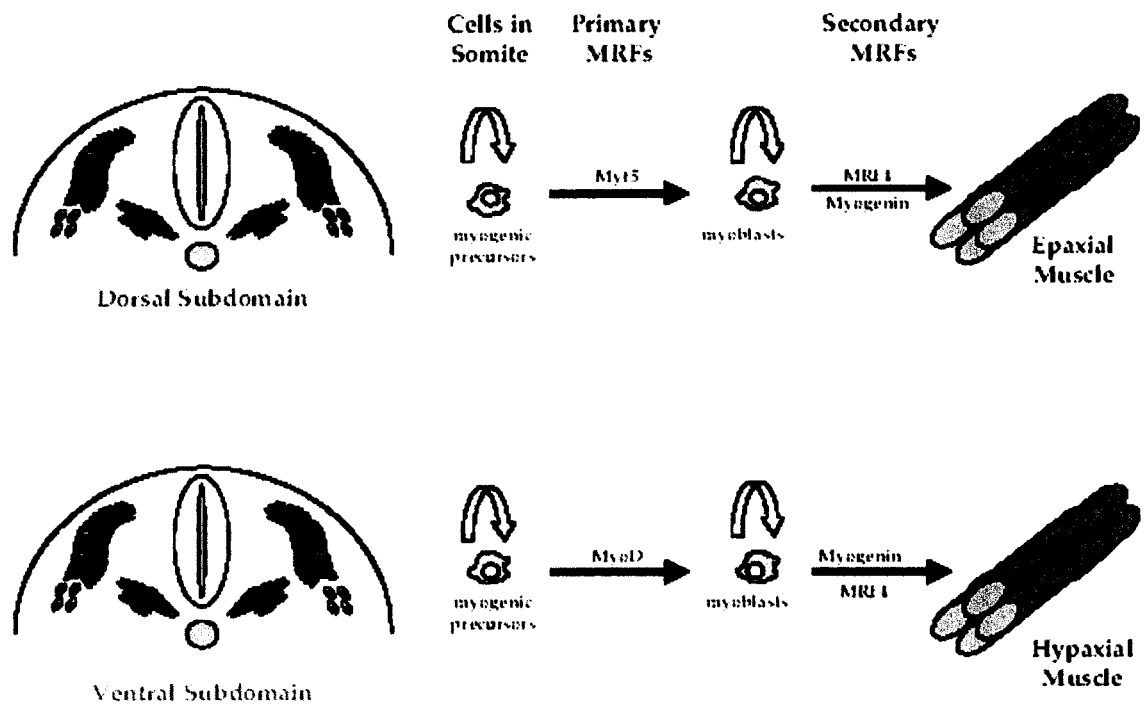


Figure 1. Schematic representation of the hierarchical genetic relationships of the MRFs in the epaxial and hypaxial myotomes. Cells from the dorsal and ventral subdomains differentiate into myoblasts by the expression of Myf5 and MyoD respectively. Subsequently both primary MRFs are co-expressed in these cells. Gene targeting experiments demonstrate that Myf5 or MyoD are required for myogenic lineage determination while myogenin and MRF4 are necessary for terminal differentiation.

mutant mice suggests that the distinct myogenic lineages can to some extent compensate for each other. The finding further supports the hypothesis that Myf5 and MyoD are not co-expressed in muscle precursor cells but rather expressed in a mutually exclusive manner (Braun and Arnold, 1996).

Several Myf5 mutant mice have been generated since the initial targeting. It was initially knocked-out by Braun *et al.* (1992) (Braun et al., 1992). These Myf5 mutant mice die perinatally due to a severe rib defect. A similar rib defect was observed when an nls-lacZ reporter gene, together with the PGK-neo selection cassette, was inserted into exon 1 of the Myf5 locus (Tajbakhsh et al., 1996). However subsequent targeted disruption of the Myf5 locus in which the PGK-neo-TK selection cassette was excised by Cre-recombinase does not have any skeletal defects and are both viable and fertile (Kaul et al., 2000).

MyoD and Myf5 are myogenic determination factors.

To investigate whether Myf5 and MyoD could functionally compensate for one another in embryonic myogenesis, compound Myf5/MyoD mutant mice were generated (Rudnicki et al., 1993), (Kaul et al., 2000). Although born alive, Myf5/MyoD compound mutant mice are immobile and die shortly after birth. These mice are devoid of both myoblasts and skeletal myofibers. Adipose and amorphous loose connective tissue occupies the spaces normally occupied by muscle (Rudnicki et al., 1993). A sequential ablation of motor neurons from the spinal cord to the brain occurs during development in Myf5^{-/-}: MyoD^{-/-} mutant embryos, highlighting the intimate connection between nervous development and skeletal myogenesis (Kablar and Rudnicki, 1999). The muscle

phenotype of the compound mutant mice is consistent with a model in which MyoD and Myf5 act as primary MRFs and are required for myogenic lineage determination (Rudnicki et al., 1993).

Interestingly, Myf5^{+/-}: MyoD^{-/-} mice unlike Myf5^{-/-}: MyoD^{+/-} mice are not viable suggesting postnatal survival of MyoD mutants requires two functional Myf5 alleles (Rudnicki et al., 1993). These mice have a substantial reduction in muscle fiber number. Although these observations confirm that Myf5 and MyoD can functionally substitute for each other during development, they suggest Myf5 and MyoD are not functionally equivalent. Similarly myogenin targeted into the Myf5 locus can partially substitute for Myf5. Myf5^{myg-ki/myg-ki}, MyoD^{-/-} mutant mice die perinatally because of reduced skeletal muscle mass (Wang and Jaenisch, 1997). The skeletal muscle formed in Myf5^{myg-ki/myg-ki}, MyoD^{-/-} mutant mice is normal suggesting the observed reduction in skeletal muscle mass is the result of inefficient recruitment of precursors into the myogenic lineages (Wang and Jaenisch, 1997).

Myogenin is essential for myoblast differentiation in vivo but not in vitro.

Two independent groups generated myogenin mutant alleles by homologous recombination-mediated gene targeting (Hasty et al., 1993; Nabeshima et al., 1993). Homozygous myogenin mutant mice die perinatally presumably because of a severe defect in the diaphragm. A significant reduction in diaphragm thickness and skeletal muscle content is observed in myogenin null mice (Hasty et al., 1993; Nabeshima et al., 1993). Unlike the MyoD^{-/-} and Myf5^{-/-} mice, myogenin mutant mice have major skeletal muscle abnormalities (Hasty et al., 1993; Nabeshima et al., 1993). A significant

reduction in skeletal muscle mass is observed throughout the body. In addition, the skeletons of myogenin^{-/-} mice show abnormal curvature of the spine and rib cage deformities. Subsequent analysis of a hypomorphic myogenin allele in which myogenin transcription is reduced to 25% of wildtype, suggests distinct myogenin threshold levels are required for myogenesis and thoracic skeletal development (Vivian et al., 1999). Muscle hypoplasia is observed in both homozygous myogenin hypomorphs and myogenin (hypo/null) mice. The severity of the hypoplasia is dependent on the level of myogenin expression. The thoracic skeletal defect observed in myogenin mutant mice appears to be due to delayed rib cartilage migration (Vivian et al., 1999).

The skeletal muscle from myogenin null neonates and embryos is extensively disorganized and there is a substantial reduction in myofibre density. However, the number of myoblasts appears normal suggesting myogenin is not required for lineage commitment but rather terminal differentiation. Consistent with this myogenin expression is upregulated during myoblast differentiation both in culture and *in vivo* (Sassoon et al., 1989; Smith et al., 1994a; Smith et al., 1994b; Wright et al., 1989). The generation of myogenin / Myf5 and myogenin / MyoD mutant mice demonstrates the function of myogenin does not overlap with either MyoD or Myf5 (Rawls et al., 1995). These results together suggest myogenin is a secondary MRF acting downstream of the primary MRFs, MyoD and Myf5.

The muscle defect in myogenin mutant mice is most severe in muscle arising from the ventral subdomain of the dermomyotome (hypaxial) with very few, if any myofibres present in these muscles (Nabeshima et al., 1993). The hypaxial dermomyotome also

gives rise to a migratory population of myogenic precursors. MyoD and myogenin are the predominate MRFs expressed in this region of the dermamyotome before and after myoblast differentiation respectively (Smith et al., 1994b). This is consistent with the hypothesis that myogenin is the predominate differentiation factor of a MyoD-dependent lineage. Myofibres are present in axial, intercostal and back muscles however the majority are disorganized and lack Z lines (Nabeshima et al., 1993). Studies by Venuti and colleagues (1995) demonstrate that these myofibres arise during primary myogenesis (Venuti et al., 1995). Suggesting primary myogenesis is unaffected by the absence of myogenin.

The levels of MRF4 transcript are markedly reduced in myogenin mutant embryos while the MyoD levels are unaffected (Hasty et al., 1993; Nabeshima et al., 1993). This reduction in MRF4 could reflect a lack of differentiated myofibres as opposed to direct regulation of MRF4 by myogenin. MRF4 can partially substitute for myogenin during embryonic myogenesis (Zhu and Miller, 1997).

Myogenin does not appear to have an essential role in myoblast differentiation *in vitro*. Continuous cell lines of myoblasts isolated from myogenin mutant embryos differentiate normally in culture. In addition, myogenin^{-/-} fibroblasts convert to myotubes by ectopic expression of MyoD (Nabeshima et al., 1993). However, analysis of primary MyoD^{-/-} myogenic cells suggests continuous culture of primary myoblasts effects differentiation potential. The differentiation defect observed in low-passage primary MyoD^{-/-} myoblasts is not observed in later-passage MyoD^{-/-} cultures (Sabourin et al.,

1999). In light of these results, it is important to characterize the differentiation potential of low-passage primary myogenin^{-/-} myoblasts.

MRF4

The gene-encoding mouse MRF4 is linked to the Myf5 gene in a head to tail orientation. Approximately 8.5 kb of sequence separates the two loci (Miner and Wold, 1990). Three laboratories have generated MRF4 mutant mice (Braun and Arnold, 1995; Patapoutian et al., 1995; Zhang et al., 1995). Although the identical PGK-neo selection cassette was used in the targeting constructs, orientation of the cassette and deleted MRF4 sequence is unique to each construct (Olson et al., 1996)]. Analysis of the role of MRF4 in embryonic myogenesis has been complicated by the close proximity of the two MRF genes. Targeted disruption of MRF4 affects Myf5 expression via a cis-acting mechanism (Floss et al., 1996; Yoon et al., 1997). The negative cis effect of MRF4 mutations on Myf5 transcription was confirmed by the generation of compound heterozygous MRF4, Myf5 mice in which the disrupted alleles are on different chromosomes. These mice have a significant reduction in Myf5 transcript compared to control animals suggesting the wildtype Myf5 allele is partially inactivated (Floss et al., 1996; Olson et al., 1996; Yoon et al., 1997). Myf5 enhancer elements have been identified in a 14 kb region spanning the MRF4/Myf5 locus (Summerbell et al., 2000). These enhancers regulate Myf5 expression in epaxial muscle precursor cells, the branchial arches and the central nervous system.

MRF4 null mice generated in Barbara Wold's laboratory die perinatally and show defects in axial myogenesis and rib structure (Patapoutian et al., 1995). In light of recent

insights into the neo cassette-dependent rib defects observed in some Myf5 mutant mice, the rib anomalies observed in the MRF4 null mice may be the result of cis effects on a yet to be identified gene (Kaul et al., 2000). A deficit in myotome development is observed in the MRF4^{-/-} mice between 9 and 11 days p.c. This corresponds to the first wave of MRF4 expression during development. A significant reduction in Myf5, MyoD and myogenin expression is observed in mutant mice at days 10 p.c. A decrease in Myf5, MyoD and myogenin levels is also observed in heterozygous mice at this time (Patapoutian et al., 1995). By 14 days p.c., myogenesis in these MRF4 mutant animals appears normal. Minor defects are observed in the intercostal muscle of neonates (Patapoutian et al., 1995). Although studies using transgenic mice have shown the MRF4 sequences deleted in the Wold mice are not required for appropriate Myf5 expression, interference of Myf5 expression by PGK-neo cannot be ruled out (Summerbell et al., 2000).

The Olson MRF4 mutant mice are viable however have malformations of the ribs including bifurcations, fusions and supernumerary processes (Zhang et al., 1995). Neonatal muscle is grossly normal although the level of embryonic myosin heavy chain is slightly decreased. Similar to the Wold MRF4^{-/-} mice, defects in myotome differentiation are observed in 10.5 days p.c. MRF4^{-/-} embryos (Vivian et al., 2000). Myogenin and Myf5 expression in the developing myotome are severely reduced indicative of retarded myotome differentiation. There is a significant increase in postnatal myogenin levels in MRF4 mutant mice. This is consistent with the notion that MRF4 is required to down-regulate postnatal myogenin expression (Zhang et al., 1995). Alternatively, increased

myogenin expression may compensate for the loss of MRF4 (Zhang et al., 1995). The sequence deleted in the Olson MRF4 allele is more extensive than either the Arnold or Wold alleles (for review see (Olson et al., 1996)). In addition, the PGK-neo cassette is inserted in the opposite orientation (opposite to Myf5 transcription). The enhancer element that regulates Myf5 epaxial somite expression is deleted in the Olson allele (Summerbell et al., 2000). The reduction in Myf5 expression in the epaxial somite may contribute to the delay in myotome differentiation observed in these MRF4 null mice.

MRF4 mutant mice generated in the laboratory of Hans Arnold die perinatally of respiratory distress due to severe malformation of the ribs (Vivian et al., 2000). Myf5 expression is disrupted in this mutant possibly because of interference by the PGK-neo cassette (Braun and Arnold, 1995; Summerbell et al., 2000). As a result, these mice are phenotypic representatives of Myf5^{-/-}; MRF4^{-/-} double knockout mice. These mice are essentially a phenocopy of the Myf5 mutant mice with a delay in early myotome formation (Braun and Arnold, 1995). The deep axial muscles are reduced in the Arnold MRF4 homozygous knockout mouse. Consistent with the hypothesis that Myf5 and MRF4 are important in regulating the development of muscles arising from the myotome (Braun and Arnold, 1995).

Compound MRF mutant mice

Compound MyoD^{-/-}; MRF4^{-/-} (Olson allele) and MyoD^{-/-}; Myogenin^{-/-} mice have been generated (Rawls et al., 1998). The muscle phenotype of a MRF4/ myogenin double mutant mouse is no more severe than the myogenin single mutant (Rawls et al., 1998). The number of residual myofibres is comparable in mice lacking myogenin or

myogenin / MRF4 suggesting neither myogenin nor MRF4 is required for primary myofibre differentiation. Interestingly continuous lines of myoblasts isolated from MRF4/myogenin double mutant mice differentiate normally *in vitro*, highlighting the differences between *in vivo* and *in vitro* myogenesis. The MRF4^{-/-}: MyoD^{-/-} mouse is a phenocopy of the myogenin mutant mouse with only residual myofibres and undifferentiated myoblasts (Rawls et al., 1998). This is consistent with the notion that terminal differentiation of myoblasts originating from the MyoD-dependent hypaxial lineage requires myogenin while terminal differentiation of myoblasts originating from the Myf5-dependent epaxial lineage requires MRF4. Myoblasts isolated from MyoD/MRF4 double mutant neonates differentiate normally *in vitro* suggesting the *in vivo* cellular environment may restrict the compensatory capacity of the remaining MRFs (Valdez et al., 2000). Mice lacking all of the MRFs except Myf5 have been generated (Valdez et al., 2000). Triple mutant myoblast fail to fuse both *in vitro* and *in vivo*. Unlike the myogenin or MRF4/MyoD mutant mice, these mice do not have residual myofibres. The observed differentiation defect in the triple mutant mice was suggested to reflect a failure to meet sufficient threshold levels of myogenic bHLH factors (Valdez et al., 2000), (Rawls et al., 1998). However, these results are consistent with the notion that individual myogenic bHLH proteins have evolved specialized functions and distinct MRFs may be required to switch on the expression of different muscle specific genes (Valdez et al., 2000). For example, MyoD and Myf5 unlike myogenin have domains that mediate chromatin remodeling, allowing for efficient activation of genes in regions of transcriptionally silent chromatin (Gerber et al., 1997).

Although a threshold level of MRF may be required for both *in vitro* and *in vivo* myogenesis, MRF embryonic expression patterns and the phenotypes of both single and compound mutant mice are consistent with a distinct myogenic lineage hypothesis. Targeting experiments demonstrate the hierarchical relationship between the MRFs. MyoD and Myf5 are primary MRFs required for the conversion of pre-myogenic cells into skeletal myoblasts. Myogenin and MRF4 are secondary MRFs required for myoblast differentiation and myotube fusion. In addition, these experiments support the notion that MyoD/myogenin and Myf5/MRF4 regulate the hypaxial and epaxial myogenic lineages respectively and that these lineages can to some extent compensate for each other.

Postnatal Myogenesis

Satellite Cells

In postnatal skeletal muscle a distinct lineage of myogenic progenitor cells, satellite cells, is responsible for growth, maintenance and repair of the muscle tissue (for review see (Seale and Rudnicki, 2000)). Satellite cells are intimately juxtaposed to the plasmalemma (sarcolemma) of mature myofibres such that a single basal lamina surrounds both the myofibre and the satellite cell. The nuclei of satellite cells are oval and are heterochromatic in comparison to myonuclei (reviewed in (Bischoff, 1994)). Satellite cells contain little cytoplasm, however have abundant plasmalemmal vesicles (reviewed in (Bischoff, 1994)). In neonatal rodents, approximately 30% of muscle nuclei represent satellite cells (Bischoff, 1994). The number of satellite cells decreases with ageing to between 3 and 5% in adult muscle (Bischoff, 1994; Gibson and Schultz, 1983;

Grounds, 1998). Normally mitotically quiescent, satellite cells are induced to proliferate in response to muscle damage and various other stresses (Seale and Rudnicki, 2000; Seale et al., 2000). After multiple rounds of division, the progeny of activated satellite cells (muscle precursor cells; mpcs) fuse to the damaged myofibres or form new fibres. Satellite cells and their daughter mpcs are distinct cell populations as defined by biological and biochemical criteria (Bischoff, 1994).

Pax7 and Satellite Cells

Pax7 is a paired homeobox transcription factor essential for satellite cell development (Seale et al., 2000). Except for in a few muscles, the muscles isolated from Pax7 mutant mice are devoid of satellite cells (Buckingham et al., 2003; Seale et al., 2000).

Furthermore differential expression of alternately spliced Pax7 transcripts correlates with muscle regenerative efficiency in different strains of mice (Kay et al., 1998; Kay et al., 1995). Consistent with the essential role of satellite cells in muscle repair, Pax7 mutant mice display a severe deficit in muscle repair following acute injury (Chargé, Seale and Rudnicki unpublished data). The specific requirement of Pax7 in satellite cell ontogeny is analogous to the essential function of a highly related family member, Pax3, in somitic myogenesis.

The Role of the MRFs in Satellite Cell Function

MRF expression pattern

The expression of the MRFs in quiescent satellite cells and during satellite cell activation, proliferation and differentiation has been assayed by both RT-PCR and transgene analysis (Cooper et al., 1999; Cornelison and Wold, 1997; Creuzet et al., 1998;

Smith et al., 1994a; Yablonka-Reuveni and Rivera, 1994). MRF expression is not detected in quiescent satellite cells by RT-PCR (Cornelison and Wold, 1997; Smith et al., 1994a; Yablonka-Reuveni and Rivera, 1994). However Myf5 (nlacZ) has been reported to be expressed in CD34 positive quiescent satellite cells (Beauchamp et al., 2000). Upon activation satellite cells quickly up regulate either MyoD or Myf5 expression prior to proliferation (Cooper et al., 1999; Cornelison and Wold, 1997; Smith et al., 1994a). Subsequently, most proliferating mpcs express both MyoD and Myf5 (Cooper et al., 1999; Cornelison and Wold, 1997). Myogenin and MRF4 are the last MRFs expressed coincidental with MPC differentiation and fusion (Cooper et al., 1999; Smith et al., 1994a; Yablonka-Reuveni and Rivera, 1994). The MRF expression pattern during satellite cell activation, proliferation and differentiation is analogous to the pattern observed during embryonic myogenesis. It is interesting to speculate that MyoD and Myf5 may regulate distinct satellite cell populations.

MyoD is required for Satellite cell function

Insights into the role of MyoD in satellite cell function came from studies in which MyoD mutant mice were interbred with *mdx* mice (Megeney et al., 1999). *mdx* mice have a loss of function point mutation in the dystrophin gene and are a model for human Duchenne and Becker muscular dystrophies (Bulfield et al., 1984; Sicinski et al., 1989). Although extensive muscle fibre necrosis is observed in young *mdx* mice, skeletal muscle integrity is maintained because of a high regenerative capacity of the tissue. The muscle of *mdx* mice is significantly hypertrophic with a predominant number of fibres containing centrally located nuclei (Anderson et al., 1987; Carnwath and Shotton, 1987; Coulton et

al., 1988). Centrally located nuclei are typical of repaired fibres (for review see (Grounds and Yablonka-Reuveni, 1993)). The severity of myopathic changes in the skeletal muscle of *mdx: MyoD^{-/-}* mice are profound in comparison to those observed in *mdx* mice and result in premature death. Compound mutant mice develop a severe dorsal-ventral curvature of the spine and have an abnormal waddling gait (Megeney et al., 1996). The muscle of *mdx: MyoD^{-/-}* mice, unlike that of *mdx* mice are not hypertrophic suggesting the loss of MyoD reduces the regenerative capacity of the tissue. This was tested directly by injury-induced regeneration experiments in MyoD null mice. MyoD-deficient muscle does not regenerate efficiently despite an increase in the number of satellite cells suggesting MyoD is required for progression through the differentiation program and in the absence of MyoD satellite cells undergo self-renewal (Megeney et al., 1996). (See figure 2).

A similar differentiation defect is observed in both primary MyoD^{-/-} myoblasts and single-fibre culture (Cornelison et al., 2000; Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). Consistent with chronic muscle regeneration *in vivo*, fibres isolated from MyoD^{-/-} muscle are abnormally branched (Cornelison et al., 2000). Although the majority of MyoD^{-/-} satellite cells in fibre culture entered the cell cycle and up regulated Myf5 expression, major defects in both myogenic gene expression and differentiation are observed (Cornelison et al., 2000). In particular MyoD^{-/-} satellite cells in single fibre culture fail to up regulate MRF4 expression and have reduced levels of m-caderin (Cornelison et al., 2000). These results suggest primary MyoD^{-/-} mpcs represent a normally transient intermediate between quiescent satellite cells and activated mpcs.

These cells provide a unique opportunity to study the early stages of satellite cell activation (Sabourin et al., 1999).

Myf5 and Regeneration.

The role of Myf5 in muscle regeneration has yet to be established. However the propensity of MyoD null Myf5 expressing mpcs for self-renewal suggests Myf5 may be involved in maintaining the satellite cell pool in adult muscle (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). Although inefficient, muscle regeneration does occur in the absence of MyoD (Megeney et al., 1996). In addition, 20% of satellite cells express Myf5 and not MyoD 3 hours post injury suggesting Myf5 expression is sufficient to activate the satellite cell developmental program (Cooper et al., 1999). Myf5 dependent regeneration may account for the regeneration observed in *mdx;MyoD^{-/-}* mice. With the recent generation of viable Myf5 null mice, the role of Myf5 in muscle repair will likely be elucidated in the near future.

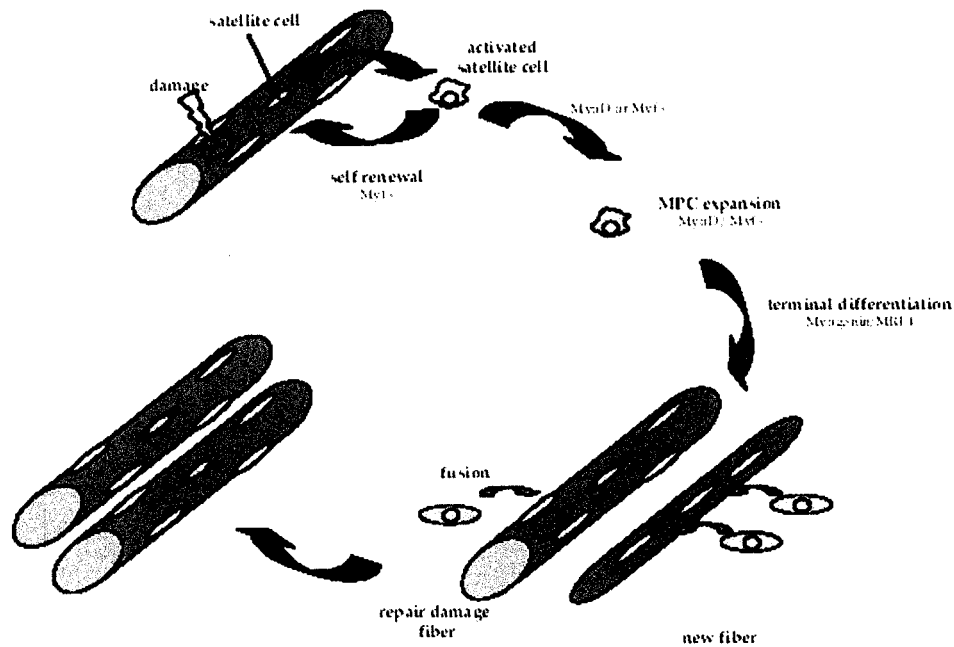


Figure 2. A schematic representation of satellite cell dependent muscle repair. Upon activation of satellite cells by muscle damage either MyoD or Myf5 expression is upregulated prior to proliferating. Subsequently, most proliferating satellite cells (myogenic precursor cells, MPCs) express both MyoD and Myf5. Expression of the secondary MRFs is coincidental with MPC differentiation and fusion.

differentiation program in mammalian cells and is insufficient to induce myoblast differentiation in myogenin null mice (Hasty et al., 1993; Molkentin et al., 1995; Nabeshima et al., 1993; Ornatsky et al., 1997; Yu et al., 1992). MEF2 factors act synergistically with the MRFs to initiate myogenesis (Black et al., 1998; Kaushal et al., 1994; Molkentin et al., 1995). This synergy has been suggested to be mediated by direct MEF2 / MRF interactions (Black et al., 1998; Molkentin et al., 1995). Because of functional redundancy between mouse MEF2 factors and cardiac insufficiency resulting in lethality, MEF2 knockout mice have been uninformative in regards to the *in vivo* role of MEF2 factors in skeletal myogenesis (Bi et al., 1999; Lin et al., 1997a; Lin et al., 1997b). However, an essential role for MEF2 factors is suggested by studies in which a dominant-negative form of MEF2A inhibits MyoD dependent conversion of 10T1/2 fibroblasts (Kolodziejczyk et al., 1999; Ornatsky et al., 1997).

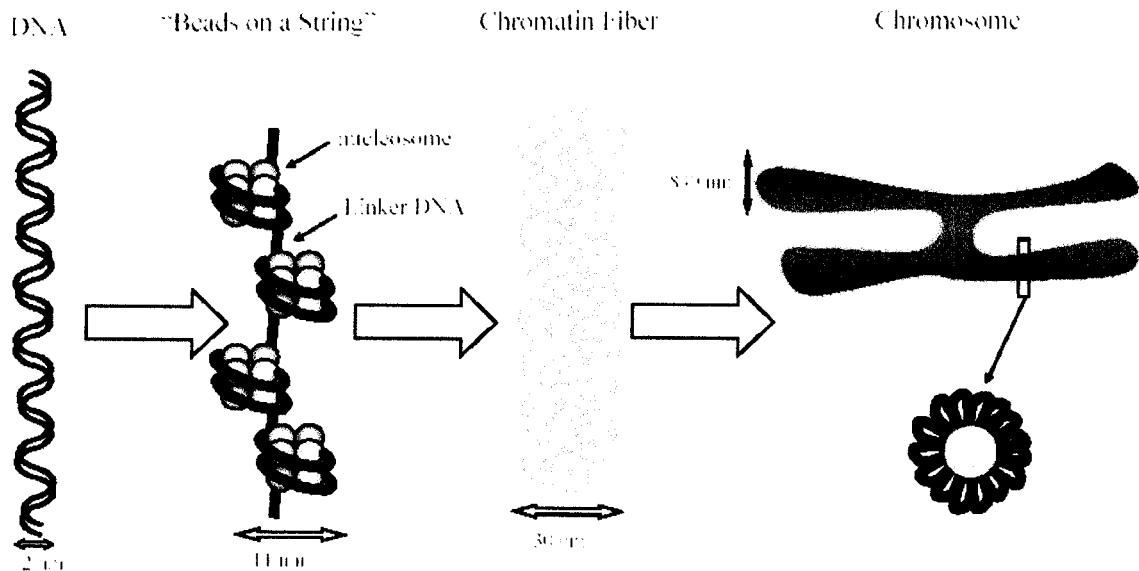


Figure 3. The appropriate regulation of transcription is dependent not only on DNA sequence but also on how the DNA is packaged or chromatin structure. In a eukaryotic cell, 146 bases of DNA double helix is wrapped $1\frac{3}{4}$ superhelical turns around a protein core consisting of histones (two each of H2A, H2B, H3, and H4). This is known as the "beads on a string" and is compacted into a 30 nm chromatin fiber by the binding of histone H1 to the nucleosome. The 30 nm fiber is in turn further condensed into 100 nm plus chromonema fiber.

Modified from www.albany.edu/~achm110/solenoidchromatin.html.

Chromatin

Chromatin Structure

Within all eukaryotic nuclei, DNA is organized into a highly dynamic and regulated structural polymer termed chromatin (for review (Fischle et al., 2003)). The nucleosome, consisting of 146 base pairs of DNA wrapped 1 3/4 times around a core octamer of histone proteins, is the basic structural unit of chromatin (see figure 3). The nucleosome core is a tripartite protein helix comprised of a histone H4 / histone H3 tetramer flanked by histone H2A / histone H2B dimers (for review (Zheng and Hayes, 2003)). The histones within the nucleosome core interact via a three α -helical “handshake” motif termed the histone fold (Rhodes, 1997). The core histones are structurally similar, highly basic proteins consisting of the histone fold motif and a N-terminal structurally undefined tail. The histone tails are subject to significant posttranslational modifications and, in part, dictate the level of chromatin condensation (Zheng and Hayes, 2003). Individual nucleosomes are separated from neighboring nucleosomes by a short segment of linker DNA between 10 and 80 bp in length. This form of chromatin is referred to as “beads on a string” and results in an approximately 10-fold compaction of the DNA (Felsenfeld and Groudine, 2003). Both *in vivo* and *in vitro* nucleosome positioning along a DNA molecule is sequence dependent (for review (Hayes and Hansen, 2001)). “Beads on a string” chromatin is compacted a further 5-fold into a 30 nm chromatin fiber by the binding of the linker histone, H1. Within the interphase nucleus of a cell, the condensation of the DNA into chromatin fiber is heterogeneous with regions of 30 nm chromatin fiber between more highly condensed

regions of 100 nm plus chromonema fiber (Horn and Peterson, 2002). Chromatin is the physiological substrate for most DNA-dependent process including transcription and DNA replication. As a result, changes in its structure have significant effects on these processes (for review (Iizuka and Smith, 2003)). The condensation of chromatin is refractory to DNA replication and transcription therefore mechanisms exist within the cell to locally de-condense the chromatin. (See figure 3).

Histone Modifications and Their Effects: Histone Code versus Charge Patch Mechanism

Covalent modifications to histone proteins affect both DNA binding to the nucleosome core and higher order nucleosome folding resulting in a change to the local chromatin structure (for review see (Wu, 1997) and (Roth et al., 2001)). The core histone proteins are subjected to an extensive array of modifications including acetylation, ADP-ribosylation, methylation, phosphorylation and ubiquitination at numerous residues. The biological role of these modifications depends not only on the type of modification but also the location of the modified site within the histone protein. However in general, hyperacetylation of histone H3 and histone H4 as well as dimethylation of histone H3-K4 are associated with open or transcriptionally active chromatin, while hypoacetylation of histone H3 and histone H4 as well as dimethylation of histone H3-K9 are the hallmarks of transcriptionally inactive chromatin. This pattern of histone modifications has been observed for the myogenin promoter (Mal and Harter, 2003). Prior to receiving pro-differentiation cues the myogenin promoter is transcriptionally inactive, hypoacetylated on histones H3 and H4 and enriched for methylation of lysine residue 9 of histone H3.

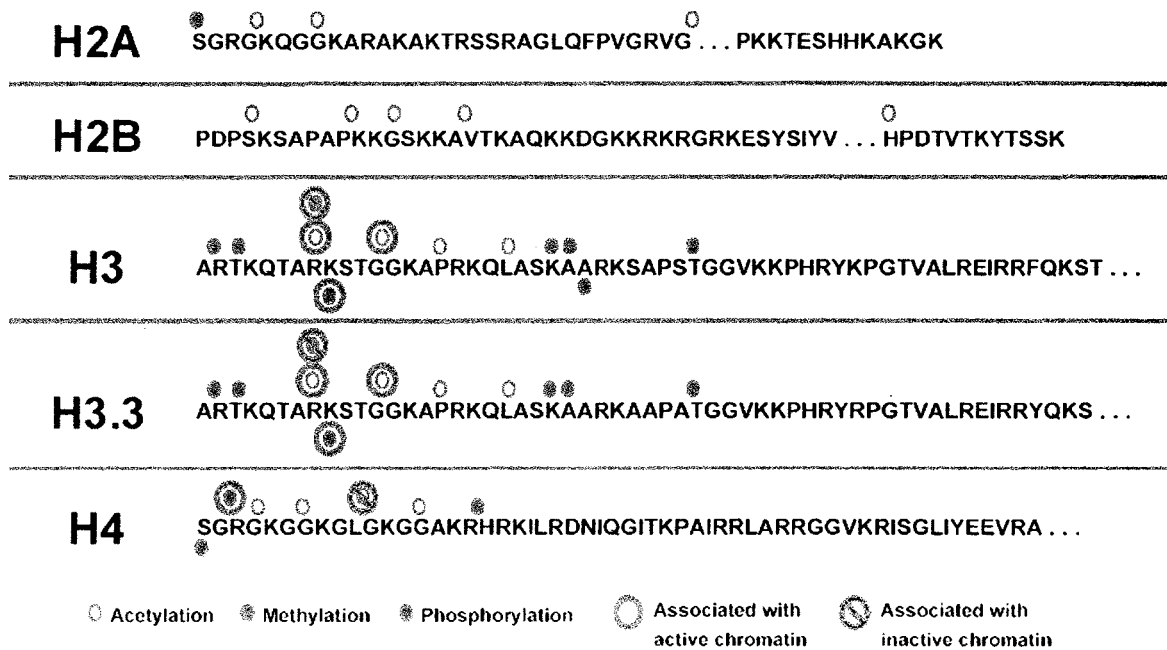


Figure 4. Core histone proteins are subject to a diverse array of post-translational modification. These modifications include acetylation, methylation, phosphorylation and ubiquitination. Each modification may have no effect, a positive effect or a negative effect on the transcriptional activity of the associated genes. Modified from (Goll and Bestor, 2002).

Subsequent to pro-differentiation cues, the myogenin promoter is hyperacetylated and transcriptionally active (Mal and Harter, 2003). (See figure 4).

Several models including the “Charge Patch Mechanism” and “Histone Code Hypothesis” have been proposed to account for the effect of histone modification on chromatin structure. In the Charge Patch Mechanism changes in the electrostatic properties of the core histones effect chromatin structure by modifying the strength of the DNA – histone interaction. A net reduction in the positive charge of the histones, for example by acetylation or phosphorylation, is predicated to weaken the strength of the DNA histone interaction and cause localized de-condensation of the chromatin (for review (Iizuka and Smith, 2003; Strahl and Allis, 2000; Wu, 1997)). Unlike the Histone Code Hypothesis, the effect of the modification is not specific to a particular residue. Support for the Charge Patch Mechanism, come from studies using *Tetrahymena* in which the effect of H2A.Z acetylation was mimicked by mutations that reduce the histones positive charge (Dou and Gorovsky, 2000; Iizuka and Smith, 2003; Ren and Gorovsky, 2001; Ren and Gorovsky, 2003). However the Charge Patch Mechanism fails to account for the different effects dimethylation of K9 and K4 of histone H3 have on chromatin function suggesting that a simple model is unlikely to account for all the effects of histone modifications on chromatin function (Iizuka and Smith, 2003).

Strahl and Allis proposed the Histone Code Hypothesis to account for the effect of multiple histone tail modifications (Strahl and Allis, 2000). In it the authors suggest “multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions”((Strahl and Allis, 2000),

p.43). Furthermore pre-existing histone modifications in this model are predicated to dictate both subsequent histone modifications and effector binding. Consistent with this is the observation that phosphorylation of histone H3-S10 promotes the acetylation of histone H3-K14 by GCN5 (Cheung et al., 2000; Lo et al., 2000). Moreover there is evidence supporting the notion that unique combinations of histone modifications define recognition motifs for distinct proteins. In particular, acetylation of Histone H4-K8 is required for the recruitment of Brg1, a component of the SWI/SNF remodeling complex while acetylation of Histone H4 at K9 and K14 is essential for the recruitment of TAFII250, a component of TFIID transcriptional complex. These results are consistent with the notion that particular histone modifications act as a receptor for particular transcriptional complexes ((Agalioti et al., 2002), for review (Iizuka and Smith, 2003)).

Chromatin Remodeling Enzymes

Histone Acetyltransferases

Histone acetyltransferases (HATs) are categorized into two classes, the nuclear HATs or type A HATs including Gcn5, PCAF, p300 and TAFII250 that are involved in transcriptional regulation and the cytoplasmic HATs or type B HATs including Hat1 that acetylate newly synthesized histones in the cytoplasm prior to nuclear import (for review (Roth et al., 2001)). Based on sequence similarities, type A HATs can be further subdivided into the Gcn5-related family, GNAT family including PCAF, the MYST family, the TFIID250 family and the p300/CBP family.

All HATs can catalyze the transfer of an acetyl group from acetyl coenzyme A to the $\epsilon\text{-NH}_3^+$ groups of lysine residues within a histone substrate; however, individual

HATs have distinct substrate preferences. *In vitro*, p300 preferentially acetylates histone H3 at K14 and K18 and histone H4 at K5 and K8 however it will acetylate histone H2A and H2B at all lysines acetylated *in vivo*. PCAF preferentially targets histone H3 at K14 and histone H4 at K5 and K8 (Schiltz et al., 1999). TAFII250 specifically acetylates histone H3 and to a lesser extent histone H2A (for review (Roth et al., 2001)). In addition to acetylating histones, several HATs including p300/CBP and PCAF target non-histone substrates such as E2F1, p53 and MyoD. The acetylation of these targets modulates their activity. In particular, the acetylation of E2F1 by PCAF stimulates its activity by increasing its affinity for DNA, its transactivating potential and its protein stability (Martinez-Balbas et al., 2000). A similar effect on DNA binding is observed following acetylation of both p53 and MyoD by CBP/p300 (Gu and Roeder, 1997; Polesskaya et al., 2000). MyoD activity on muscle reporter plasmids is dependent on this acetylation event since non-acetylatable mutants are non functional (Polesskaya et al., 2000).

CBP, p300 and GCN5 are essential for embryonic development since mice with targeted disruption of any of these HATs die *in utero* while the function of PCAF appears to be redundant since mice deficient in this HAT have no obvious phenotype (Xu et al., 2000; Yamauchi et al., 2000; Yao et al., 1998). HATs not only play a critical role in transcriptional regulation but also are essential for appropriate DNA repair and replication (for review (Hasan and Hottiger, 2002)).

Histone Methyltransferases and Demethylases

Histone methyltransferases share a highly conserved SET domain which catalyses the methylation event (for review (Marmorstein, 2003). To date over 70 genes have been identified with this motif suggesting that mammalian cells have a large repertoire of histone methyltransferases. Members of the Suv39 family of histone methyltransferases (HMTase) catalyze the dimethylation histone H3 at K9. This modification creates a binding site for HP1, a protein required for heterochromatin formation and is therefore generally associated with transcriptionally silent heterochromatic subdomains (Lachner and Jenuwein, 2002; Lachner et al., 2001). Histone H3 methylation is essential for the appropriate expression of developmental genes, inactivation of G9a, the dominant H3-K9 HMTase, results in growth and developmental defects and ultimately embryonic lethality (Tachibana et al., 2002; Xin et al., 2003).

The enzymatic demethylation of histones was first described in 1973 (Bannister et al., 2002; Lachner and Jenuwein, 2002; Paik and Kim, 1973). Although a histone demethylase has yet to be identified, there is evidence for an active and rapid gene-specific reversal of histone methylation (for review see (Bannister et al., 2002)). Sequence analysis of the Elp3 histone acetyltransferase indicates it may contain a second catalytic domain capable of demethylating histones suggesting the demethylation of histone H3-K9 is linked to its acetylation, however, the role of Elp3 as a demethylase has yet to be confirmed experimentally (Chinenov, 2002).

Histone Deacetylases

Histone deacetylases (HDACs) are a functional diverse group of proteins which catalyze the remove of acetyl residues from both histone and non-histone substrates. Based on sequence similarities HDACs can be categorized into three groups; class I HDACs include HDAC1, HDAC2, HDAC3 and HDAC8, class II HDACs include HDAC4, HDAC5, HDAC6 and HDAC7 and class III HDACs include the SIR2 related proteins SIRT1 to SIRT7 (for review (Khochbin et al., 2001) and (Thiagalingam et al., 2003)). HDACs have been implicated in a variety of cellular functions including cell cycle progression, transcriptional regulation DNA replication and damage response (Thiagalingam et al., 2003).

Class I HDACs share a common catalytic domain and are expressed in a wide variety of tissues. Members of this family, HDAC1 and HDAC2 associate with pRB and are required for appropriate cell cycle progression (Zhang et al., 2000). In addition, these HDACs are components of several co-repressor complexes include the N-CoR and SMRT complexes (for review (Jepsen and Rosenfeld, 2002)). Except for HDAC3, class I HDACs are strictly nuclear proteins (Takami and Nakayama, 2000).

Class II HDACs are evolutionarily divergent from Class I HDACs. Unlike there Class I counterparts, these HDACs are only expressed in specific tissues and are shuttled between the cytoplasm and nucleus. Class II HDACs are targeted to specific promoters through a direct interaction with the MEF2 family of transcription factors and have been shown to inhibit myogenesis (Verdin et al., 2003).

ATP-Dependent Chromatin Remodeling Motors

SNF2, ATP-dependent chromatin remodeling motors can be subdivided into several families including the SWI/SNF subfamily (Brg1), ISWI (SNF2H) subfamily and Chromo-domain containing subfamily (for review (Becker and Horz, 2002; Fry and Peterson, 2001; Fyodorov and Kadonaga, 2001; Narlikar et al., 2002; Peterson, 1996; Peterson and Workman, 2000; Vignali et al., 2000; Wang et al., 1996). The SWI/SNF family of molecular motors includes two highly similar proteins, Brg1 and Brahma. Brg1 and Brahma share 75% amino acid identity and have similar activities in *in vitro* assays suggesting that they may be functionally redundant (Kadam et al., 2000; Khavari et al., 1993; Phelan et al., 1999; Wang et al., 1996). However targeted disruption of Brg1 and Brahma highlight the functional differences between these two ATP-dependent chromatin remodeling proteins. Mice deficient in Brg1 die early in embryogenesis during the periimplantation stage suggesting Brahma is not able to compensate for the loss of Brg1 (Bultman et al., 2000). Mice deficient in Brahma develop normally however are significantly larger than wildtype counterparts consistent with a defect in the control of cellular proliferation (Reyes et al., 1998).

The homology between the SNF/SWI and ISWI subfamilies is limited to the ATPase or helicase domains, which consists of a bipartite ATP binding motif (figure 5). Although both SNF2H (ISWI) and Brg1 (SNF/SWI) can hydrolyze ATP and remodel linear arrays of nucleosomes, there are significant functional differences between the two proteins (Aalfs et al., 2001). In particular, SNF2H unlike Brg1 can neither remodel mononucleosomes nor alter the topology of nucleosomal plasmids (Aalfs et al., 2001). In

addition, Brg1 but not SNF2H can efficiently remodel tightly packed nucleosome arrays suggesting the targets of these SNF2 family members are distinct (Aalfs et al., 2001; Fan et al., 2003). These results are consistent with the notion that the mechanisms by which SNF/SWI and ISWI family members remodel chromatin are distinct (for review (Narlikar et al., 2002)). In particular unlike the mechanism used by SNF/SWI family members, the mechanism employed by ISWI family members appears to require nucleosome sliding (for review (Narlikar et al., 2002)).

Members of the SNF2 family of proteins are the ATPase component of large protein complexes. The Brg1 complex has as many as 16 subunits and a molecular weight of 2 MDa. Brg1 containing complexes have been implicated in a diverse array of cellular activities including cell cycle progression, transcriptional activation and repression (Sif et al., 2001; Zhang et al., 2000).

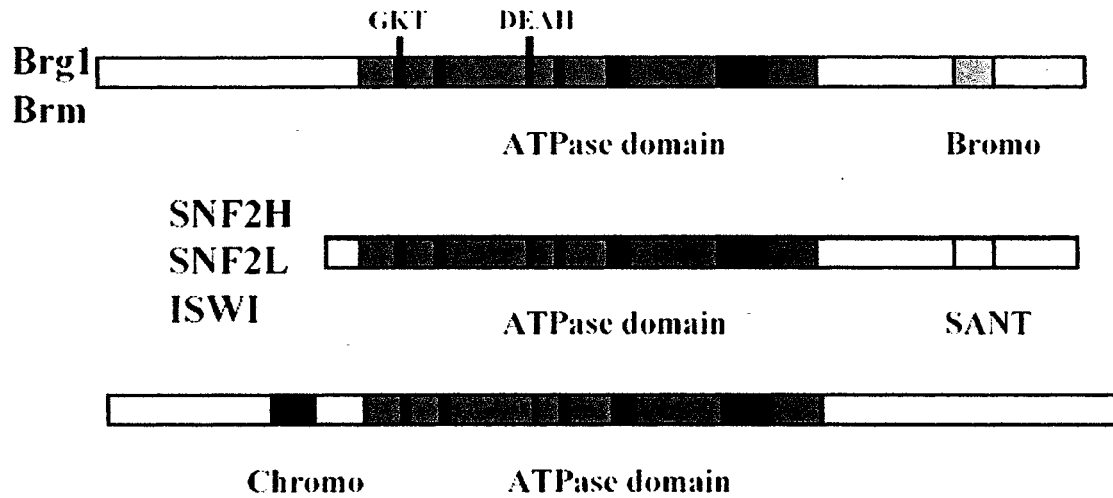


Figure 5. The SNF2 Family of chromatin remodeling motors. Modified from (Kingston and Narlikar, 1999).

The ISWI motors have been identified in multiple complexes ranging in size from 200 to 700 kDa and having up to 6 subunits. Recently, ISWI motors have been identified as a component of the RSF and ACF chromatin remodeling complexes (Bozhenok et al., 2002; Collins et al., 2002; Hakimi et al., 2002; LeRoy et al., 2000; Strohner et al., 2001). These include ACF1 (ATP-utilizing chromatin assembly and remodeling factor 1) and WICH (WSTF-ISWI chromatin remodeling complex) complexes (Bozhenok et al., 2002; Collins et al., 2002; Hakimi et al., 2002; LeRoy et al., 2000). As part of these complexes, SNF2H participates in various nuclear processes including the replication of highly condensed heterochromatin and sister chromatid cohesion during mitosis (Collins et al., 2002; Hakimi et al., 2002). In addition, SNF2H through its activity in the nucleolar remodeling complex (NoRC) complex is required to repress rRNA transcription (Santoro et al., 2002; Strohner et al., 2001). The NoRC complex establishes a heterochromatin structure around the promoter of ribosomal RNA genes via the recruitment of methyltransferases and the histone deacetylase containing Sin3 co-repressor complex (Santoro et al., 2002; Zhou et al., 2002).

Chromatin remodeling and the MRFs

Activation of the myogenic differentiation program requires the alleviation of condensed chromatin structure. Both MyoD and Myf5 remodel chromatin in the regulatory regions of skeletal muscle specific genes (Gerber et al., 1997). Two domains, a carboxy terminal domain and a cysteine/histidine rich domain located between the activation domain and the bHLH domain mediate MyoD-dependent chromatin remodelling (Gerber et al., 1997). Interestingly, a number of previously identified MyoD

binding partners (including COUP-TFII, N-CoR, p300 and PCAF) can modulate chromatin structure (Bailey et al., 1999; Bailey et al., 1998; Puri et al., 1997b; Sartorelli et al., 1997; Sartorelli et al., 1999). The interaction of MyoD with binding partners that can modulate chromatin structure likely provides a mechanism for targeting chromatin remodelling to muscle specific targets.

The MyoD repressor COUP-TFII is an orphan nuclear receptor that recruits histone deacetylases via its interaction with N-CoR and other co-repressors (Bailey et al., 1998). It directly binds the N-terminal activation domain of MyoD. Interestingly MyoD also directly interacts with N-CoR through its bHLH domain (Bailey et al., 1999). In addition to recruiting histone deacetylases, COUP-TFII represses MyoD activity by competitively inhibiting the binding of p300 as a result this repression can be alleviated by over expression of p300 (Bailey et al., 1998). It and other orphan nuclear receptors including REV-erb α and RVR expression are down regulated during myogenic differentiation and inhibit MyoD –mediated transcription (see (Bailey et al., 1999; Bailey et al., 1998)). These orphan nuclear receptors negatively regulate muscle differentiation and appear to have a role in maintaining the proliferative state of myoblasts (Bailey et al., 1998; Burke et al., 1996; Downes et al., 1995; Muscat et al., 1995).

Interestingly, MyoD recruits the histone acetyltransferase p300 and PCAF (Lau et al., 1999; Puri et al., 1997b; Sartorelli et al., 1997). CBP/p300 and PCAF interact directly with MyoD and are required for MyoD-dependent transactivation (Puri et al., 1997a; Puri et al., 1997b; Sartorelli et al., 1997; Yuan et al., 1996). A dominant negative p300 that is unable to bind MyoD inhibits both MyoD dependent myogenic conversion of

10T1/2 fibroblasts and transactivation by MyoD (Sartorelli et al., 1997). This is consistent with an essential role for endogenous p300 as a coactivator of MyoD. Both CBP/p300 and PCAF acetylate MyoD on two evolutionarily conserved lysine residues at the boundary of the bHLH domain (Polesskaya et al., 2000; Sartorelli et al., 1999). Acetylation of MyoD by either CBP/p300 or PCAF increases both *in vitro* DNA binding affinity and activity of muscle specific promoters (Polesskaya et al., 2000; Sartorelli et al., 1999). Conservation substitutions of these lysine residues with non-acetylable arginines inhibit MyoD activity (Polesskaya et al., 2000; Sartorelli et al., 1999). Recently an inhibitor of p300 histone acetylase activity, EID-1, was identified as a repressor of MyoD function (MacLellan et al., 2000). EID-1 is a 187 amino acid E1A like protein preferentially expressed in adult cardiac and skeletal muscle tissue (MacLellan et al., 2000). Skeletal muscle specific transcription is inhibited when EID-1 is over expressed and appears to be due to its ability to bind and inhibit p300's histone acetylase activity (MacLellan et al., 2000).

Previously SNF2 family members, including Brg1 have been implicated in muscle differentiation. MyoD-dependent conversion of fibroblasts to the myogenic lineage is inhibited by ectopic expression of a dominant negative Brg1 suggesting Brg1 is essential for MyoD-mediated muscle differentiation. However Brg1 is not required for MyoD-dependent cell cycle arrest. Brg1 has also been implicated in Myf5 and MRF4 dependent transcription (de la Serna et al., 2001a; de la Serna et al., 2001b; Roy et al., 2002).

MEF2 and Chromatin Remodeling

In addition to modulating MRF transcriptional activity, histone-modifying enzymes are critical in regulating the activity of a second family of transcription factors essential in myogenesis. The MEF2 family consists of MADS-box containing transcription factors that act synergistically with the MRFs to activate the muscle differentiation program (Black et al., 1998; Black and Olson, 1998; Kaushal et al., 1994; Molkenkin et al., 1995). Class II HDACs, including HDAC4 and 5 bind directly to MEF2 and repress MEF2-dependent transcription. Release from this repression is a prerequisite for muscle differentiation and is mediated by CaMK (calcium/calmodulin-dependent kinase) pathway dependent nuclear export of class II HDACs (McKinsey et al., 2000; McKinsey et al., 2001a; McKinsey et al., 2001b). Although evidence suggests CBP/p300 may be required to activate MEF2-dependent transcription, a definitive role for this HAT as a MEF2 co-activator in skeletal muscle has yet to be established (McKinsey et al., 2001a).

Project Outline

Chromatin is actively remodeled during both myoblast specification and differentiation. It has been clearly demonstrated that both ATP-dependent chromatin remodeling proteins and histone modifying enzymes have critical roles in myogenesis. The main objective of this thesis is to further characterize the role of chromatin remodeling in myoblast specification and differentiation. Such a characterization, in addition to furthering our understanding of the myoblast differentiation process and highlighting the importance of histone modifying enzymes to the differentiation process,

provides lessons that are generally applicable to other cell culture models of differentiation.

Objective 1: The first objective was to characterize the role of the ATP-dependent chromatin remodeling motor Snf2h in myoblast differentiation. Snf2h is a ubiquitously expressed member of the ISWI subfamily of nucleosome remodeling factors. Snf2h was identified as a MyoD-interacting protein in a yeast two-hybrid screen. This interaction was confirmed using *in vitro* binding assays. Ectopic expression of an ATPase dead form of hSNF2H in C2C12 myoblasts (C2C12 [hSNF2H (211KtoR)]) accelerated the differentiation process while having no affect on either growth rate or cell cycle kinetics. The chromatin structure surrounding the myogenin (a MyoD target gene) E boxes is more accessible in C2C12 [hSNF2H (211KtoR)] compared to control C2C12 cells. Taken together these results suggest that MyoD actively recruits chromatin remodeling complexes to the promoters of target genes. Snf2h appears to be required to suppress the expression of differentiation specific genes under growth conditions.

Objective 2: Although in general, the histones associated with actively transcribed genes are thought to be hyperacetylated and those associated with inactive genes are thought to be hypoacetylated, this has not been assessed on a genome-wide scale. Our goal was to develop a methodology that would allow for the unbiased assessment of chromatin modifications throughout the genome. By combining chromatin immunoprecipitation with Affymetrix SNP microarray technology, the changes in histone modifications occurring at over ten thousand loci were assessed simultaneously. This data was compared with transcript data generated from Affymetrix transcript microarray analysis

to examine the correlation between histone modification and transcriptional status. Using this data, we generated a temporal picture of genome-wide changes to a variety of histone modifications occurring during myoblast differentiation.

Objective 3: Activated MEK1 inhibits MyoD dependent transcription while inhibition of MEK1 by U1026 promotes myogenic differentiation. We undertook a study to assess the role of chromatin remodeling factors in MEK1-dependent inhibition of MyoD activity. It was demonstrated that inhibition of MEK1 increased MyoD associated histone acetyltransferase activity by approximately two-fold leading to hyperacetylation of muscle specific promoters. This data is consistent with the notion that MEK1 modulates MyoD-dependent transcription by regulating the chromatin remodeling proteins associated with MyoD.

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CHAPTER 2: Overexpression of an ATPase Defective SNF2H in C2C12 Myoblasts Accelerates Differentiation.

Foreword

The following chapter entitled “Overexpression of an ATPase Defective SNF2H in C2C12 Myoblasts Accelerates the Differentiation” describes the role of the ISWI homology Snf2h in myoblasts differentiation. Our data suggests, Snf2h activity is required to repress differentiation specific genes during proliferation. C.M. Palmer completed the majority of the work and wrote the manuscript. L.A. Sabourin constructed all yeast two hybrid vectors including the library used in this screen. L.A. Sabourin and C.M. Palmer completed the yeast two-hybrid screen. M.A. Lazzaro, and D.J. Picketts provided Flag-hSNF2H (WT) vector. M.A. Rudnicki supervised the project.

Title Page

**Overexpression of an ATPase Defective SNF2H in
C2C12 Myoblasts Accelerates Differentiation.**

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Abstract

To further understand the mechanisms by which MyoD activity is regulated, we used MyoD as bait in a repressed transactivator yeast two-hybrid screen of a mouse embryonic day 10.5 library. We identified the ISWI nucleosome remodeling factor, Snf2h as a MyoD interacting protein. *In vitro*, the MyoD basic DNA binding domain interacted with amino acids 475 to 588 of mouse Snf2h. Overexpression of an inactive form of hSNF2H [hSNF2H (K211R)] in C2C12 myoblasts accelerated the differentiation process, causing early expression of both myogenin and myosin heavy chain however did not affect either growth rate or cell cycle kinetics. The chromatin structure surrounding the myogenin (a MyoD target gene) E boxes in growing control and C2C12 [hSNF2H (K211R)] myoblasts was assessed using nuclease access assays. The chromatin structure surrounding the myogenin E boxes was more open in C2C12 [hSNF2H (K211R)] compared to control C2C12 cells. Microarray analysis of growing C2C12 [hSNF2H (K211R)] and control C2C12 cells suggested the myoblast differentiation program was partly derepressed in cells expressing hSNF2H (K211R). These results are consistent with the notion that functional Snf2h complexes are required to repress differentiation specific genes during growth and subsequently maintain the myoblast proliferative state.

Introduction

MyoD is a dominant acting myogenic regulatory factor with a central role in myoblast specification and differentiation (for review (Sabourin and Rudnicki, 2000)). It has the capacity to initiate the entire myogenic program in non-muscles cells. MyoD activity is regulated by both post-translational modifications and at the level of protein-protein interactions including both the regulation of heterodimerization and the recruitment of co-factors (Li et al., 1992; Liu et al., 1998; Polesskaya et al., 2001a; Puri and Sartorelli, 2000).

Chromatin is actively remodeled during myogenic specification and differentiation. Histone acetyltransferases, histone deacetylases and ATP-dependent chromatin remodeling motors have been implicated in both myoblast specification and differentiation. MyoD specifically targets chromatin remodeling machinery to the regulatory regions of skeletal muscle specific genes (Bailey et al., 1999; Gerber et al., 1997; Mal and Harter, 2003; Mal et al., 2001; Puri et al., 2001). A carboxyl terminal and a cysteine/histidine rich domain located between the activation domain and the bHLH appear to mediate this activity (Gerber et al., 1997). MyoD interacts with a number of proteins (including HDAC1, COUP-TFII, N-CoR, p300 and PCAF) that modulate chromatin structure to either promote or inhibit transcription (Bailey et al., 1999; Bailey et al., 1998; Polesskaya et al., 2001a; Puri et al., 1997b; Sartorelli et al., 1997; Sartorelli et al., 1999).

The histone acetyltransferases p300 and PCAF directly interact with MyoD and are essential for MyoD-dependent transactivation (Lau et al., 1999; Polesskaya et al.,

2000; Polesskaya et al., 2001a; Puri et al., 1997a; Puri et al., 1997b; Sartorelli et al., 1997; Yuan et al., 1996). Inhibition of p300 by a dominant negative version of the protein negates both MyoD transactivation and MyoD dependent conversion of 10T1/2 fibroblasts suggesting endogenous p300 is an essential MyoD coactivator (Sartorelli et al., 1997). COUP-TFII is an orphan nuclear receptor that interacts with MyoD and recruits histone deacetylases via its interaction with N-CoR and other co-repressors. This interaction represses MyoD transcriptional activity (Bailey et al., 1998). Repression of MyoD transcription is also achieved by the direct interaction of HDAC1 with the MyoD bHLH domain (Mal and Harter, 2003; Mal et al., 2001; Puri et al., 2001). The negative regulation of MyoD and muscle differentiation by these chromatin remodeling proteins may be required for the maintenance of the myoblast proliferative state (Bailey et al., 1999; Bailey et al., 1998; Burke et al., 1996; Downes et al., 1995; Muscat et al., 1995).

During chromatin remodeling, nucleosomes are mobilized by members of the SNF2 family of ATP-dependent chromatin remodeling motors. Based on homology SNF2 proteins are classified as members of either the Brg1 subfamily, ISWI subfamily or Chromo-domain containing subfamily (for review (Becker and Horz, 2002; Fry and Peterson, 2001; Fyodorov and Kadonaga, 2001; Narlikar et al., 2002; Peterson, 1996; Peterson and Workman, 2000; Vignali et al., 2000; Wang et al., 1996). Functional differences between the ISWI and Brg1 subfamilies suggest they have distinct substrates (Aalfs et al., 2001). In particular, Brg1 has the unique capacity to efficiently remodel tightly packed nucleosome arrays (Aalfs et al., 2001; Fan et al., 2003). ISWI subfamily members can destabilize reconstituted nucleosomes facilitating the binding of activator

proteins to consensus DNA binding sites (Aalfs et al., 2001). Previously SNF2 family members, including Brg1 have been implicated in MyoD-mediated muscle differentiation but not MyoD-dependent cell cycle arrest (de la Serna et al., 2001a; de la Serna et al., 2001b).

We have used a repressed transactivator (RTA) yeast two-hybrid system to screen an embryonic day 10.5 library for novel MyoD interacting proteins (Hirst et al., 2001). Using this system, we identified Snf2h as a potential MyoD interacting protein. Expression of a dominant negative version of hSNF2H in C2C12 myoblasts accelerated the differentiation process without affecting either growth rate or cell cycle kinetics. Our data appears to support the notion that Snf2h functions to suppress the expression of differentiation specific genes under growth conditions.

Materials and Methods

Yeast Inverse Two-Hybrid.

The yeast repressed transactivator two-hybrid screen performed was modified from the methodology originally described by (Hirst et al., 2001).

Plasmids and Yeast Strains.

GAL4 DNA binding domain-MyoD fusion was expressed from the ADH1 promoter on a low copy number Trp selectable plasmid derived from Ycplac22 (YcpMyoD). To generate the TUP1-E12 fusion protein, the amino-terminal portion (residues 1-200) of TUP1 was PCR amplified and subcloned into the unique BglII site of pRS425 (*LEU2*) (Christianson et al., 1992) through primer-derived BamHI/BglII sites. Yeast TUP-E12 expression vectors were generated by subcloning PCR products. PCR fragments corresponding to the E12 bHLH region (nucleotides 822-1332) and the bHLH region lacking helix 2 (nucleotides 822-1129) were cloned in frame downstream of the TUP1 amino-terminal region in pRS425 generating pTUP-E12bHLH and pTUP-E12bHLH Δ H2. Plasmid TUP-E12bHLH-R was constructed by cloning the E12 bHLH fragment in the reverse orientation. Expression from the TUP1 fusion plasmids is controlled by a yeast PGK-1 promoter-terminator cassette (Masuda et al., 1994).

MaV103 was maintained on YPAD plates and individual colonies were grown in liquid cultures and transformed with the various plasmids as described (Gietz et al., 1992; Gietz et al., 1995). The genotype of MaV103 has been described previously (Vidal et al., 1996). MaV103 strain was kindly provided by Marc Vidal (Harvard). Following transformation with the indicated plasmids, equivalent aliquots of transformation

mixtures were plated on synthetic complete (SC) medium lacking the appropriate amino acids, in the presence or absence of 5-fluoroorotic acid (5-FOA; Sigma).

Construction of pTRD1 and expression library screening.

The library expression plasmid pTRD1 (*LEU2*) was derived from pGAD10 (Clontech). Briefly, the GAL4 activation of pGAD10 was replaced by inserting a TUP1 EcoRV/BamHI fragment containing residues 1-200 into a BamHI/blunt-ended HindIII site (upstream of GAL4 AD), leaving unique convenient restriction sites downstream of TUP1 for cloning purposes. To construct the mouse embryonic library, total RNA was extracted from pooled 10.5-day embryos and twice selected through oligo dT cellulose to isolate poly-A⁺ mRNA (Sambrook, 1989). Adapter-ready double stranded cDNA was synthesized (Clontech, Two Hybrid cDNA library construction kit) and ligated into EcoRI-restricted and dephosphorylated pTRD1. The primary library, containing a total of 1.2×10^6 independent clones, was amplified and stored at -80°C . Plasmid DNA was prepared for transformation according to the manufacturer's instructions. Library screens were performed as described (Gietz et al., 1997). Briefly, a subclone expressing the GAL4 DB-MyoD fusion was transformed with library DNA and plated out on SC/-Leu/-Trp selective medium containing 0.025% 5-FOA and colonies were allowed to grow for 4-6 days. PCR inserts from selected clones were sequenced and analysed using BLAST searches.

Beta-galactosidase assays.

Liquid β -galactosidase assays were performed as described in (Gietz et al., 1997) in triplicate on three independent clones for each plasmid vector combination.

***In vitro* binding assays.**

For *in vitro* binding studies, SNF2H was subcloned into Myc-tagged pcDNA3 vector and *in vitro* translated using T7 polymerase TNT system (Promega) in the presence of 35 [S]-methionine. For binding assays, the various GST-MyoD fusions bound to glutathione Sepharose beads (Pharmacia) were pre-blocked in the presence of 1% bovine serum albumin for 15 min at room temperature. Labelled SNF2H product was then added and incubated for an additional 15 min and then washed three times in NETN buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris and 0.1% NP-40). The samples were boiled in SDS sample buffer and analysed by 12% SDS-PAGE and autoradiography. Full length flag-tagged human SNF2H was described previously (Lazzaro and Picketts, 2001). Truncated GST-MyoD fusions were constructed by cloning PCR products in-frame with GST into pGEX4T-1 (Pharmacia). The MyoD N-terminal truncation consists in amino acids 1-95, the MyoD-basic domain construct encompasses residues 95-122 and finally, the C-terminal fragment comprises amino acids 174-318.

Transfection of C2C12 myoblasts with hSNF2H Plasmid and Generation of Stable C2C12 (hSNF2H) Clones.

Subconfluent C2C12 myoblasts were transfected with pFlag-SNF2H (neo) or empty vector using lipofectamine as per the manufacturers instructions. The cultures were refed with growth media (DMEM supplemented with 15% fetal calf serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin) containing 800 μ g of G418 until colonies formed. The resulting colonies were picked, and expression confirmed by

western blot using antibody directed against the flag epitope. Positive clones were expanded for further analysis.

Growth Rate and Differentiation Time Course.

The growth kinetics was analyzed by enumerating the cells at 24 hrs, 72 hrs and 120 hrs after seeding at a density of 10^5 in growth media (each in triplicate). The cell cycle profiles were determined by fluorescence activated cell sorting (FACS) as described previously and confirmed by anti-phosphorylated Histone H1 and H3 immunohistochemistry. Determination of cell cycle stages by immunohistochemistry was performed according to manufacturers instructions (Upstate Biotechnology).

To assess the differentiation potential of the C2C12 clones, cells were seeded into growth media at a density of 10^4 cells / well of a 2-well chamber slide or 10^5 cells / 10 cm tissue culture dish and cultured until 75% confluence prior to the addition of differentiation media (DMEM supplemented with 2% horse serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin). Differentiation media was changed every two days. Each day one chamber slide for each clone was fixed with 4% paraformaldehyde, cells were permeabilized by 0.2% Triton-X and immunostained for myogenin (M-225; Santa Cruz, sc-576) and myosin heavy chain (MF20 supernatant) at a dilution of 1/50 and 1/5 respectively.

Nuclease Access Assay.

The nuclease access assay was performed as described elsewhere (Gerber et al., 1997). Briefly, cells were washed with PBS and trypsinized. Cell pellets were washed with ice-cold RSB buffer (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM MgCl₂). Cells were

resuspended in ice-cold lysis buffer (RSB buffer + 0.1% NP40) to a density of 1.5×10^6 / mL and incubated on ice for 10 min. The nuclei were isolated by centrifugation at 2000 RPM for 5 min at 4°C and wash once with lysis buffer. 10^6 nuclei were resuspended in 200 μ L of 1 x NEB 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol) and digested with 40 U of BanI for 30 min at 37°C. The reaction was terminated by the addition of 200 μ L of stop buffer (0.6M NaCl, 20mM Tris Base at pH 7.4, 10mM EDTA, 1% SDS and 2mg/mL Proteinase K) and deproteinated overnight at 50°C. DNA was purified and digested to completion with indicated restriction enzymes. Digested DNA was subjected to Southern blot analysis. See figure legend for details regarding the probe.

Results

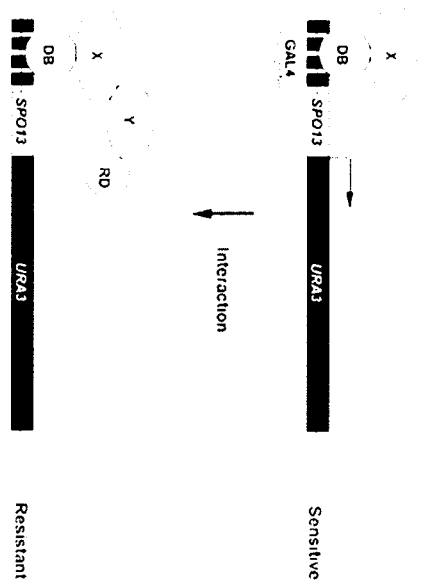
A MyoD-Specific Repressed Transactivator Yeast Two-Hybrid Screen.

MyoD activity is regulated at the level of protein-protein interactions (Puri and Sartorelli, 2000). To identify proteins which interact with and potentially regulate MyoD function, a GAL4-DNA binding domain / MyoD fusion (GAL4-MyoD) was used in a repressed transactivator yeast two-hybrid screen. Full length MyoD is a transcriptional activator and therefore is not amenable for use in a traditional yeast two-hybrid screen in which the expression of a reporter gene, required for growth on minimal media, is dependent on an interaction between the “bait”-binding domain fusion and “prey”-activation domain fusion. However, it has been previously demonstrated that a repressed transactivator (RTA) yeast two-hybrid system is an appropriate tool for examining the interaction of a transactivator protein including full length MyoD with its binding partners (Hirst et al., 2001) (figure 1A).

The RTA yeast two-hybrid system utilizes the URA3 reporter gene whose expression is toxic under specific growth conditions (Hirst et al., 2001). The URA3 gene encodes for orotidine-5'phosphate decarboxylase, an enzyme required for uracil biosynthesis (Denis-Duphil, 1989). Since orotidine-5'phosphate decarboxylase catalyzes the release 5-fluorouracil, a toxic metabolite, from 5-fluoroorotic acid (5-FOA), URA3 can be utilized as a yeast selectable marker (Boeke et al., 1984). Thus activation of the URA3 reporter gene by a transactivator such as GAL4-MyoD results in a 5-FOA-sensitive phenotype (FOA^S).

Figure 1. Repressed Transactivator (RTA) Yeast Two-Hybrid. (A) Binding of a transactivator/GAL4-DNA binding domain fusion (X-DB) to GAL4 binding sites induces the reporter gene (*URA3*) whose expression is toxic in the presence of 5-FOA (sensitive). Interaction between protein X and a protein Y-repression domain fusion (Y-RD) alters the chromatin conformation due to nucleosome assembly. Reduced expression of the reporter gene allows a selective growth advantage on medium containing 5-FOA (resistant). (B) FOA^S phenotype in SPAL::*URA3* yeast cells is titratable. A strong FOA^S phenotype (0.025% 5-FOA) is observed in MaV103 cells transformed with the GAL4-DB/MyoD fusion, indicating high level of expression of the SPAL::*URA3* reporter. Co-transformation with the *LEU2*-based TUP-E12bHLH fusion results in a FOA^R phenotype up to 0.05% 5-FOA.

A



B

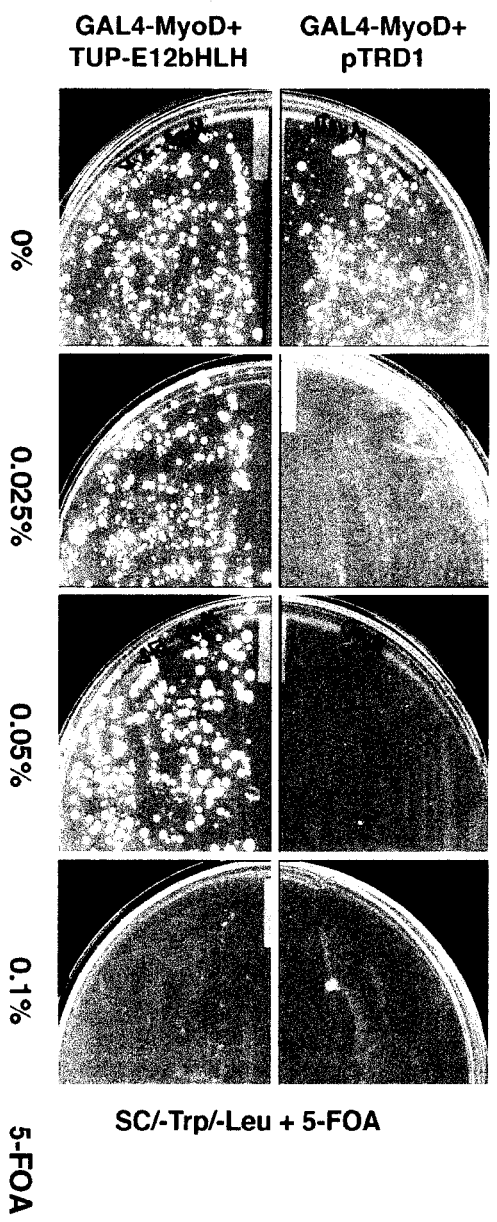
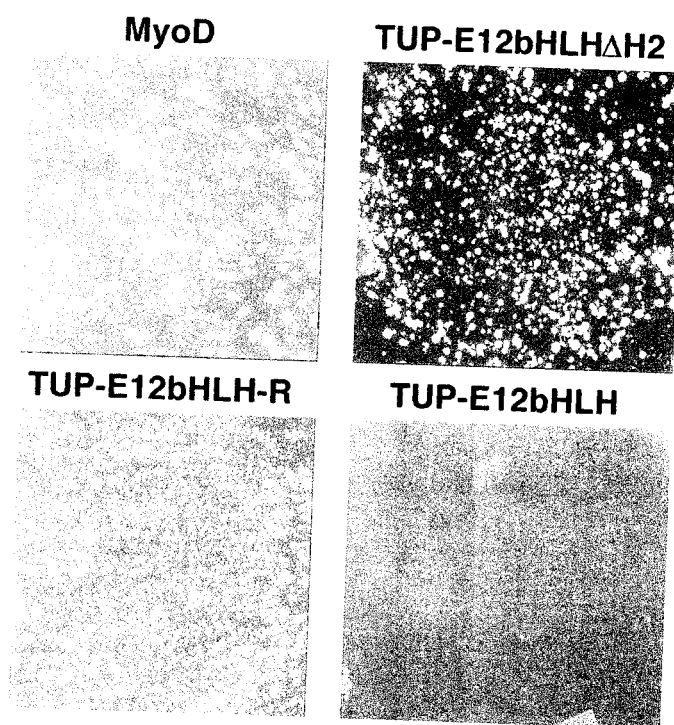
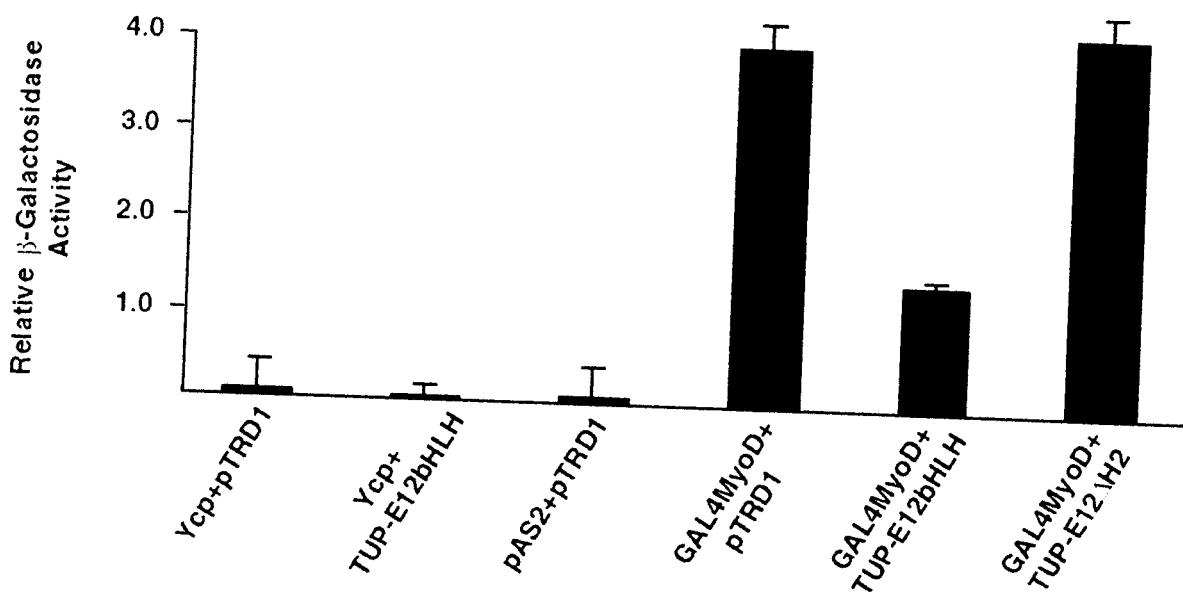


Figure 1 continued. (C) Conversion of a His⁺ to His⁻ phenotype following co-transformation of Y190 with GAL4-DB/MyoD and TUP-E12bHLH. Following transformation, equivalent aliquots of transformation mixtures were plated on synthetic complete medium lacking leucine and tryptophan in the presence of 20 mM 3-aminotriazole (3-AT). No colonies developed when MyoD was co-transformed with TUP-E12bHLH. Growth was observed on plates co-transformed with MyoD and TUP-E12bHLH-R (reverse E12bHLH orientation) or TUP-E12bHLHΔH2, a non-dimerizing form of E12 lacking the second helix. (D) Repression of *GAL1::LacZ* in MaV103. Liquid β-galactosidase assays were performed in triplicate for three independent clones co-transformed with the indicated plasmids. A 60-70% reduction in LacZ activity was consistently observed when TUP-E12bHLH was co-transformed with GAL4-DB/MyoD. Ycp and pAS2 are control *TRP1*-based GAL4-DB empty vectors. pTRD1 is a control TUP1 amino-terminus empty vector (*LEU2*).

C



D



Down regulation of the URA3 reporter gene occurs when the transactivating bait interacts with a protein that is fused to the yeast TUP1 amino-terminal transcriptional repression domain (Carrico and Zitomer, 1998; Edmondson et al., 1996; Huang et al., 1997; Keleher et al., 1992; Marquez et al., 1998; Wahi and Johnson, 1995). This confers a selective growth advantage on minimal media containing 5-FOA (see figure 1).

The yeast strain MaV103 (kindly provided by Marc Vidal; Harvard) carries the URA3 reporter gene under the transcriptional control of ten GAL4 binding sites upstream of the sporulation-specific SPO13 promoter (Wang et al., 1987), allowing for both tight regulation of the reporter gene and reduced basal expression (Vidal et al., 1996). Therefore, in the absence of GAL4 binding domain-dependent transactivation, the GAL410-SPO13::URA3 (SPAL10::URA3) reporter confers a very tight FOA-resistant (FOA^R) phenotype. Upon expression of a GAL4 DB-MyoD fusion, MaV103 is sensitive to 5-FOA (FOA^S). However, co-transformation with the MyoD interacting partner, E12 fused to the amino-terminus repression domain of TUP1 results in repression of URA3 and a FOA^R phenotype (Figure 1B).

To test the extent of the SPAL10::URA3 gene repression upon MyoD-E12 interaction, GAL4-MyoD was co-transformed with a TUP1-E12bHLH fusion, or a TUP1 control vector, into Mav103. Titration of the interaction on increasing concentrations of 5-FOA showed a FOA^R phenotype in cells co-transformed with both plasmids, up to 0.05% 5-FOA, indicative of TUP-mediated repression of the URA3 reporter.

Repression of two other MaV103 reporter genes, GAL1::LacZ and GAL1::HIS3, was also demonstrated in co-transformation assays. As shown in figure 1C, expression of

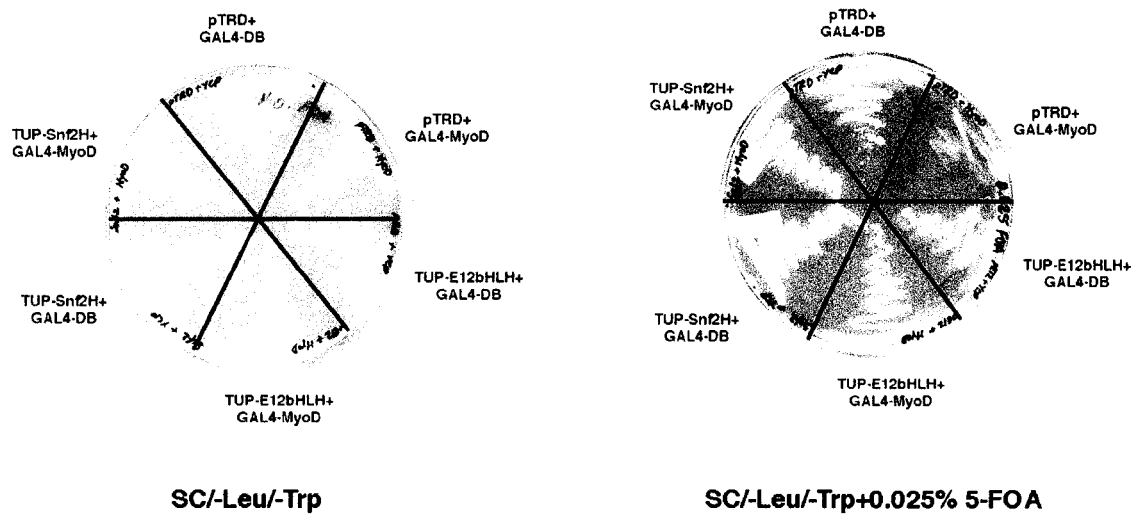
GAL4-MyoD in MaV103 induced a strong His⁺ phenotype that could overcome the presence of 20 mM 3-aminotriazole (3-AT). However co-transformation of GAL4-MyoD and TUP-E12bHLH resulted in the loss of the His⁺ phenotype, suggesting repression of the GAL1::HIS3 reporter. A strong His⁺ phenotype was maintained in cells co-transformed with GAL4-MyoD and TUP-E12bHLH Δ H2, a non-interacting E12 mutant, or a plasmid encoding TUP-E12bHLH in the reverse orientation (TUP-E12bHLH-R). When the relative β -galactosidase activity was evaluated from these co-transformants, a 60-70% reduction in LacZ activity was observed in cells expressing TUP-E12bHLH, but not in cells expressing the E12 mutant TUP-E12bHLH Δ H2 (Figure 1D). Again, the partial repression of GAL1::LacZ may reflect the leakiness of the GAL1 promoter. No activity was observed in the absence of GAL4-MyoD.

Identification of Snf2h as a MyoD interacting protein using an RTA

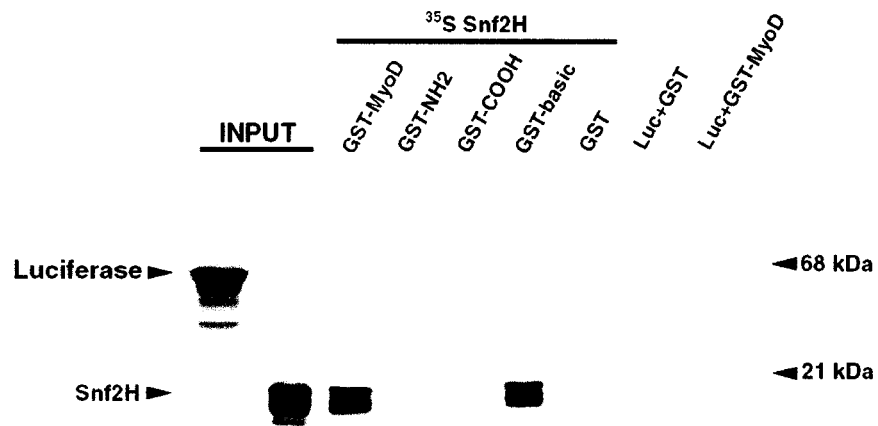
To identify novel proteins that bind to and potentially regulate MyoD function during embryonic myogenesis, Gal4-MyoD was used as bait in a RTA screen of a library containing the TUP1 chromatin repression domain fused to random-primed cDNA from e10.5 day BalbC mouse embryos. Approximately one million transformants were screened for resistance to 0.025% 5-FOA. Of 5000 clones resistant to 5-FOA, 200 were sequenced. One of the clones identified as a candidate MyoD interacting protein was Snf2h. Ectopic expression of this fragment of Snf2h in mouse cells was toxic. Since Snf2h is homologous to the yeast ISWI proteins, high-level expression of this fragment of Snf2h in yeast may not be compatible with viability. This may account for the failure to isolate more than one yeast clone containing Snf2h.

Figure 2. Snf2h and MyoD interact *in vivo* and *in vitro*. (A) *In vivo* interaction between GAL4-Db/MyoD and TUP-Snf2h. TUP-Snf2h plasmid was co-transformed with various control plasmids in addition to GAL4-DB/MyoD. Colonies from the initial plating on SC/-Leu/-Trp plates were streaked on SC/-Leu/-Trp medium and SC/-Leu/-Trp containing 0.025% 5-FOA and allowed to grow at 30°C for 3 days. Growth was observed when TUP-SNF2H was co-transformed with GAL4-DB/MyoD compared to GAL4-MyoD alone. Yeast expressing GAL4-DB grows on media containing 5-FOA since the URA3 reporter gene is not expressed. No interaction was observed between MyoD and TUP1 or Snf2h and GAL4-DB. (B) Snf2h binds MyoD *in vitro*. To test binding between MyoD and SNF2H, *in vitro* translated ³⁵[S]-Snf2h was incubated with immobilized GST-MyoD or MyoD truncations, washed and resolved by SDS-PAGE followed by autoradiography. Binding of Snf2h was observed to full length MyoD (GST-MyoD) and to the basic DNA binding domain (GST-basic). Weak or no binding was observed to either MyoD amino or carboxy-terminus (GST-NH2 and GST-COOH, respectively). Snf2h showed no binding to GST alone. As controls, luciferase was incubated with GST alone or GST-MyoD; no binding was detected.

A



B



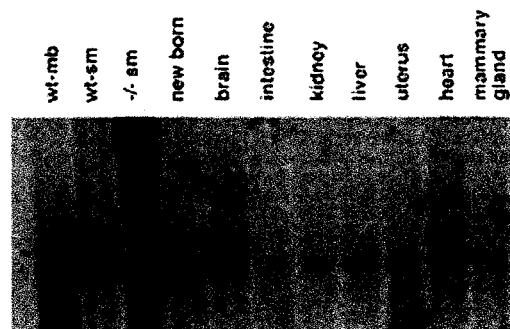


Figure 3. *Snf2h* is widely expressed in mouse tissues. Wt-mb, wildtype mouse myoblasts; sm, skeletal muscle; -/- sm, MyoD null skeletal muscle. Northern blot was probed with *Snf2h* fragment isolated during RTA screen

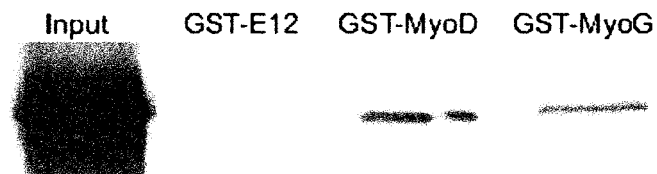


Figure 4. SNF2H binds MyoD *in vitro*. To test binding between MyoD and full length SNF2H, *in vitro* translated ³⁵[S]-SNF2H was incubated with immobilized GST-MyoD, GST-myogenin (MyoG) or GST-E12, washed and resolved by SDS-PAGE followed by autoradiography. Binding of SNF2H was observed to full length MyoD (GST-MyoD) and myogenin (GST-MyoG). Weak or no binding was observed to E12 (GST-E12).

The Snf2h clone isolate had a frame shift mutation in the promoter resulting in low level expression in yeast. In this report, we describe the characterization of the role of SNF2H in myogenesis.

Snf2h is a widely expressed member of the ISWI subfamily of nucleosome remodeling factors (Chapter 1, Fig. 6; Fig. 3). ISWI subfamily members can destabilize reconstituted nucleosomes facilitating the binding of activator proteins to consensus DNA binding sites. Transformation of MAV103 with GAL4-MyoD and TUP-Snf2h conferred resistance to 5-FOA concentrations as high as 0.05% while resistance to 5-FOA was not observed when Gal4-MyoD was transformed into MAV103 with the empty Tup1 vector (Fig. 2A). Together, these results confirm the specific nature of the repression of MyoD-dependent activation of the URA3 reporter by the TUP-Snf2h fusion. The Snf2h fragment identified in the RTA yeast two-hybrid screen encompassed amino acids 475-588 of the mouse sequence. This region of Snf2h contains a large portion of the SNF2 ATPase domain located in the C-terminus (amino acids 512 to 596) (Fig. 2C).

MyoD binds SNF2H *in vitro*

To test the binding between MyoD and Snf2h, *in vitro* translated ³⁵[S]-Snf2h (113 amino acid fragment identified in RTA screen) was incubated with immobilized GST-MyoD or various MyoD truncations, washed and resolved by SDS-PAGE followed by autoradiography. Snf2h was capable of binding full length MyoD (GST-MyoD) and the basic DNA binding domain of MyoD (GST-basic, amino acids 95-122). High affinity binding was not observed to either the amino-terminus or carboxy-terminus of MyoD (GST-NH2 and GST-COOH, respectively) (Fig. 2B). Similarly, full length human

SNF2H (hSNF2H) bound full length MyoD and myogenin *in vitro* (Fig. 4). Although Snf2h and MyoD interacted in both the RTA yeast two-hybrid and a GST *in vitro* binding assay, we failed to co-immunoprecipitate SNF2H with MyoD from C2C12 myoblasts suggesting that the interaction is transient in nature. Since MyoD has been shown to interact with other chromatin remodeling proteins, it prompted us to further evaluate this interaction.

Expression of SNF2 Family Members During Myoblast Differentiation

In addition to the interaction between MyoD and SNF2H that we identified, Brg1 and Brahma are essential for MyoD-mediated muscle differentiation but not MyoD-dependent cell cycle arrest (de la Serna et al., 2001a; de la Serna et al., 2001b). As such, we examined the expression pattern of a variety of ATP-dependent chromatin remodeling motors from each of the SWI/SNF, ISWI and chromo-domain containing subfamilies, by semi-quantitative RT-PCR during C2C12 myoblast differentiation (Fig. 5). Brm and Brg1, members of the SWI/SNF family of the ATPase dependent chromatin remodeling motors are expressed throughout myoblast differentiation with maximal mRNA expression observed following the induction of differentiation by serum withdrawal consistent with their role during myoblast differentiation as described previously. Snf2h is expressed in both cycling myoblasts and differentiating myotubes with maximum levels observed subsequent to serum withdrawal. A similar expression pattern was observed for Etl-1, the founding member of distinct subfamily of SNF2 proteins (Soininen et al., 1992). The expression of the chromodomain containing SNF2 motor, Chd-1 was constant throughout differentiation.



Figure 5. Snf2h and Brg1 are expressed in both proliferating and differentiating myoblasts, with maximum expression observed subsequent to mitogen withdrawal. The expression of SNF2 family members during myoblast differentiation was analyzed by semi-quantitative RT-PCR. Total RNA was prepared from either proliferating or differentiating myoblasts. PCR primers and conditions are as described previously (Machida et al., 2001). G, growth media, 1 through 5 indicate days in differentiation media.

Since Snf2h has a similar pattern of expression as Brg1 and bromha and these SNF2 proteins have previously been shown to have a role in myoblast differentiation, we decided to investigate the role of Snf2h in myoblast differentiation.

MyoD, Myf5, and Snf2h protein expression is regulated during the cell cycle.

In proliferating myoblasts, the expression of MyoD and Myf5 are cell cycle regulated (Kitzmann et al., 1998). MyoD protein expression is highest in mid-G1, and subsequently decreases to its minimum level at the G1/S boundary prior to increasing. Myf5 protein levels decrease to minimal levels during G1, rebound at the end of G1 to a level that remains stable until mitosis (Kitzmann et al., 1998). To test if MyoD and Snf2h were co-expressed during the cell cycle C2C12 myoblasts were synchronized as described by Kitzmann et al (1998). Similar to Kitzmann *et al*, we observed cyclic expression of both MyoD and Myf5. Interestingly Snf2h was co-expressed with MyoD during the cell cycle, consistent with role for Snf2h in regulating MyoD activity (figure 6).

Generation of a dominant negative hSNF2H.

To determine the role of Snf2h in muscle differentiation we generated C2C12 myoblast cell lines that overexpressed either wildtype or dominant negative hSNF2H. Replacement of the ATP binding lysine residue of ISWI family members with an arginine residue has previously been shown to generate a dominant negative protein (Hakimi et al., 2002). A dominant negative version of a flag tagged hSNF2H was constructed by overlapping PCR in which lysine residue 211 was replaced with arginine. C2C12 myoblasts were stably transfected with either wildtype hSNF2H or hSNF2H (K211R).

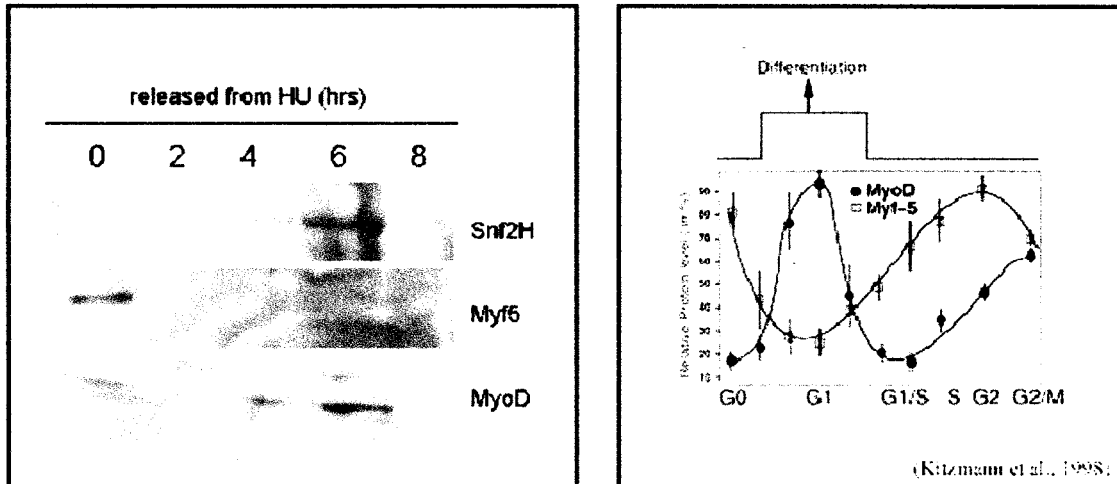


Figure 6. Expression of Snf2h, MyoD and Myf5 proteins are cell cycle regulated. Consistent with a role in regulating MyoD activity Snf2h and MyoD are co-expressed during the cell cycle.

LADEMGLGKTLQTISLL → LADEMGLGRTLQTISLL

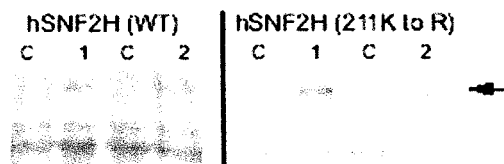


Figure 7. C2C12 myoblasts clones that expressed either wildtype hSNF2H or hSNF2H (K211R) were analyzed by Western blot against the Flag epitope. The ATP-binding lysine in hSNF2H was mutated to arginine using overlapping PCR. C212 myoblasts were transfected with either empty pCI-neo vector or pCI-neo/Flag-tagged hSNF2H (WT), or pCI-neo/Flag-tagged hSNF2H (K211R). Transformants were selected using G418 and individual clones isolated. Expression from clones was confirmed by Western blot analysis using antibodies directed against the Flag epitope.

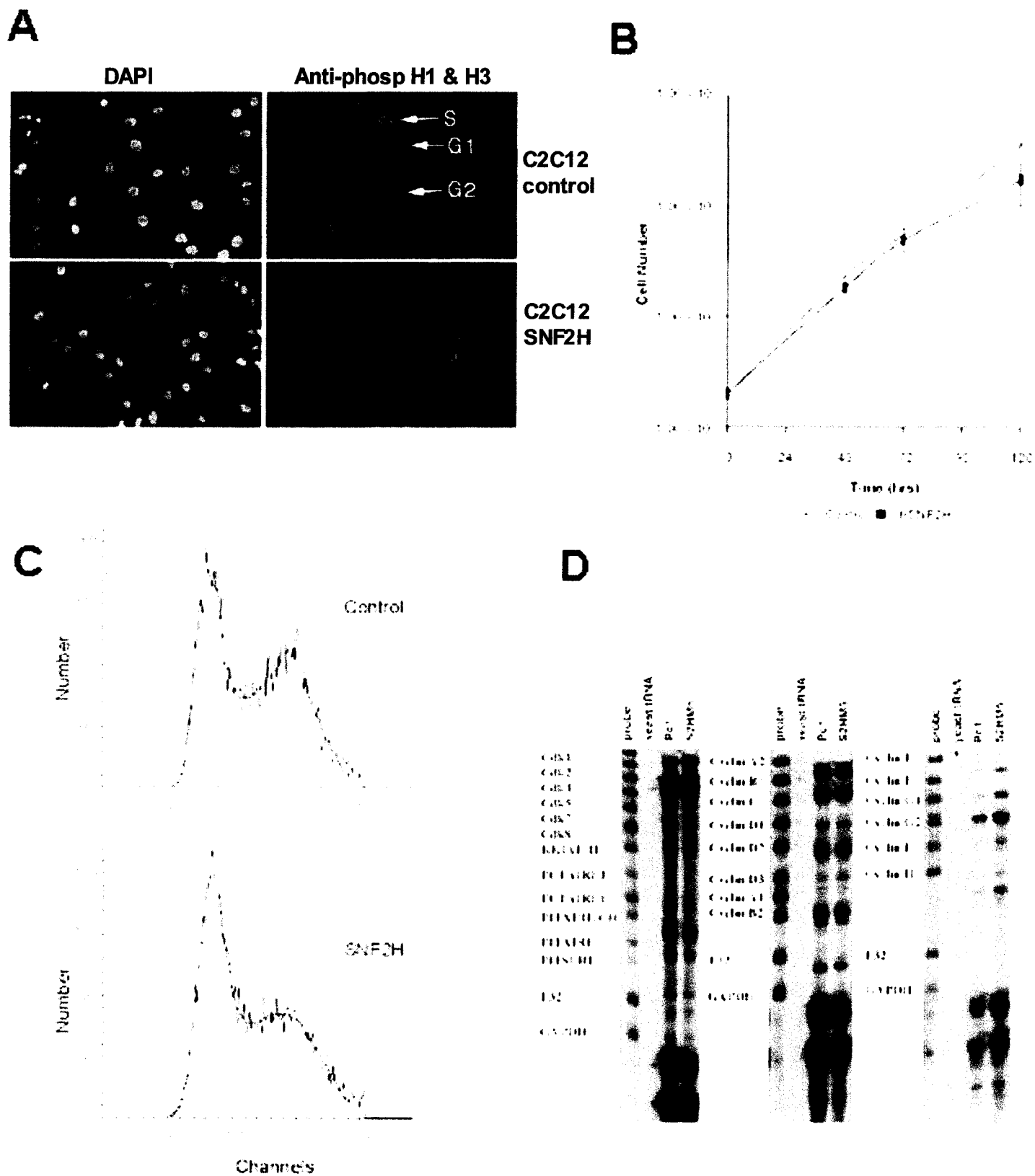
Cells were subjected to G418 selection. Since only between 10% and 15% of G418 resistant C2C12 myoblasts expressed low levels of either wildtype hSNF2H or hSNF2H (K211R) we choose to use isolate clones as opposed to using pools of transfectants. This result suggests high-level expression of hSNF2H is incapability with viability.

Expression of either dominant negative or wildtype hSNF2H was confirmed using antibodies directed against the Flag epitope (figure 7). A total of 4 independent clones, 2 independent clones expressing dominant negative hSNF2H (C2C12 [hSNF2H (K211R)]) and 2 independent clones expressing wild type hSNF2H (C2C12 [hSNF2H (WT)]) were expanded for further analysis. In each set, (C2C12 [hSNF2H (WT)]) and C2C12 [hSNF2H (K211R)], independent clones behaved similarly.

Dominant negative SNF2H does not affect the growth rate of C2C12 myoblasts.

The growth rate of control C2C12 myoblasts was comparable to the growth rate of C2C12 myoblasts overexpressing the dominant negative form of hSNF2H (see figure 8B). In addition the cell cycle distribution (see figure 8A and C) or the expression of cell cycle regulators as determined by RNase protection (see figure 8D) was not significantly altered in hSNF2H (K211R) overexpressors consistent with the notion that Snf2h activity is not required for myoblast growth.

Figure 8. Inhibition of Snf2h activity has no effect on myoblast proliferation or cell cycle. (A, B, C & D) The cell cycle profiles [phospho-histone staining (A) and FACs (C)] and expression of cell cycle regulators (as determined by RNase protection) are not significantly different between overexpressing SNF2H (K211R) (S2HM6) and control C2C12 myoblasts (Pc1). (B) There was no difference in growth rate between C2C12 overexpressing SNF2H (K211R) and control C2C12 myoblasts.



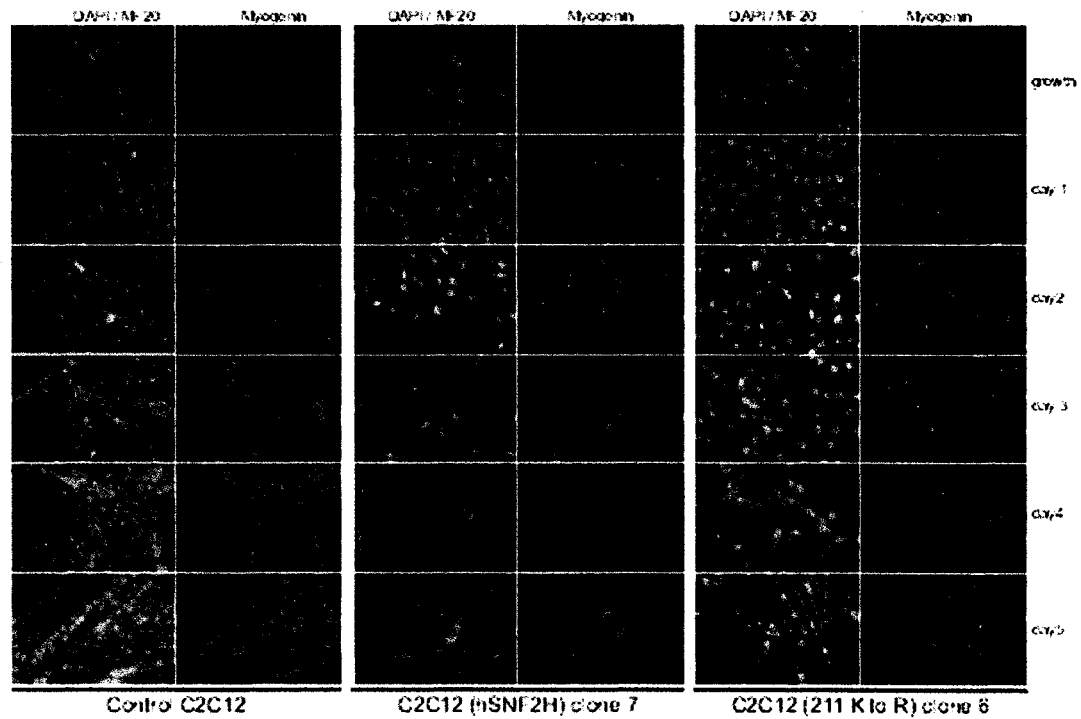


Figure 9. C2C12 clones were induced to undergo terminal differentiation by mitogen withdrawal. Differentiation was assessed immunocytochemical using antibodies directed against the differentiation markers myogenin and myosin heavy chain. MF20 is a mouse monoclonal antibody directed against myosin heavy chain. Inhibition of Snf2h activity accelerated C2C12 myoblast differentiation.

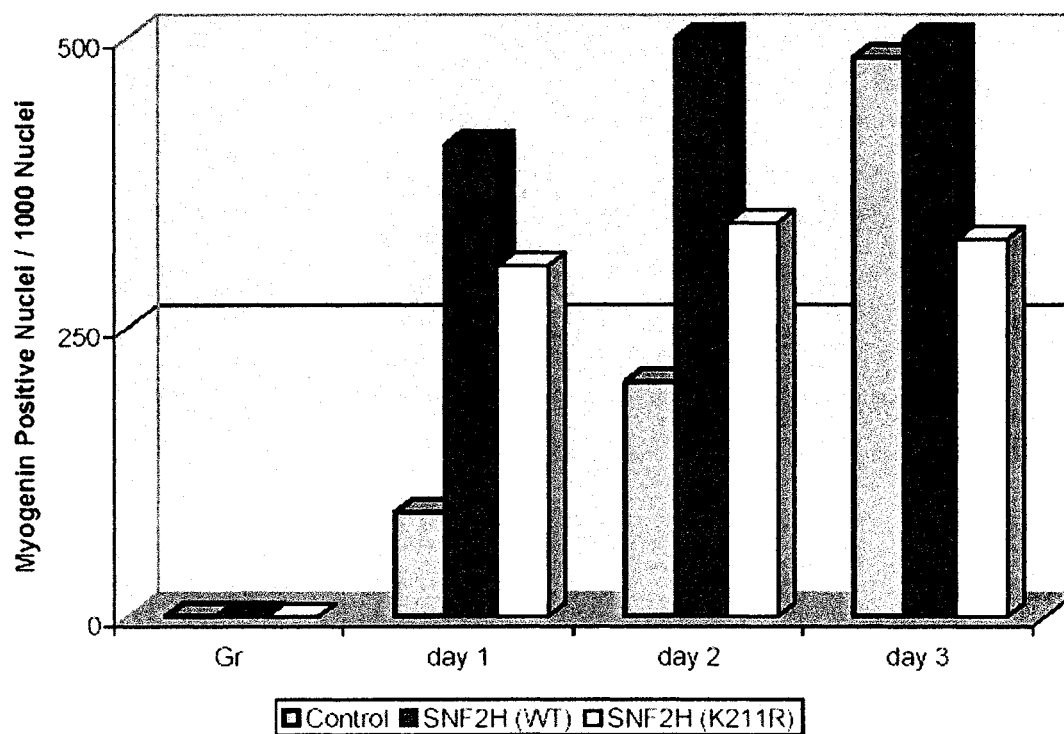


Figure 10. Differentiation is accelerated in C2C12 myoblasts expressing either hSNF2H (WT) or hSNF2H (K211R) as indicated by early expression of myogenin.

Ectopic Expression of SNF2H accelerates myoblast differentiation.

Ectopic expression of either hSNF2H (WT) or hSNF2H (K211R) resulted in earlier expression of both myogenin and myosin heavy chain protein (figure 9 and figure 10). In C2C12 myoblasts ectopically expressing hSNF2H (WT) compared to control C2C12 myoblasts, there was a 4.5-fold and 3.4-fold higher number of myogenin positive cells after 24 hrs and 48 hrs in differentiation media respectively. Similarly, in C2C12 myoblasts ectopically expressing hSNF2H (K211R) compared to control C2C12 myoblasts, there was a 3.4-fold and 1.7-fold higher number of myogenin positive cells after 24 hrs and 48 hrs in differentiation media respectively. Although accelerated, the later differentiation process in C2C12 [hSNF2H (WT)] appears to be abnormal with very few multinucleated myotubes formed after 5 days in differentiation media. In addition, there was a marked reduction in cell number suggesting that appropriate Snf2h activity is essential for normal myoblast differentiation. The defect in multinucleated myotube formation was less severe in C2C12 myoblasts expressing hSNF2H (K211R) suggesting ectopic expression of hSNF2H (WT) more effectively squelches Snf2h activity.

The chromatin surrounding the myogenin promoter is more accessible in myoblasts expressing hSNF2H.

Upregulation appears to be at the transcriptional level since myogenin mRNA is upregulated in cell ectopically expressing SNF2H (Figure 11). To determine if the early expression of myogenin mRNA was due to premature remodeling of the myogenin promoter, the chromatin structure surrounding the myogenin (a MyoD target gene) E boxes in growing control and C2C12 [hSNF2H (K211R)] was assessed using a nuclease

access assays (see Figure 11C). A BanI restrictions site located between the two E boxes within the myogenin promoter is more accessible to cleavage when the myogenin gene is transcriptional active (Gerber et al., 1997). This BanI was more readily cleaved in chromatin isolated from C2C12 [hSNF (K211R)] cells as compared to control C2C12 myoblasts. This is consistent with the notion that the chromatin structure surrounding the myogenin E boxes is more open in growing C2C12 in which Snf2h activity (Murre et al., 1989) has been inhibited.

To further ascertain the role of Snf2h during myoblast differentiation, the transcriptional profiles of growing myoblasts over expressing the ATPase dead form of SNF2H was compared to control myoblasts using Affymetrix microarrays. The Affymetrix microarray data confirmed the aberrant expression of muscle differentiation specific genes in growing overexpressors including IGF2, alpha 1 procollagen, alpha-actinin-2 and myosin alkalin light chain (Table 1). Interesting, a downregulation of decorin was observed in C2C12 [hSNF2H (K211R)] compared to control cells. Previously, expression of an antisense directed against decorin was shown to accelerate myoblast differentiation (Riquelme et al., 2001)(Table 2). Together our results suggest Snf2h activity may be required to maintain myoblasts in a proliferative state.

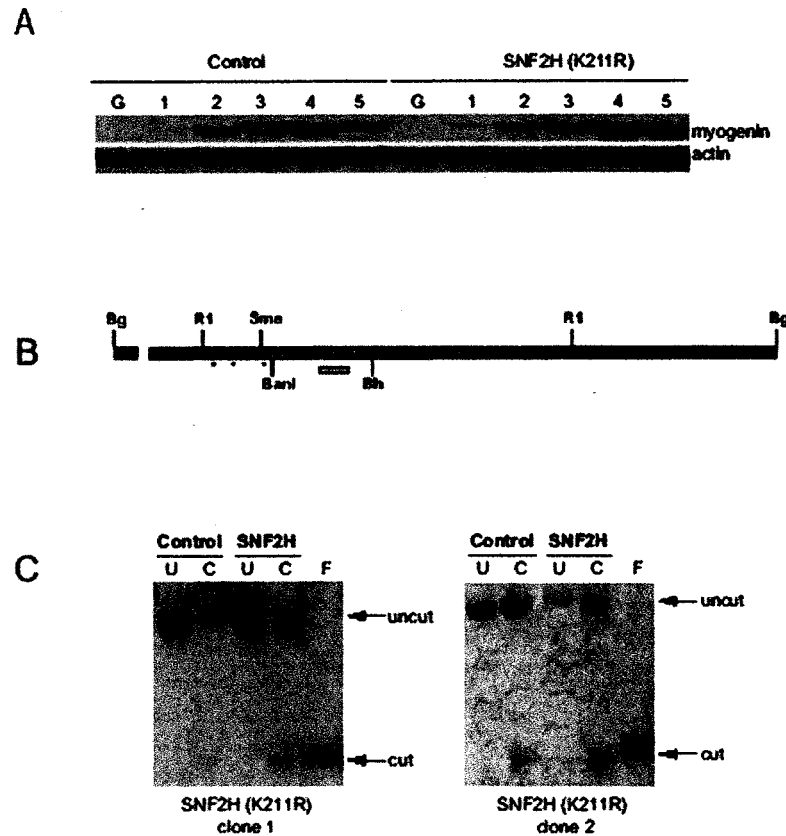


Figure 11. (A) Myogenin protein and mRNA are expressed prematurely in C2C12 myoblasts overexpressing SNF2H (K211R). (C) The E box within the myogenin promoter is more accessible to cleavage with BanI in C2C12 myoblasts overexpressing SNF2H (K211R) compared to control C2C12 myoblasts. Southern blot analysis assessing the accessibility of a BanI site located within the myogenin promoter. Isolated nuclei were subjected to limited digestion with BanI as described previously (Gerber et al., 1997). DNA was purified and digested to completion with BamHI/EcoRI and probed with the fragment depicted in the schematic (B). These results are consistent with the promoter being in a more active chromatin configuration in the SNF2H (K211R) C2C12 myoblasts.

Gene Name	Fold Increase	Description
Adhalin	9.4	•50 kDa dystrophin-associated-glycoprotein (Matsumura et al., 1999)
IGF2	8.8	•Overexpression promotes myogenic differentiation
MPTPdelta (type A)	7.1	•Protein tyrosine phosphatase, receptor type D
alpha-actinin-2 associated LIM protein	5.0	•actin binding protein. localizes to Z bands
sFRP-2	5.0	•secreted frizzled related protein sFRP-2 •Wnt antagonist (Jones and Jomary, 2002)
Cardiac actin	4.8	• major actin in skeletal muscle cell differentiation in vitro (Sieck and Regnier, 2001)
RyR1	3.2	•skeletal muscle ryanodine receptor(Fill and Copello, 2002)
NLRR-1	17.0	•leucine-rich-repeat protein
DAN	15.4	•TGF β antagonists(Dionne et al., 2001)
Mox-2	14.0	•Associates with Pax-3(Mankoo et al., 1999)
MLC1F/MLC3F	6.4	•myosin alkalin light chain (fast skeletal muscle isoform)

Table 1. A list of mRNAs whose expression is increased in growing C2C12 [hSNF2H (K211R)] compared to control myoblasts.

Gene Name	Fold Decrease	Description
Decorin	52.6	<ul style="list-style-type: none"> •Antisense accelerates muscle differentiation •Sequesters TGF-beta(Riquelme et al., 2001)
Clast1	12.9	<ul style="list-style-type: none"> •CD40-activated gene
Integrin alpha 5 subunit	3.5	<ul style="list-style-type: none"> •Expression decreases during myoblasts differentiate into bipolar myocytes(Blaschuk et al., 1997; Steffensen et al., 1992)
Ceruloplasmin	6.9	<ul style="list-style-type: none"> •Principle copper-containing protein in serum(Hellman and Gitlin, 2002)
Ptz-17	7.3	<ul style="list-style-type: none"> •pentynetetrazol-related mRNA

Table 2. A list of mRNAs whose expression is decreased in growing C2C12 [hSNF2H (K211R)] compared to control myoblasts.

Discussion

We employed an RTA yeast two-hybrid screen of an embryonic day 10.5 library to identify proteins, which bind to and potentially regulate MyoD function during early embryonic myogenesis. In the RTA yeast two-hybrid screen, we identified Snf2h as a novel MyoD interacting protein. This interaction was subsequently confirmed using *in vitro* binding assays yet could not be confirmed *in vivo*. The inability to co-immunoprecipitate MyoD and Snf2h could indicate the interaction is transient in nature. Since Snf2h does not appear to effect MyoD-dependent transactivation in reporter assays or bind to MyoD / E protein heterodimers on naked DNA (figure 12 and 13), we hypothesize that MyoD targets Snf2h to particular promoters and that this does not require a sustained interaction. Expression of both an ATPase dead form of hSNF2H and wildtype hSNF2H in C2C12 myoblasts resulted in early expression of differentiation markers consistent with the notion that it may be required for the maintenance of the myoblast proliferative state and the suppression of differentiation specific genes (figure 9-10). Since Snf2h functions in a multi-protein complex, the similar effects of both hSNF2H (WT) and hSNF2H (K211R) on myoblast differentiation are not surprising. Although hSNF2H (K211R) would inhibit the active of Snf2h-containing complexes directly, the ectopic expression of hSNF2H (WT) likely inhibits complex activity by sequestrating other components.

Snf2h activity appears to be required to maintain the repressive chromatin structure around the myogenin promoter during growth (figure 11C, model figure 14). This suggests that Snf2h functions, in concert with MyoD, to target either co-repressor

complexes or HDAC1 to the myogenin promoter. Both co-repressor complexes and HDAC1 are targeted to differentiation specific promoters by a direct interaction with MyoD and have been implicated in the repression of these genes prior to the receipt of appropriate pro-differentiation cues (Bailey et al., 1999; Bailey et al., 1998; Mal and Harter, 2003; Mal et al., 2001; Puri et al., 2001).

A role for SNF2H and its homologs in transcriptional repression is well established (Deuring et al., 2000) (Santoro et al., 2002; Strohner et al., 2001; Zhou et al., 2002). In particular, SNF2H through its activity in the nucleolar remodeling complex (NoRC) complex is required to repress rRNA transcription (Santoro et al., 2002; Strohner et al., 2001; Zhou et al., 2002). The NoRC complex establishes a heterochromatin structure around the promoter of ribosomal RNA genes via the recruitment of methyltransferases and the histone deacetylase containing Sin3 co-repressor complex (Santoro et al., 2002; Zhou et al., 2002). Interestingly, the Sin3 co-repressor complex through its interaction with HDAC1 and N-CoR has previously been implicated in MyoD mediated repression (Bailey et al., 1999; Mal et al., 2001; Puri et al., 2001).

The expression of differentiation specific genes not only requires the alleviation of repression by HDAC1 and co-repressor complexes, it requires the activity of histone acetyltransferases such as CBP/p300 and PCAF (Polesskaya et al., 2000; Polesskaya et al., 2001b; Puri et al., 1997b; Sartorelli et al., 1999). Through a direct interaction with MyoD, histone acetyltransferase activity is directly targeted to differentiation specific promoters (Polesskaya et al., 2000; Polesskaya et al., 2001b; Puri et al., 1997b; Sartorelli et al., 1999). Since myogenin expression was not observed in growing C2C12 [hSNF2H

(WT)] or C2C12 [hSNF2H (K211R)] but was expressed in these cells after the withdrawal of mitogens, the regulation of MyoD activity by HATs appears to be occurring appropriately suggesting Snf2h activity is not required for this aspect of MyoD transcriptional regulation.

This and previous studies have implicated various ATPase dependent chromatin remodeling motors in the regulation of myoblast differentiation (de la Serna et al., 2001a; de la Serna et al., 2001b; Roy et al., 2002). The roles of Brg1 and Snf2h in myoblast differentiation appear to be distinct. Brg1 is essential for the induction of muscle-specific gene expression (de la Serna et al., 2001b). Unlike Brg1, Snf2h does not appear to play an active role in the induction of differentiation markers, but does appear to be essential for repression of these markers during growth. The following model highlights the unique functions of the ISWI and SNF2 family of ATP-dependent chromatin remodeling motors during myoblast proliferation and differentiation (figure 14). Under growth conditions, MyoD in association with Snf2h targets co-repressor complexes to muscle-specific promoters, inhibiting transcription. After pro-differentiation cues are received, the co-repressor complexes switch from MyoD as a binding partner to pRB (Puri et al., 2001). The association of pRB with the co-repressor complexes ultimately leads to the repression of E2F-dependent promoters and cell cycle arrest (Lai et al., 2001; Lai et al., 1999). Brg1 and Brahma, likely targeted to muscle specific promoters by a direct association with MyoD, are required to alleviate the repressive chromatin structure surrounding these promoters and activate transcription.

The results presented here, demonstrate a previously unknown role of Snf2h in the regulation of myoblast differentiation. Snf2h appears to be essential for the repression of differentiation specific genes during growth. Our results in association with reports demonstrating the requirement of Brg1 in the induction of differentiation specific genes highlight the distinct functions of these two ATPase dependent chromatin remodeling motors in myogenesis.

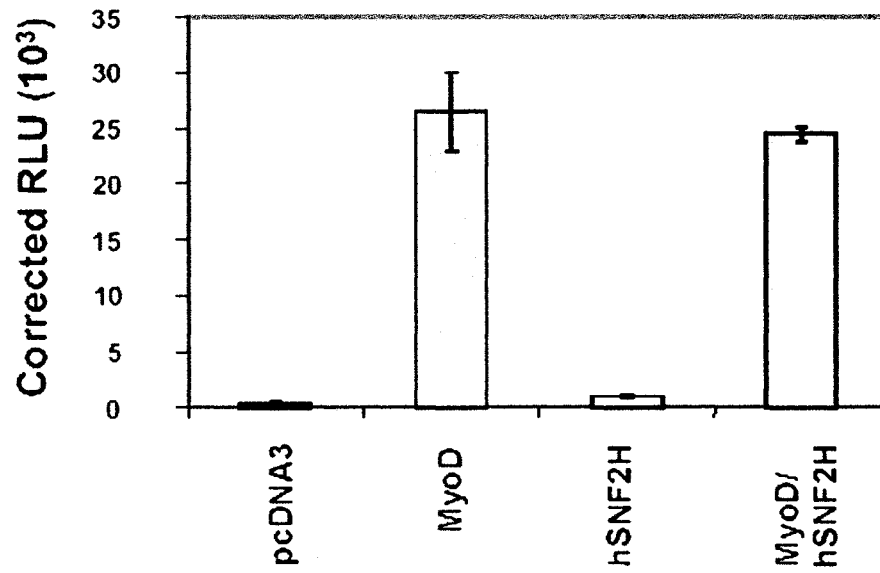


Figure 12. 4RSV-luciferase activity in 10T1/2 mouse fibroblasts transfected with MyoD and/or hSNF2H.

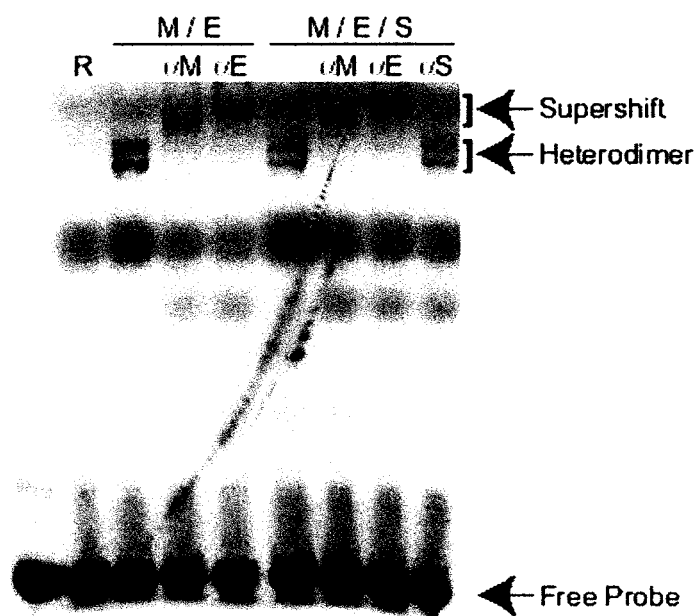
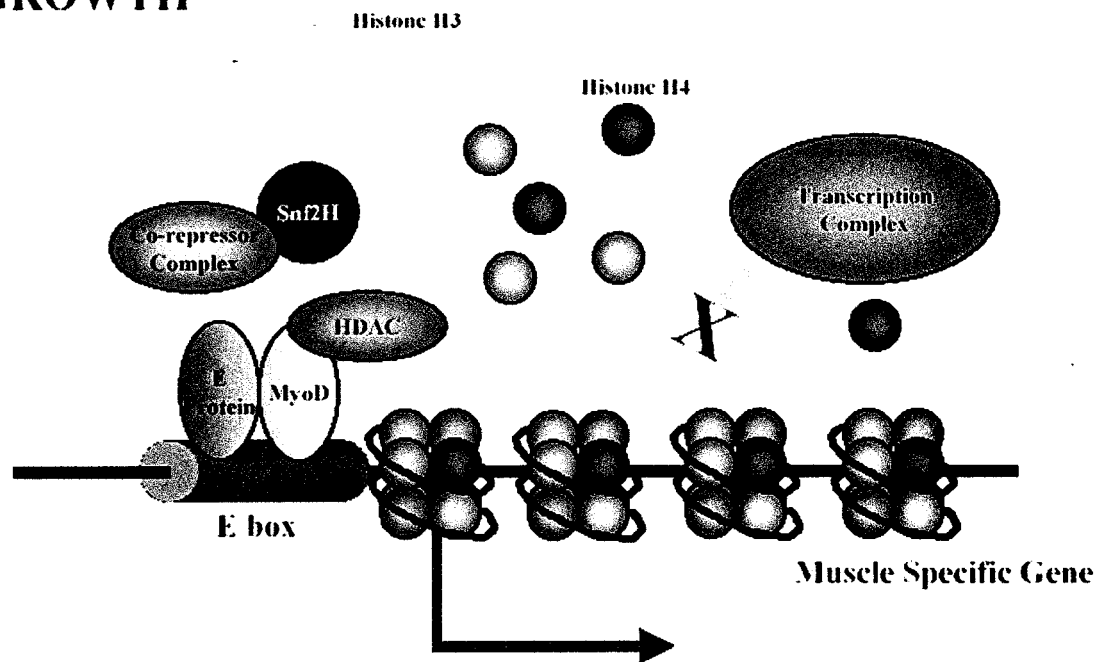


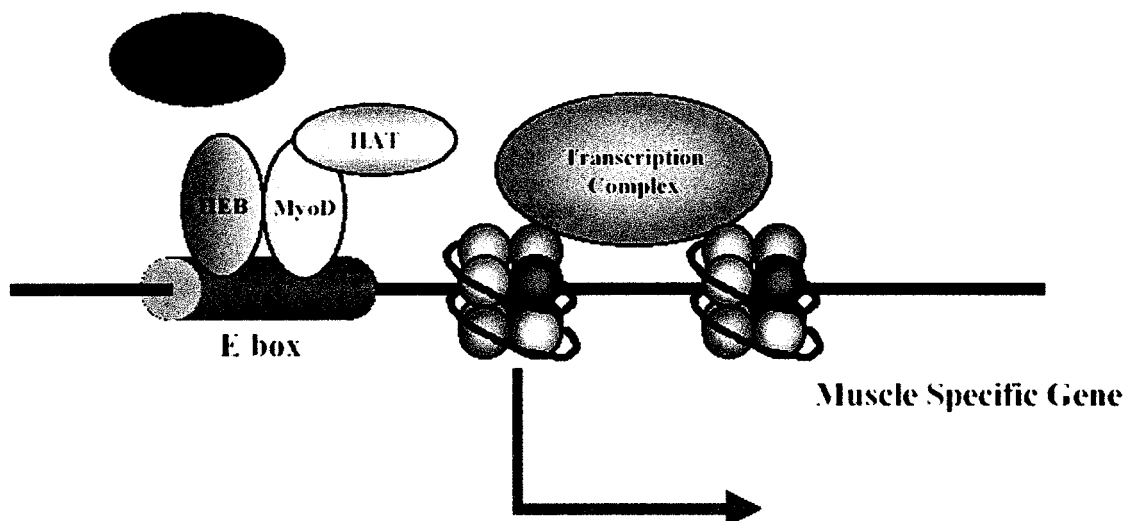
Figure 13. SNF2H (S) does not bind MyoD (M) / E12 (E) heterodimers associated with E boxes. *In vitro* translated SNF2H, MyoD and E12 were used in an EMSA. MyoD / E12 heterodimers readily bound E box containing probe. The heterodimers were supershifted with antibody directed against MyoD (α M) or E12 (α E). SNF2H had no effect on heterodimer binding to E boxes.

Figure 14. Model describing the function of Snf2h in myoblasts. We hypothesize that Snf2h is required during growth to repress transcription of differentiation specific genes. It may act by facilitating the binding of co-repressor complexes. Inhibition of Snf2h activity results in expression of differentiation markers since the chromatin surrounding these promoters is not repressive. During differentiation, the SNF2 family member Brg1 and histone acetyltransferases are essential for MyoD-dependent transactivation of muscle-specific genes such as myogenin.

GROWTH



DIFFERENTIATION



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CHAPTER 3: Myoblasts and Myotubes Have Distinct Patterns of Histone Modifications

Foreword

The following chapter, entitled “Myoblasts and Myotubes Have Distinct Patterns of Histone Modifications” examines the changes to histone H4 acetylation and histone H3-K9 dimethylation that occur during myoblast differentiation. In addition, this work describes a methodology to combine chromatin immunoprecipitation techniques with Affymetrix whole genome assay microarray technology. Claire Palmer completed the majority of the work including all cell culture; ChIP assays; probes generation and data analysis in this study and in addition wrote the manuscript. G. Palidwor, C.J. Porter, N.A. Sanche wrote software to annotate the data (CEL) files. K. Sheikheleslamy hybridized, washed and read microarray chips. M.A. Rudnicki supervised this project and reviewed the manuscript.

Title Page

**Myoblasts and Myotubes have Unique Patterns of Histone Modifications
As Assessed by ChIP on Chip Technology**

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Abstract

Covalent modifications to histone proteins including acetylation and methylation change the local chromatin structure by modulating the affinity of DNA for the nucleosome core, by affecting higher order nucleosome folding and by facilitating the binding of chromatin modifying enzymes. We have developed a protocol to combine ChIP and Affymetrix SNP array technologies to assay the changes in histone H4 acetylation and histone H3-K9 dimethylation occurring during myoblast differentiation on a genome-wide scale. The patterns of genome-wide histone modifications in proliferating myoblasts were distinct from the patterns observed in myotubes. Approximately 7% and 10% of assessed SNPs were specifically hyperacetylated in myoblasts and myotubes respectively. In myotubes but not myoblasts, there was a correlation between histone acetylation and transcription. Approximately 4% of assessed SNPs were specifically hypermethylated in both myoblast and myotubes. In myoblasts but not myotubes, there was a weak inverse correlation between H3-K9 dimethylation and transcription. Our results are consistent with a model in which a combination of distinct histone and DNA modifications together defines the transcriptional competence of a particular gene. In addition, our results are consistent with the notion that unique mechanisms are targeting histone-modifying enzymes to particular loci in myoblasts and myotubes.

Introduction

In cell culture, proliferating myoblasts can be induced to exit the cell cycle and terminally differentiate into elongated multinucleated myotubes by the removal of mitogens. During the differentiation process the nuclear structure and cytoskeleton are re-organized. Concurrent with the morphological changes occurring during myoblast differentiation are significant changes in gene expression. Subsequent to the receipt of pro-differentiation cues, the expression of genes required for cell cycle progression is repressed while the expression of differentiation-specific genes including muscle structural proteins is activated. This coordinated repression and activation of specific genes is regulated, in part, by the Myogenic Regulator Factors (MRFs) and the MEF2 family of transcription factors and their interactions with widely expressed transcriptional co-activators and repressors (reviewed in (McKinsey et al., 2001; Megeney and Rudnicki, 1995; Puri et al., 2001)). These co-factors include histone acetyltransferases (HATs) and histone deacetylases (HDACs), which together by modulating the acetylation status of the core histones regulate chromatin structure (for review (Wu, 1997)).

The MRFs are dominant-acting basic helix loop helix transcription factors capable of initiating the myogenic differentiation program in non-muscle cells (Megeney and Rudnicki, 1995). This family of transcription factors includes MyoD, Myf5, myogenin and MRF4. The individual roles of the MRFs have been, in part, defined by gene targeting experiments. These experiments suggest the MRFs can be subdivided into two categories; the primary MRFs (MyoD and Myf5), essential for myoblast specification and the secondary MRF (MRF4 and myogenin), required for myoblast differentiation

(reviewed in (Megeny and Rudnicki, 1995)). MyoD and Myf5 can alleviate repressive chromatin structure in the promoters of muscle specific genes (Gerber et al., 1997).

MyoD through a direct interaction with either HATs or HDACs has the capacity to act as both a transcriptional activator and repressor respectively. These interactions target histone modifying activity directly to muscle specific promoters (Mal and Harter, 2003; Polesskaya et al., 2001a; Puri et al., 2001; Sartorelli et al., 1997).

The histone acetyltransferases, CBP/p300 and PCAF, are critical cofactors of the MRFs (Polesskaya et al., 2000; Polesskaya et al., 2001b; Puri et al., 1997a; Puri et al., 1997b; Sartorelli et al., 1997; Sartorelli et al., 1999; Yuan et al., 1996). CBP/p300 activity is required for MyoD-mediated cell cycle arrest and the expression of markers of terminal differentiation (Polesskaya et al., 2001b; Puri et al., 1997a). Under growth conditions, MyoD acts as a repressor targeting HDAC1 to muscle specific promoters (Mal and Harter, 2003). Subsequent to pro-differentiation cues, HDAC1 disassociates from MyoD and forms a complex with hypophosphorylated pRB allowing for both activation of differentiation specific genes and the repression of growth specific genes (Puri et al., 2001).

In addition to modulating MRF transcriptional activity, histone-modifying enzymes are critical in regulating the activity of a second family of transcription factors essential in myogenesis. The MEF2 family consists of MADS-box containing transcription factors that act synergistically with the MRFs to activate the muscle differentiation program (Black et al., 1998; Black and Olson, 1998; Kaushal et al., 1994; Molkentin et al., 1995). Class II HDACs, including HDAC4 and 5 bind directly to MEF2

and repress MEF2-dependent transcription. Release from this repression is a prerequisite for muscle differentiation and is mediated by CaMK (calcium/calmodulin-dependent kinase) pathway dependent nuclear export of class II HDACs (McKinsey et al., 2000; McKinsey et al., 2001). Although evidence suggests CBP/p300 may be required to activate MEF2-dependent transcription, a definitive role for this HAT as a MEF2 co-activator in skeletal muscle has yet to be established (McKinsey et al., 2001).

The coordinated activity of HATs and HDACs is central to the differentiation process. Gene specific histone modifications can be determined using chromatin immunoprecipitation assays. In a chromatin immunoprecipitation assay, DNA-protein interactions as well as protein-protein interactions are reversibly fixed by formaldehyde-induced crosslinks, essentially giving a snapshot of the events occurring within the cell at the time of fixation (Orlando, 2000). By performing ChIP assays at various stages during differentiation, a temporal picture of chromatin remodeling during the differentiation process can be obtained. Although the ChIP assay has revolutionized the study of chromatin structure its usefulness is primarily limited to assessing histone modifications occurring at a limited number of loci. Recently ChIP assays have been combined with dotted CpG island microarrays to globally assess both changes in chromatin structure and transcription factor binding (Fontemaggi *et. al.* (2002); Liu *et. al.* (2002); Robyr *et. al.* (2002)). However to date, commercially available oligonucleotide microarrays have not been used to analyze DNA from ChIP assays; with the development of human single nucleotide polymorphism (SNPs) microarrays this platform is now amendable for use with ChIP protocols. We have developed the methodology to combine these two

technologies. Using the ChIP on SNP methodology, we have simultaneously assessed the changes in histone modifications that occur during myoblast differentiation at 10,043 chromosomal locations and compared this data to transcriptional maps generated from microarray data. Our data clearly indicates that in general a combination of distinct histone modifications and not a single histone modification defines the transcriptional competence of a particular gene. Furthermore, our results suggest histone-modifying enzymes are targeted to particular loci and that the targeting mechanism in myoblasts is distinct from that in myotubes.

Materials and Methods

Cell Culture

Human skeletal muscle cells (SkMc, Clonetics, CC-2561) were grown in skeletal muscle basal media (SKBM, Clonetics, CC-3161) supplemented with hEGF, insulin, BSA, fetuin, dexamethasone and gentamicin/amphotericin-B (SkGM, SingleQuots, Clonetics, CC-4139) as per manufactures instructions. At 75% confluence, SkMc cells were induced to differentiate into multinucleated myotubes by mitogen withdrawal. Cells were maintained in differentiation media (DMEM supplemented with 2.5% horse serum) for 5 days prior to harvesting.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were preformed using the ChIP Assay kit from Upstate Biotech (Cat# 17-295) as per manufactures instructions. Briefly, 10^7 cells were crosslinked for 10 minutes at room temperature with formaldehyde (1% formaldehyde final concentration). Crosslinking reaction was terminated by the addition of 0.125 M glycine. Cells were washed twice with ice-cold PBS (10 mM phosphate-buffered saline, pH 7.2) prior to being harvested. Cell pellets were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) supplemented with protease inhibitors (Mini Complete, Roche, Cat.# 1836153) and 10 mM sodium butyrate. Lysis was allowed to proceed for 10 minutes on ice prior to sonication. Lysates were sonicated until DNA was sheared to between 0.5 and 2 kb. Chromatin was sheared by sonication to an average length of 500 base pairs. To achieve this lysates were sonicated for 4 15-second pulses

with a 1.5 mm step probe equipped sonicator set to a magnitude of 30%. Lysates were cooled on dry ice between pulses. Sonicated lysates were cleared by centrifugation for 10 minutes at 13,000 rpm at 4°C. Cleared lysates were diluted 10-fold in 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl, pH8.1, 167 mM NaCl and incubated with Salmon Sperm DNA /Protein A Agarose-50% slurry (Upstate Biotech, Cat# 16-157C) to reduce non-specific background for 30 minutes at 4°C with rotation. Either 5 µL of Anti-Acetylate Histone H4 (Upstate Biotech, Cat# 06-866) or Anti-dimethyl-Histone H3 (Lys9) (Upstate Biotech, Cat# 07-212) was added to the lysates (per 10⁶ cell equivalents). Immune complexes were recovered with Salmon Sperm DNA/Protein A Agarose and washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH8.1, 150 mM NaCl); once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.1, 500 mM NaCl); once with LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris HCl, pH 8.1) and twice with TE. Immune complexes were eluted with 1% SDS, 0.1 M NaHCO₃ for 15 min at room temperature. DNA-protein crosslinks were reversed for 4 hrs at 65°C with 0.2 mM NaCl. Samples were deproteinated and DNA isolated using Qiaquick columns as described by the manufacture (Qiagen). DNA was eluted from columns with ddH₂O.

Generation of Biotinylated Target

The GeneChip Mapping Assay steps (Adaptor ligations, PCR, fragmentation, labeling) in the Affymetrix protocol were modified to accommodate the chromatin IP procedure as follows. Briefly, precipitated DNA (from 2.5 x 10⁶ cells) was digested to

completion with Xba1. 6.25 pmol of Xba adaptor and digested DNA were ligated for 120 min. at 16°C. Ligations were diluted 10 fold in ddH₂O prior to first round PCR reaction. 10 µL of diluted ligation was amplified with Xba primer using an MJ Research thermocycler in a 100 µL PCR reaction. One-tenth of this reaction was subsequently re-amplified using the Xba primer for a total of ten PCR reactions. PCR reactions were pooled and purified using Qiaquick PCR purification kit. DNA was eluted from the Qiaquick column with 55 µL of EB. 20 µg of purified PCR product was digested with Affymetrix Fragmentation Reagent to an average size of 50 bp. Fragmented target was biotinylated overnight at 37°C using Biotin-N6-ddATP (Perkin Elmer) and terminal transferase.

Target Hybridization, Washing, Scanning and Staining

Target hybridization, washing, scanning and staining were performed as recommended by Affymetrix.

Data Analysis

The signal (θ) for each SNP on the array is equal to

$$\theta = \frac{\text{avg}(PM-MM)_1 + \text{avg}(PM-MM)_2 + \text{avg}(PM-MM)_N}{N}$$

Where PM is equal to the intensity of the perfect match oligonucleotide (for both A and B alleles, unless otherwise stated), MM is equal to the intensity of the mismatch oligonucleotide (for both A and B alleles, unless otherwise stated) and is therefore a

measure of noise, avg is the average of the 28 *PM/MM* oligonucleotide sets present on the array that interrogate each SNP. N is equal to the number of replicates. θ was calculated from intensities obtained from a minimum of three arrays, each analyzing DNA from independent ChIP assays. Background relative signal was set at a value of 10. The p value was determined using a two-tailed t test assuming unequal variance. Raw data was scaled prior to analysis to compensate for chip-to-chip variation in hybridization. Outliers as determined by Affymetrix MAS 5.0 were removed prior to analysis. Relative signal was plotted against chromosomal location. SNP locations are from Golden Path, June 2002.

Affymetrix Transcript Data Analysis

Three independent RNA samples from either growing SkMc cells or differentiating SkMc myotubes was analyzed using Affymetrix U133A human microarrays.

Results

ChIP on SNP methodology

The differentiation process requires both the alleviation of condensed chromatin structure at muscle specific promoters such as the myogenin promoter and the condensation of chromatin associated with growth specific genes (Crowder and Merlie, 1988a; Crowder and Merlie, 1988b; Gerber et al., 1997; Pandey and Kanungo, 1984; Wiid et al., 1988). Covalent modifications to histone proteins affect both DNA binding to the nucleosome core and higher order nucleosome folding resulting in a change to the local chromatin structure (for review see (Wu, 1997) and (Roth et al., 2001)). In general, hyperacetylation of histone H3 and histone H4 are associated with open or transcriptionally active chromatin, while hypoacetylation of histone H3 and histone H4 as well as methylation of lysine residue 9 (K9) of histone H3 are the hallmarks of transcriptionally inactive chromatin. This pattern of histone modifications has been observed for the myogenin promoter (Mal and Harter, 2003). Our goal in this study was to extend the analysis of histone modifications occurring during myoblast differentiation genome-wide. To facilitate this, we have combined chromatin immunoprecipitation methodology (ChIP) with Affymetrix microarray technology. Using this methodology we have investigated the genome-wide changes in histone H4 acetylation and histone H3 lysine 9 (K9) dimethylation occurring during myoblast differentiation (Figure 1).

In a ChIP assay, specifically modified chromatin is immunoprecipitated by antibodies directed against the modified histone. By combining ChIP with microarray

technology the number of loci examined is significantly increased and the survey is unbiased. The Affymetrix WGA 10K microarray consists of a series of oligonucleotides that specifically hybridize to defined single nucleotide polymorphisms (SNPs) within the human genome and therefore can be used to assess specific chromosome location (Figure 2). The Affymetrix WGA 10K microarray allows for the examination of 10,043 SNPs distributed over the 22 autosomes and the X chromosome.

To characterize the changes to histone modifications occurring at multiple loci during myoblast differentiation we used the combined ChIP on SNP methodology outlined in Figure 1. The relative signals are from the average of three independent experiments. Prior to analysis, data from each of the WGA 10K arrays was scaled such that total array intensities were similar between arrays. This compensates for any variation in hybridization between arrays and is similar to what is standard procedure for transcript microarray analysis. SNPs with significantly different relative signals were determined using a two-tailed t test assuming unequal variance (see Materials and Methods for details). To obtain sufficient quantities of DNA to generate biotinylated targets from DNA isolated from immunoprecipitated chromatin, it was necessary to amplify the input DNA with two successive rounds of PCR; this however is unlikely to effect representation since at least for cDNA it has been clearly established that representation is maintained faithfully during exponentially amplification (Iscove et al., 2002).

Figure 1. In the schematic the ChIP on SNP methodology is outlined. Briefly, human cells are crosslinked with formaldehyde to fix protein-DNA complexes. The crosslinked chromatin is isolated and sheared to an average length of 1000 bp by sonication. The sheared crosslinked chromatin is immunoprecipitated with antibodies directed against modified histones. The antibody-chromatin complexes are recovered by protein A-agarose and washed repeatedly. The chromatin is eluted and crosslinks reversed. The chromatin is deproteinated and the DNA is isolated. Specific primers are ligated onto the DNA and it is subject to two rounds of amplification. 20 µg of amplified DNA are digested to an average length of 50 bp and biotinylated using terminal transferase. Biotinylated target is hybridized overnight to the Affymetrix 10K SNP array. The array is processed and relative signal determined.

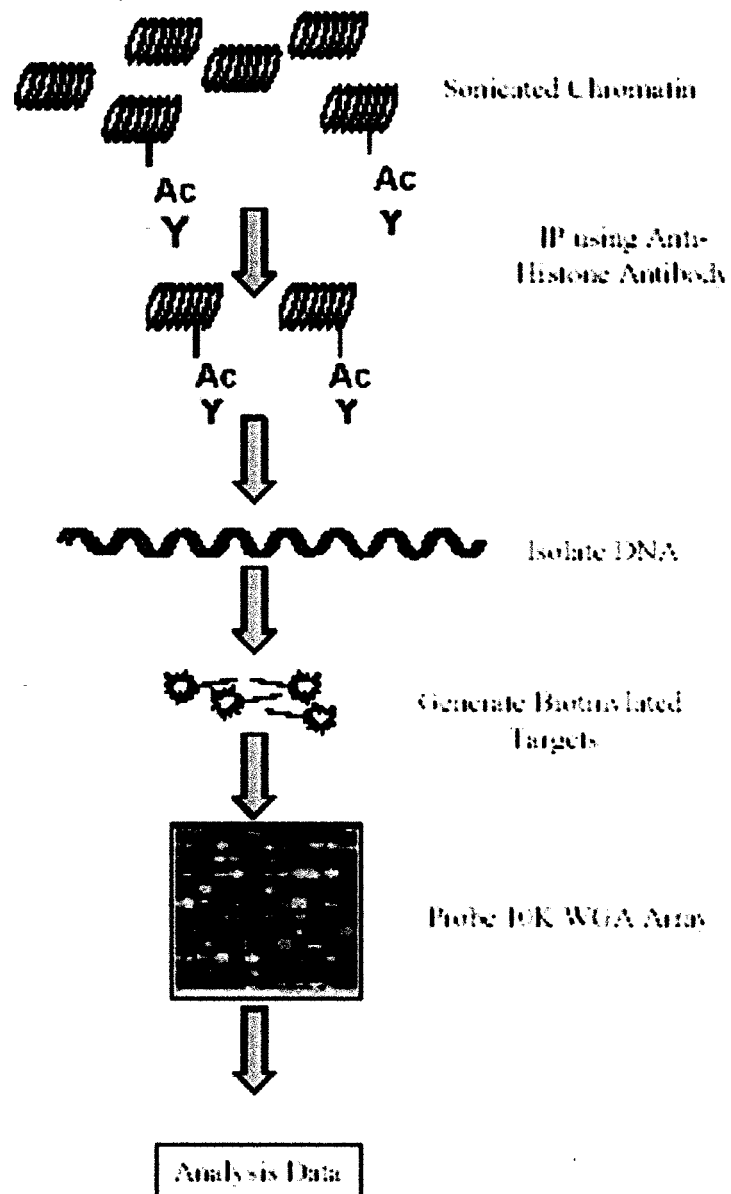
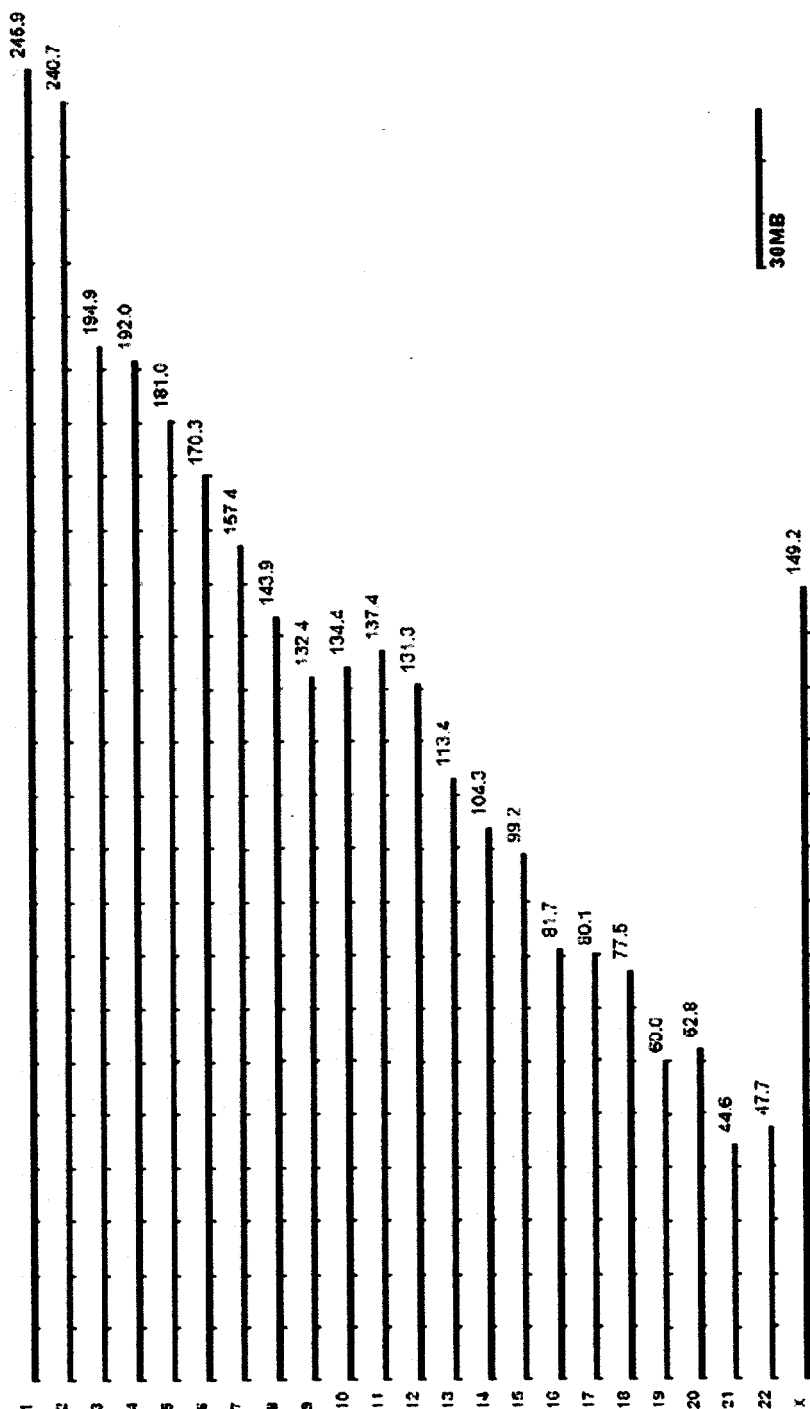


Figure 2. Chromosomal distribution of SNPs assessed by the Affymetrix WGA 10K SNP array. The WGA 10K array interrogates 10,043 SNPs distributed over all 22 autosomes and the X chromosome. The average inter-SNP distance is 250 kb. Generally, the individual SNPs are highly informative with an average heterozygosity of 0.39.



Myoblasts and myotubes have distinct pattern of histone H4 acetylation.

Histone H3 is a substrate for CBP/p300 and to a lesser extent PCAF, which preferentially targets histone H3 at lysine residue 14 (Schiltz et al., 1999). Lysine residue 5 and 8 of histone H4 are preferentially acetylated by p300 while PCAF predominantly targets lysine residue 8 (Schiltz et al., 1999). The pattern of histone H4 acetylation was determined by plotting relative signal against chromosomal location of the SNP (figure 3). Consistent with the previously determined role of histone acetyltransferase and histone deacetylases in myoblast differentiation, the pattern of histone H4 acetylation in myoblasts is distinct from the pattern of histone H4 acetylation observed in myotubes (figure 4). Of the 10,043 SNPs assessed, approximately 7% of SNPs or 691 were specifically hyperacetylated in myoblasts and not myotubes while approximately 10% or 996 of SNPs were specifically hyperacetylated in myotubes and not myoblasts. Although the total of number of SNPs acetylated above background increased slightly upon differentiation (28% compared to 32% of total SNPs assessed), the genome-wide level of acetylation as assessed by total signal was reduced by approximately 50% upon differentiation and is consistent with the observed reduction in total acetylated histone H4 protein after differentiation (figure 3, 4 and 5).

Figure 3. (A) Using the ChIP on SNP methodology the genome-wide pattern of histone H4 acetylation was assessed. Acetylation of histone H4 has previously been suggested to be a hallmark of transcriptional active chromatin. The pattern of histone H4 acetylation in myoblasts is distinct from the pattern of histone H4 acetylation observed in myotubes. (B) Summary of chromosomal distribution of hyperacetylated SNPs in both myoblasts and myotubes. Approximately 7% and 10% of SNPs assessed were hyperacetylated on histone H3 in myoblast and myotubes respectively.

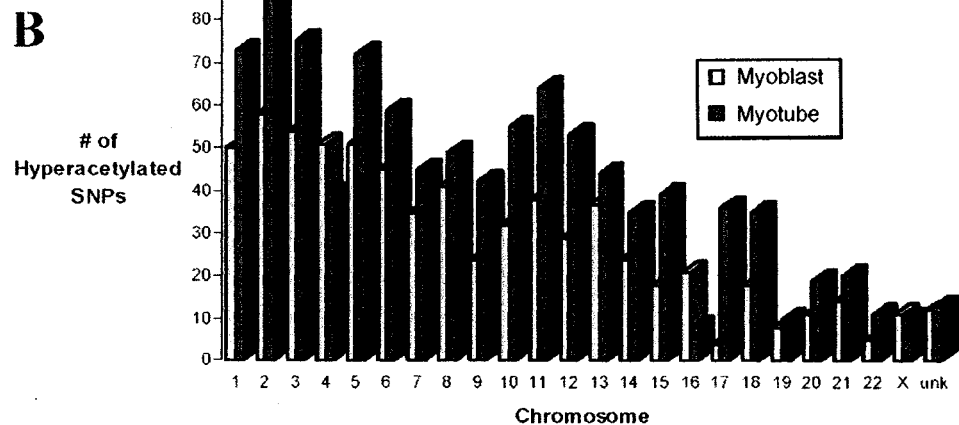
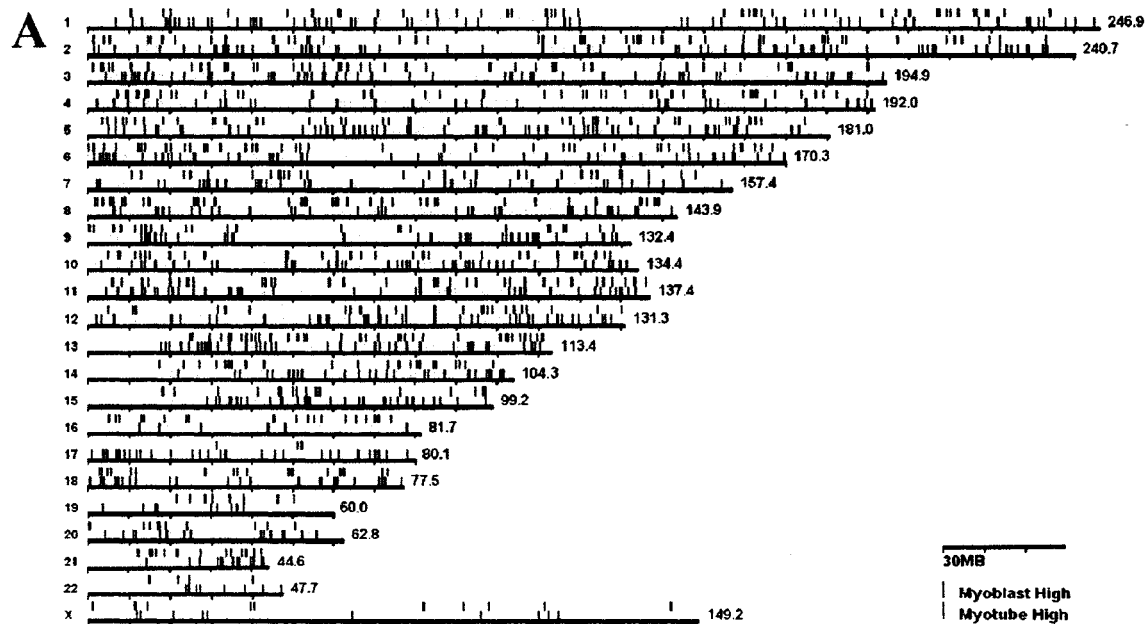


Table 1. Summary of chromosomal distribution of hyperacetylated SNPs in both myoblasts and myotubes.

Chromosome	Hyperacetylated SNPs (Myoblasts) $p < 0.05$	Hyperacetylated SNPs (Myoblasts) $0.05 < p < 0.1$	Hyperacetylated SNPs (Myotubes) $p < 0.05$	Hyperacetylated SNPs (Myotubes) $0.05 < p < 0.01$	Number of SNPs Interrogated
1	31	19	46	27	748
2	37	21	56	34	850
3	30	24	42	33	701
4	30	21	27	12	667
5	30	21	44	28	686
6	23	22	40	19	689
7	26	9	26	19	528
8	23	18	33	16	499
9	15	9	24	18	469
10	16	16	27	28	537
11	27	11	42	22	573
12	16	13	33	20	461
13	19	18	23	21	420
14	21	3	24	11	369
15	12	6	26	13	285
16	13	8	7	1	214
17	2	2	24	12	166
18	10	8	23	12	276
19	7	1	4	6	90
20	4	7	10	9	190
21	9	5	8	12	170
22	3	2	9	2	76
X	4	7	7	3	177
Unknown	6	6	6	7	202

Figure 4A. Using the ChIP on SNP methodology the genome-wide pattern of histone H4 acetylation was assessed and a detailed plot of the changes in histone H4 acetylation occurring on chromosomes 1 to 8 during differentiation was generated. Relative signal is an average of the relative signal determined in three independent experiments. Blue, Myoblasts; Pink, Myotube; Triangle, $p < 0.05$; Square, $0.05 < p < 0.1$.

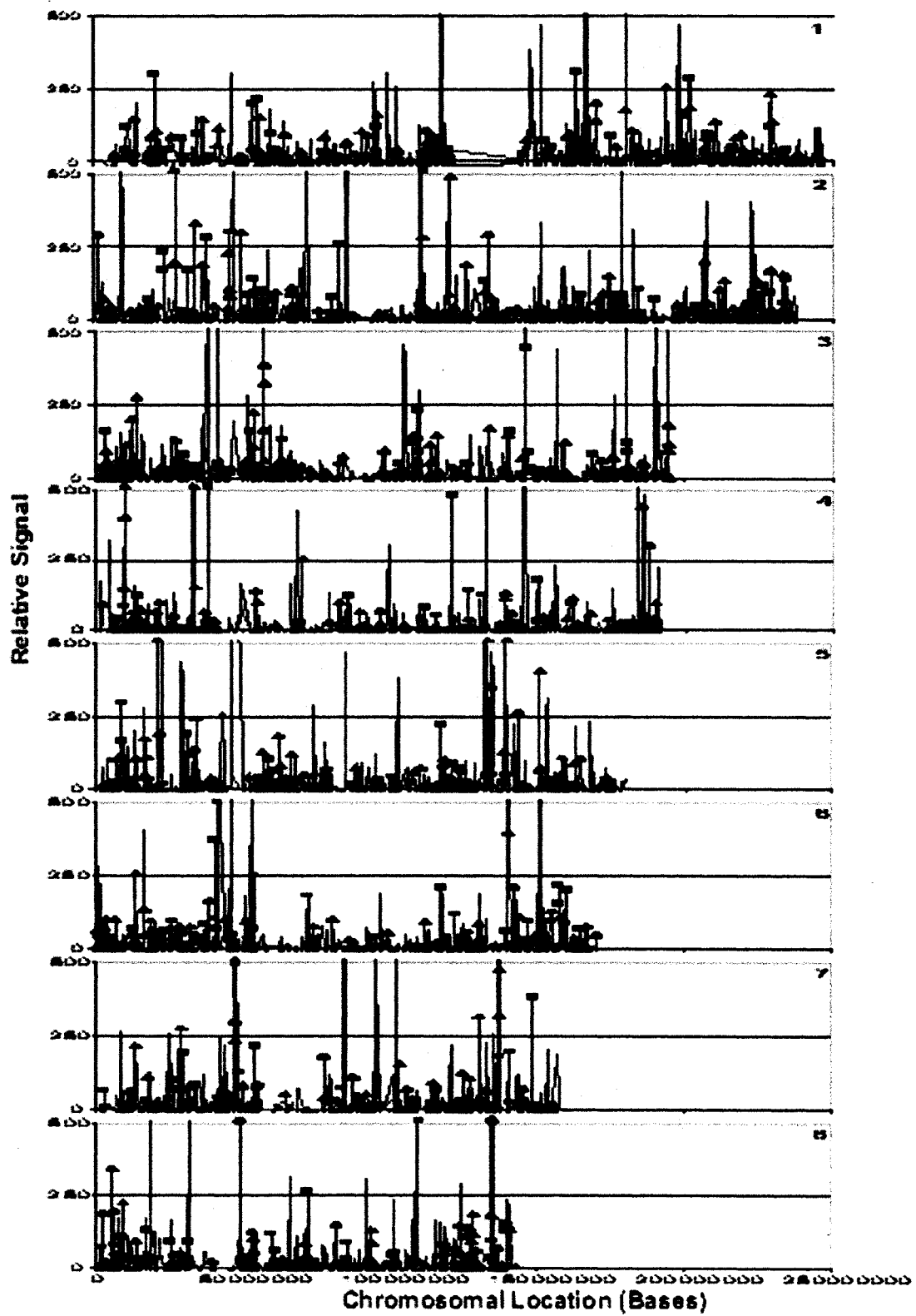


Figure 4B. Using the ChIP on SNP methodology the genome-wide pattern of histone H4 acetylation was assessed and a detailed plot of the changes in histone H4 acetylation occurring on chromosomes 9 to 16 during differentiation was generated. Relative signal is an average of the relative signal determined in three independent experiments. Blue, Myoblasts; Pink, Myotube; Triangle, $p < 0.05$; Square, $0.05 < p < 0.1$.

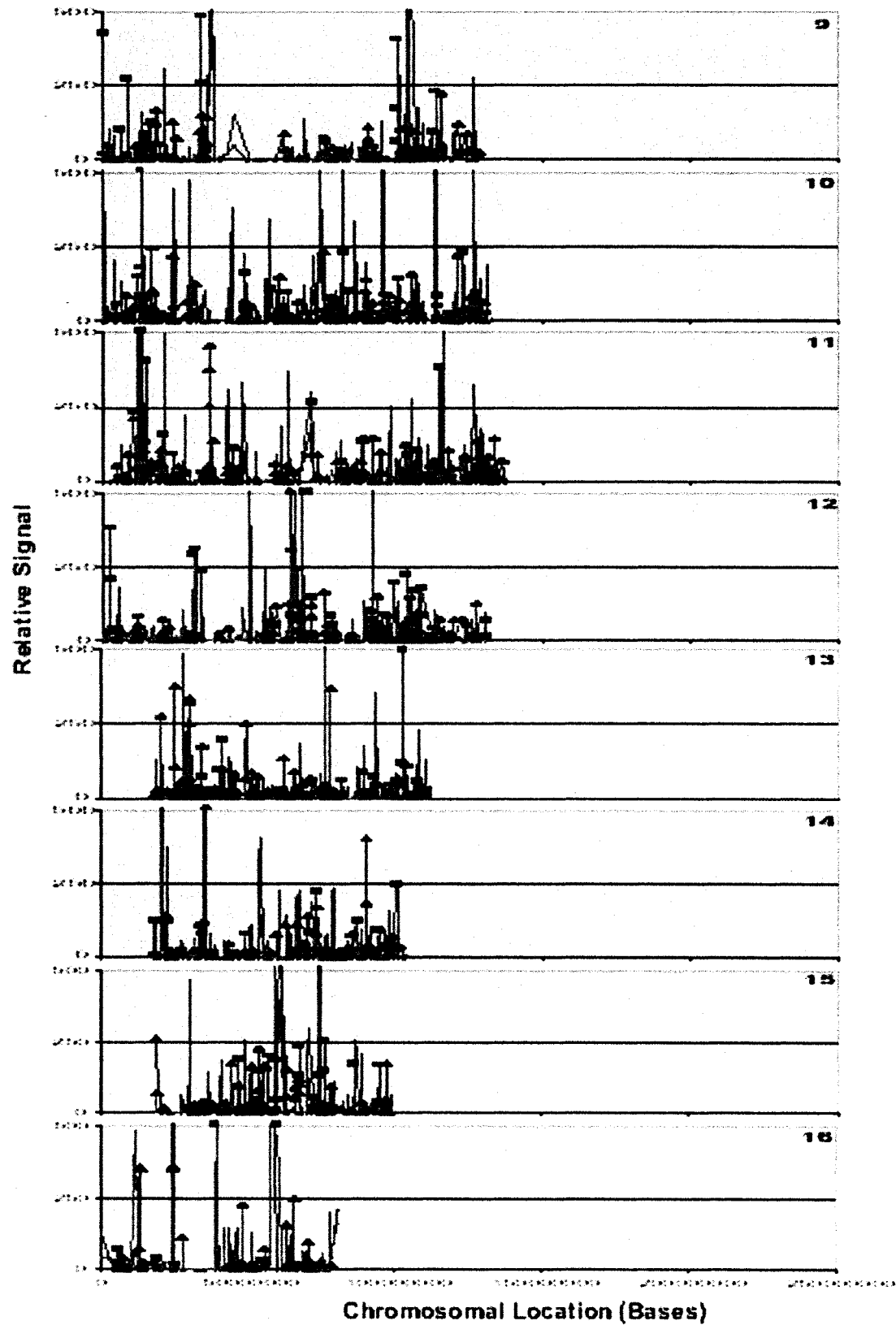
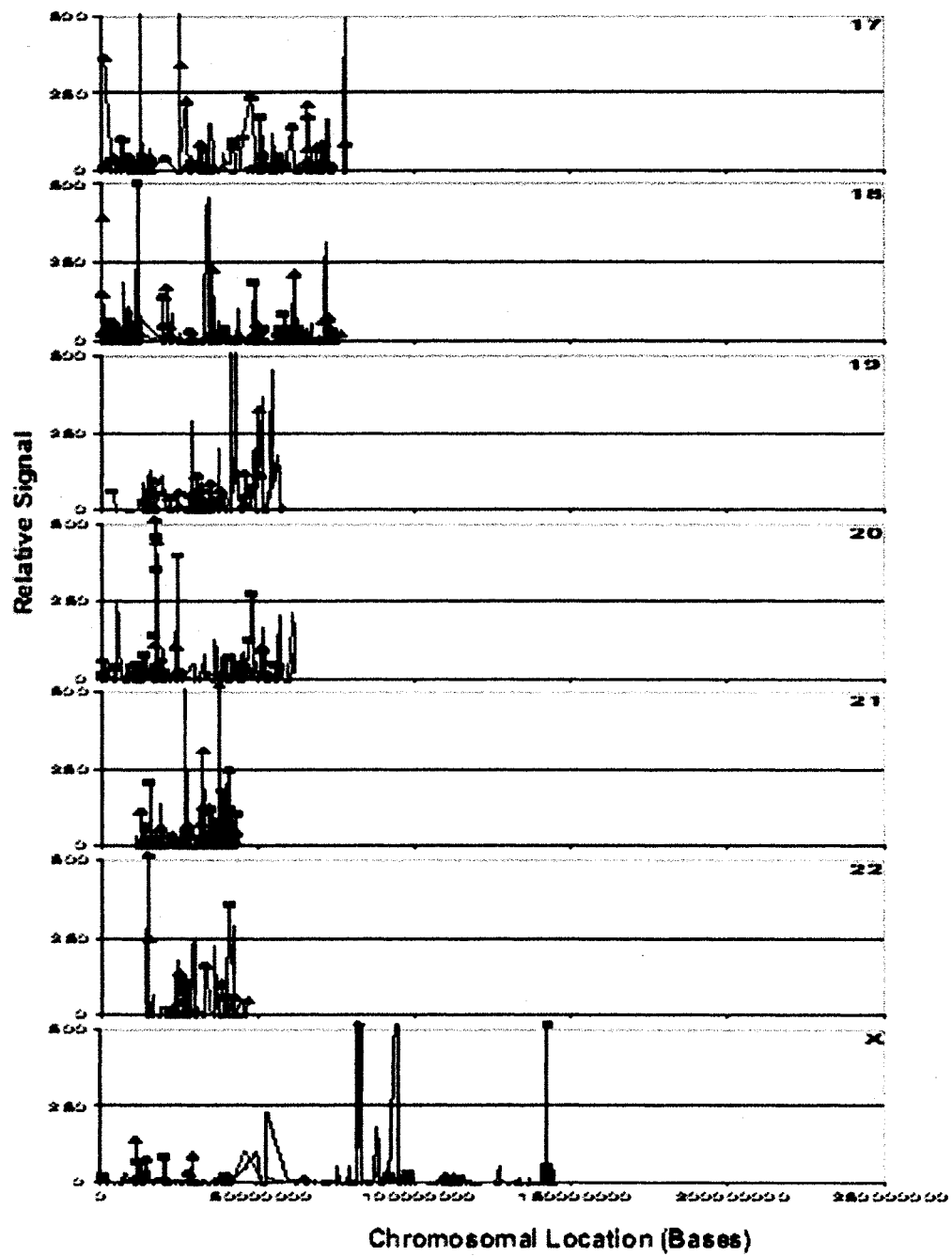


Figure 4C. Using the ChIP on SNP methodology the genome-wide pattern of histone H4 acetylation was assessed and a detailed plot of the changes in histone H4 acetylation occurring on chromosomes 17 to X during differentiation was generated. Relative signal is an average of the relative signal determined in three independent experiments. Blue, Myoblasts; Pink, Myotube; Triangle, $p < 0.05$; Square, $0.05 < p < 0.1$.



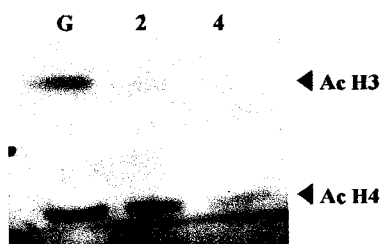


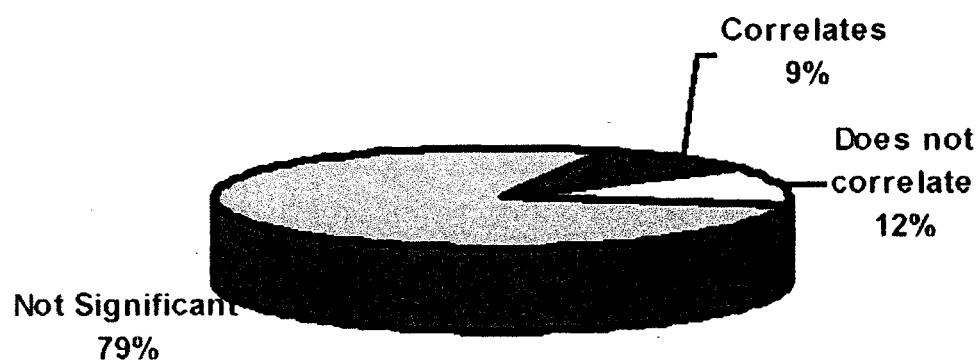
Figure 5. Histone acetylation decreases during differentiation. 10 μ g of acid soluble protein was analyzed by western blot (G, growth; 2, 2 days in differentiation media; 4, 4 days in differentiation media. Equal loading of histones was confirmed by brilliant blue staining of replicate gel.

In myotubes, hyperacetylation of histone H4 correlates with transcriptional activity.

Histone H4 hyperacetylation is generally associated with transcriptionally active genes and newly replicated chromatin, therefore increases in histone H4 hyperacetylation of a particular gene should correlate with increased transcription from that gene. To investigate the association of histone H4 hyperacetylation with transcriptional activity we analyzed the expression of 390 genes with probes on both the WGA 10K array and U133A human transcript array in which there was an observed significant change in the level of histone H4 acetylation following differentiation. Either no significant change ($p > 0.05$) in transcriptional status or no expression in myoblasts and myotubes was observed for approximately 80% (306/390) of these genes. Of the 84 genes with significant changes ($p < 0.5$) occurring in both histone H4 acetylation and transcriptional activity, only in 36 or 43% of these genes did the change in transcriptional status correspond with the expected change histone H4 acetylation (figure 6A). However, if these genes were subdivided based on transcriptional activity into Myoblast High and Myotube High, there was a correspondence between hyperacetylation of histone H4 in myotubes and transcriptional activity (72% (34/47) correlated) (figure 6B).

Figure 6. (A) Whereas previous studies have clearly linked hyperacetylation of histone H4 with transcriptional activation, we did not observe a strong correlation between increased acetylation in the subset of SNPs located in genes and elevated levels of transcription or decreased acetylation and a reduction in transcription. (B) However, if these genes were subdivided based on transcriptional activity into Myoblast High and Myotube High, there was a correlation between hyperacetylation of histone H4 in myotubes and transcriptional activity [72% (34/47) correlated].

A



B

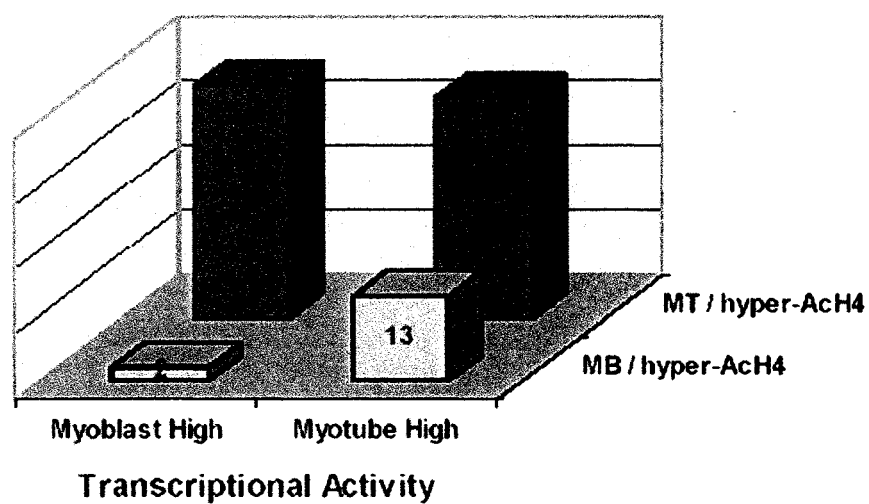


Table 2. Analysis of co-precipitating adjacent SNPs suggests the regions of hyperacetylation are extended in myoblasts compared to myotubes.

<i>Chromosome</i>	<i>Co-Precipitating Adjacent Pairs (myoblast, myotube)</i>	<i>Co-Precipitating Adjacent Triples (myoblast, myotube)</i>	<i>Co-Precipitating Sets (>3) (myoblast, myotube)</i>
1	10, 4	4, 0	0, 0
2	10, 3	2, 1	0, 0
3	12, 2	3, 0	0, 0
4	4, 3	1, 2	0, 0
5	8, 1	1, 0	0, 0
6	6, 1	3, 0	0, 0
7	1, 0	1, 1	0, 0
8	0, 0	0, 0	0, 0
9	7, 0	1, 0	0, 0
10	7, 0	3, 0	0, 0
11	8, 0	3, 0	1, 0
12	10, 1	2, 0	0, 0
13	2, 1	0, 0	0, 0
14	5, 1	1, 0	0, 0
15	4, 1	1, 0	0, 0
16	2, 1	0, 0	0, 0
17	8, 0	1, 0	1, 0
18	4, 0	0, 0	1, 0
19	0, 0	0, 0	0, 0
20	3, 0	1, 0	0, 0
21	3, 0	1, 0	0, 0
22	0, 0	1, 0	0, 0
X	0, 0	0, 0	0, 0

The regions of hyperacetylation are extended myoblasts compared to myotubes.

To assess whether histone H4 hyperacetylation occurred in distinct highly localized regions or extended over larger chromosomal segments, we analyzed the frequency with which adjacent or nearest neighbor SNPs (within 10,043 set analyzed) co-precipitated. Over 30% of hyperacetylated SNPs in myoblasts were adjacent to one other hyperacetylated SNP while only 2% of hyperacetylated SNPs in myotubes were adjacent one other hyperacetylated SNP suggesting that larger genomic regions are hyperacetylated in proliferating myoblasts compared to myotubes. In approximately one half the incidences (55%), co-precipitating adjacent SNPs were located within the same gene (table 2). This is consistent with the notion that following differentiation the amount of transcriptionally “open” chromatin is reduced.

Myoblasts and Myotubes have distinct patterns of Histone H3-K9 dimethylation.

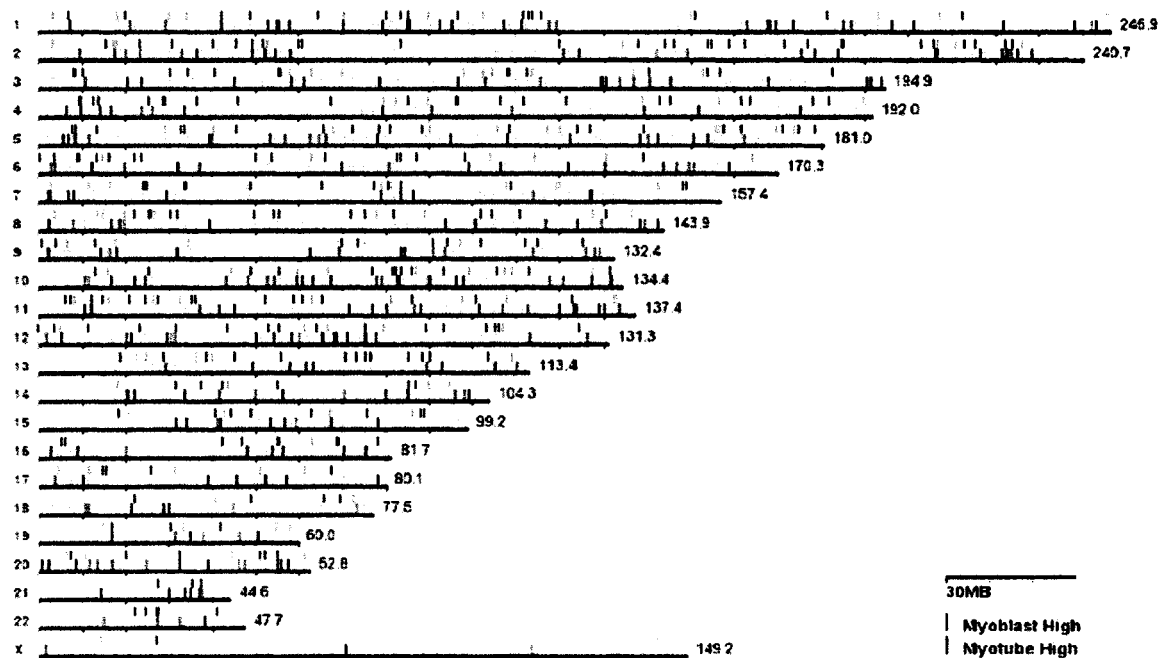
Histone H3 is dimethylated at lysine 9 (K9) by members of the Suv39 family of histone methyltransferases (HMTase). This modification is generally associated with transcriptionally silent heterochromatic subdomains (Lachner and Jenuwein, 2002). Although a histone demethylase has yet to be identified, there is evidence for an active and rapid gene-specific reversal of histone methylation (for review see (Bannister et al., 2002)).

Using the ChIP on SNP methodology the genome-wide pattern of dimethylated histone H3-K9 was assessed. The pattern of histone H3-K9 dimethylation in myoblasts is distinct from the pattern of histone H3-K9 dimethylation observed in myotubes (figure 7).

Approximately 4% of the total SNPs assessed were specifically hypermethylated in either myoblasts or myotubes (figure 8). Although the number of SNPs assessed with relative signal above background doubled after differentiation, the genome-wide level of histone H3-K9 dimethylation as assessed by total signal did not change significantly.

Figure 7. (A) Using the ChIP on SNP methodology the genome-wide pattern of histone H3-K9 dimethylation was assessed. The pattern of histone H3-K9 diMe in myoblasts is distinct from the pattern of H3-K9 diMe observed in myotubes. (B) Summary of chromosomal distribution of hypermethylated SNPs in both myoblasts and myotubes. Approximately 4% of SNPs assessed were hypermethylated in both myoblasts and myotubes.

A



B

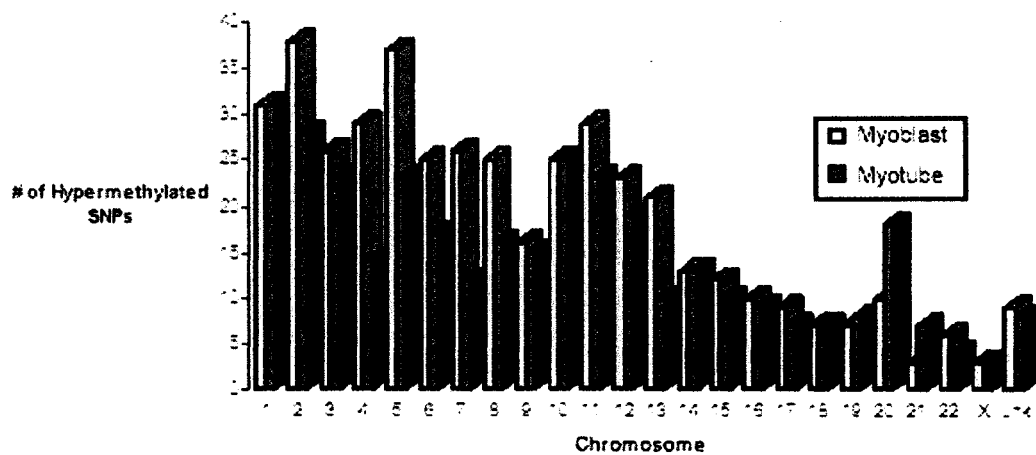


Table 3. Summary of chromosomal distribution of hypermethylated SNPs in both myoblasts and myotubes.

Chromosome	Hypermethylated SNPs (Myoblasts) $p < 0.05$	Hypermethylated SNPs (Myoblasts) $0.05 < p < 0.1$	Hypermethylated SNPs (Myotubes) $p < 0.05$	Hypermethylated SNPs (Myotubes) $0.05 < p < 0.01$	Number of SNPs Interrogated
1	17	14	10	21	748
2	28	10	13	15	850
3	21	5	12	11	701
4	15	14	8	6	667
5	21	16	12	11	686
6	17	8	5	12	689
7	19	7	7	5	528
8	17	8	7	9	499
9	9	7	5	10	469
10	15	10	11	14	537
11	22	7	11	12	573
12	14	9	7	12	461
13	14	7	5	5	420
14	7	6	6	7	369
15	8	4	5	5	285
16	5	5	6	3	214
17	5	4	1	6	166
18	4	3	2	5	276
19	7	0	6	2	90
20	7	3	11	7	190
21	0	3	2	5	170
22	4	2	2	2	76
X	2	1	1	2	177
Unknown	5	4	5	3	202

Figure 8A. Using the ChIP on SNP methodology the genome-wide pattern of histone H3-K9 dimethylation was assessed and a detailed plot of the changes in histone H3-K9 dimethylation occurring on chromosomes 1 to 8 during differentiation was generated. Relative signal is an average of the relative signal determined in three independent experiments. Blue, Myoblasts; Pink, Myotube; Triangle, $p < 0.05$; Square, $0.05 < p < 0.1$.

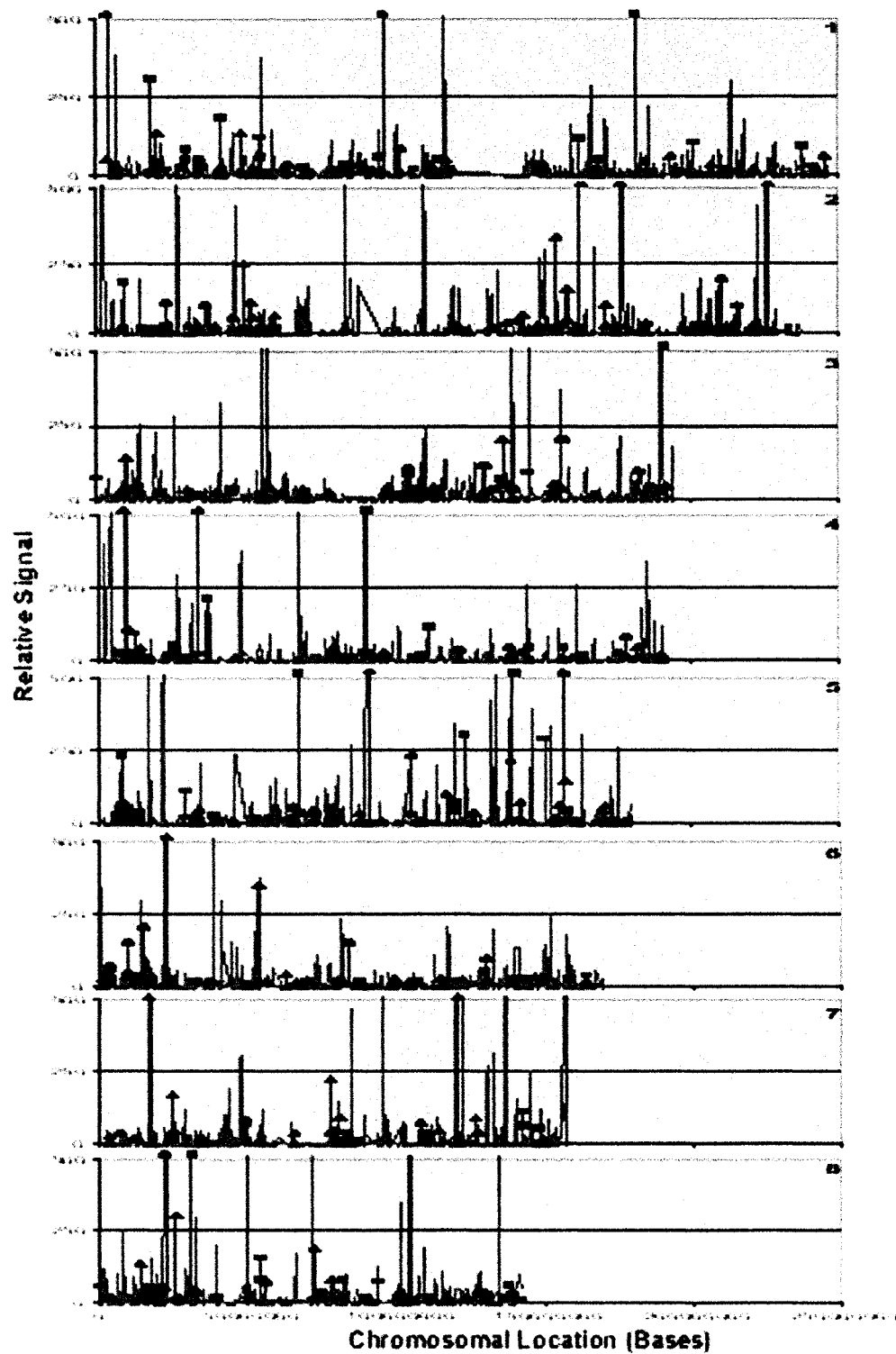


Figure 8B. Using the ChIP on SNP methodology the genome-wide pattern of histone H3-K9 dimethylation was assessed and a detailed plot of the changes in histone H3-K9 dimethylation occurring on chromosomes 9 to 16 during differentiation was generated. Relative signal is an average of the relative signal determined in three independent experiments. Blue, Myoblasts; Pink, Myotube; Triangle, $p < 0.05$; Square, $0.05 < p < 0.1$.

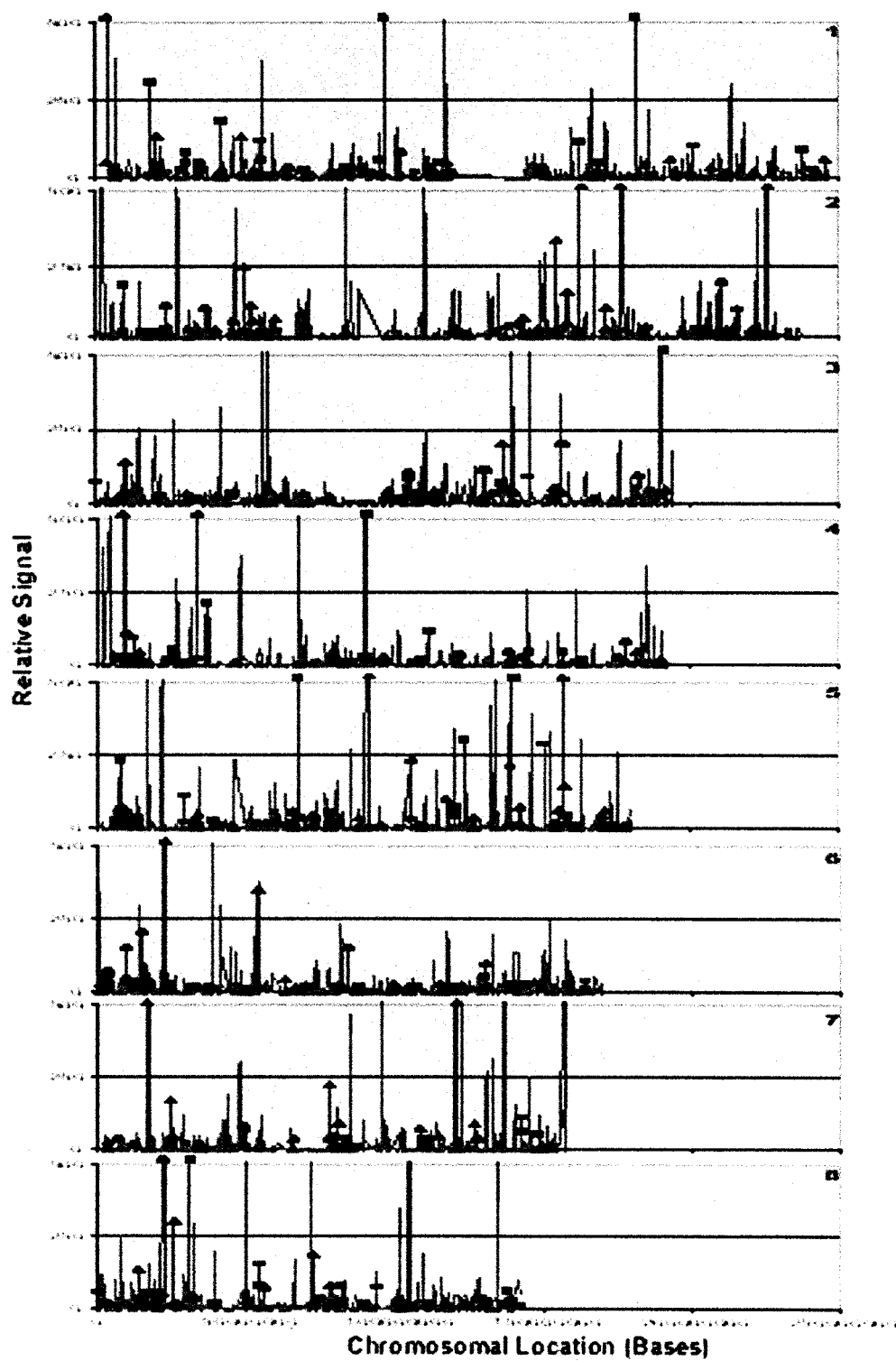
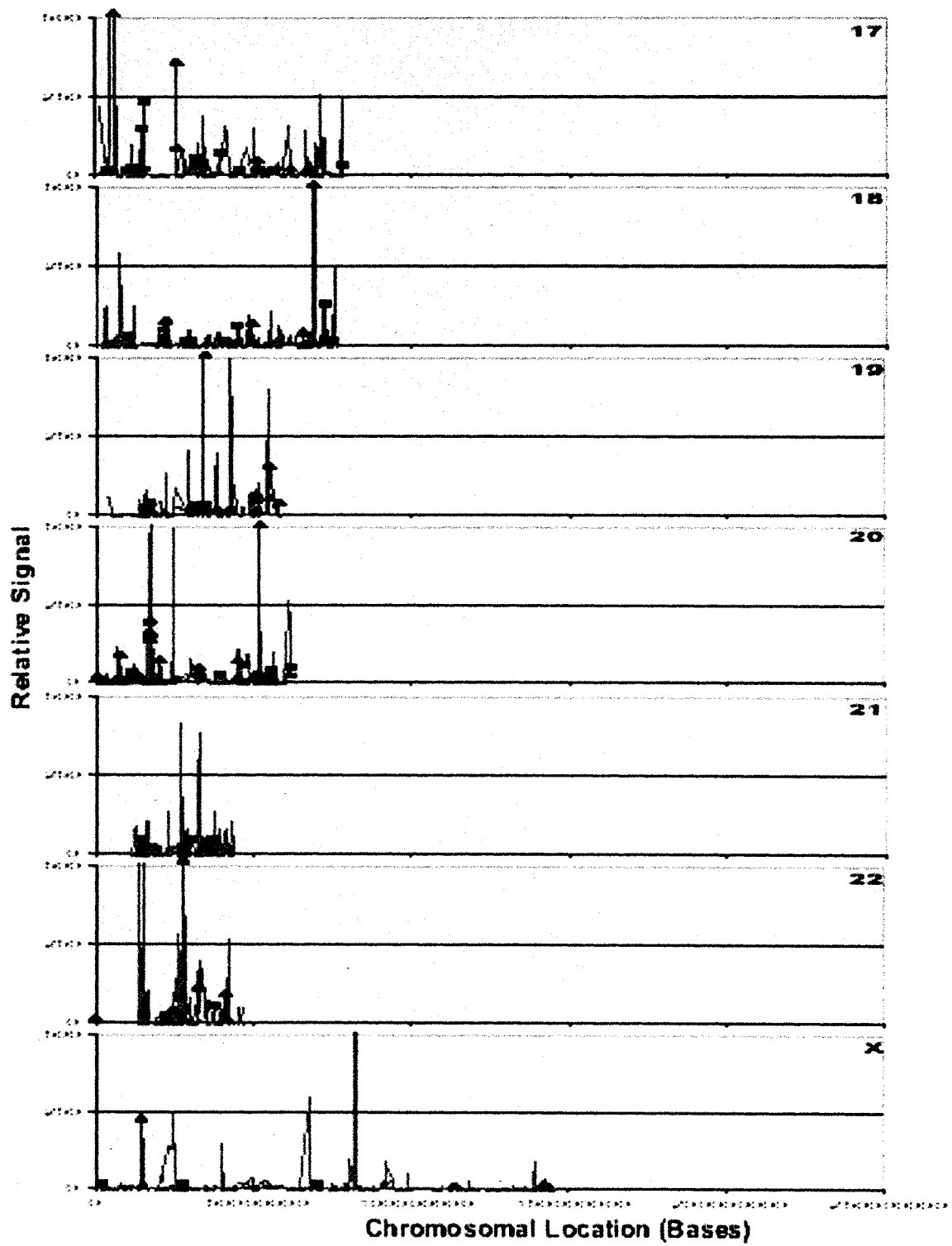


Figure 8C. Using the ChIP on SNP methodology the genome-wide pattern of histone H3-K9 dimethylation was assessed and a detailed plot of the changes in histone H3-K9 dimethylation occurring on chromosomes 17 to X during differentiation was generated. Relative signal is an average of the relative signal determined in three independent experiments. Blue, Myoblasts; Pink, Myotube; Triangle, $p < 0.05$; Square, $0.05 < p < 0.1$.



In myoblasts dimethylation of histone H3-K9 inversely correlates with transcriptional activity.

Histone H3-K9 dimethylation is generally associated with transcriptionally inactive genes and heterochromatin (Lachner and Jenuwein, 2002). Therefore we would predict an increase in histone H3-K9 dimethylation should correlate with decreased transcription and vice versa. To investigate if the changes in H3-K9 demethylation inversely correlated with changes to transcriptional status of the gene in question, we analyzed the expression of 156 genes with probes on both the WGA 10K array and U133A human transcript array in which there was an observed significant change in the dimethylation of histone H3-K9. Either no significant change ($p > 0.05$) to the transcriptional status or no expression in myoblasts and myotubes was observed for approximately 84% (131/156) of these genes. Of the 25 genes with significant changes ($p < 0.05$) occurring in both histone H4 acetylation and transcriptional status, only in 14 (56%) of these genes did the change in transcriptional status correlate with the expected change histone H3-K9 dimethylation (see figure 9A). However, the inverse correlation between hyperdimethylation of H3-K9 and transcriptional activity increase to 67% (8/12) if genes in which the transcriptional activity was repressed subsequent to differentiation (see figure 9B).

Figure 9. To investigate if the changes in H3-K9 diMe inversely correlated with changes to transcriptional activity, we analyzed the expression of 156 genes with probes on both the WGA 10K and U133A array in which there was an observed significant change in the H3-K9 diMe. Either no significant change in transcription or no expression in myoblasts and myotubes was observed for approximately 84% of these genes. In 56% of genes with a change in both transcriptional activity and methylation, the change in transcriptional status inversely correlated with the change H3-K9 diMe. However, the inverse correlation increased to 67% (8/12) if analysis was restricted to genes in which the transcriptional activity was repressed subsequent to differentiation.

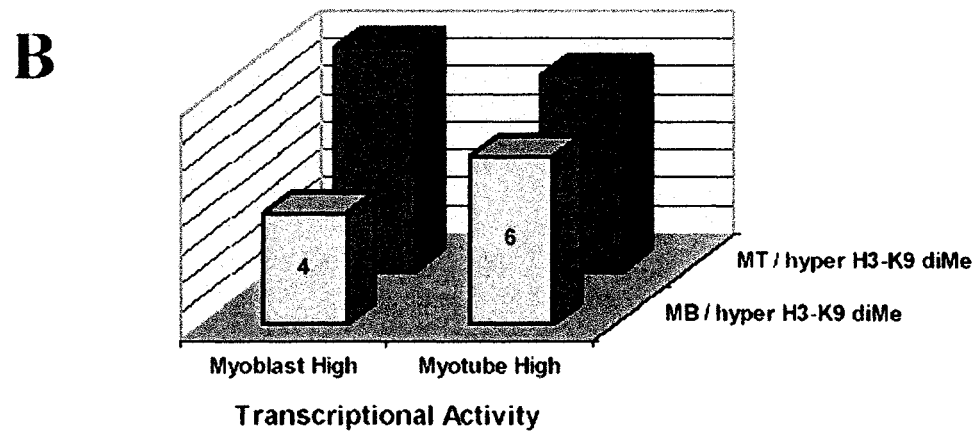
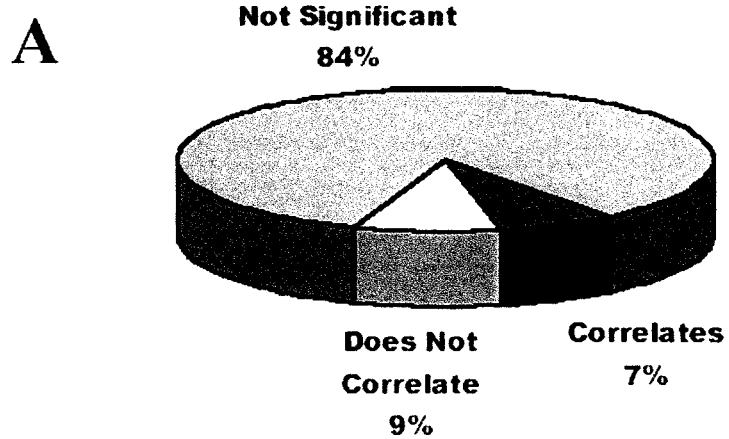


Table 4. Analysis of co-precipitating adjacent SNPs suggests there are no extended regions of hypermethylation in either myoblasts or myotubes.

<i>Chromosome</i>	<i>Co-Precipitating Adjacent Pairs (myoblast, myotube)</i>	<i>Co-Precipitating Adjacent Triples (myoblast, myotube)</i>	<i>Co-Precipitating Sets (>3) (myoblast, myotube)</i>
1	2, 0	0, 0	0, 0
2	1, 1	0, 0	0, 0
3	2, 1	0, 0	0, 0
4	1, 1	0, 0	0, 0
5	2, 1	0, 0	0, 0
6	0, 0	0, 0	0, 0
7	1, 2	1, 0	0, 0
8	1, 1	0, 0	0, 0
9	0, 0	0, 0	0, 0
10	1, 0	0, 0	0, 0
11	1, 2	0, 0	0, 0
12	0, 0	0, 0	0, 0
13	1, 1	0, 0	0, 0
14	2, 1	0, 0	0, 0
15	0, 1	0, 0	0, 0
16	1, 0	0, 0	0, 0
17	0, 0	0, 0	0, 0
18	1, 0	0, 0	0, 0
19	0, 2	0, 0	0, 0
20	0, 1	0, 0	0, 1
21	0, 1	0, 0	0, 0
22	0, 0	0, 0	0, 0
X	0, 0	0, 0	0, 0

No extended regions of histone H3-K9 dimethylation were detected in myoblasts or myotubes.

Approximately 4% of hypermethylated SNPs in myoblasts and myotubes were adjacent to one other hypermethylated SNP. This suggests that in the chromosomal regions assessed in this study there were no large heterochromatin regions or large expansion of H3-K9 dimethylation domains (table 4).

The patterns of Histone H4 acetylation in both myoblasts and myotubes are distinct from the patterns of Histone H3-K9 dimethylation.

During myoblast differentiation the chromosomal locations (SNPs) targeted for changes to the level of histone H4 acetylation are generally distinct from those targeted for changes to H3-K9 dimethylation (compare figure 3 and figure 7). Approximately 14% (1412/10043) of the SNPs assessed had significant changes in only histone H4 acetylation, approximately 5% (548/10043) of SNPs had significant changes in only histone H3-K9 dimethylation while significant changes in both acetylation and methylation were observed for 2% (224/10043) of SNPs assessed. Of the 224 SNPs with significant changes in both histone H4 acetylation and H3-K9 dimethylation, at 25 SNPs the reduction in H3-K9 dimethylation, which occurred during myoblast differentiation, was accompanied by an increase in H4 acetylation. At 17 of the 224 SNPs the reversed occurred, an increase in H3-K9 dimethylation was accompanied by a decrease in H4 acetylation. Preliminary studies suggest the pattern of histone H3 acetylation shares more overlap with the pattern of histone H4 acetylation than it does with histone H3-K9

dimethylation (see appendix). This is consistent with the notion that histone H3 and H4 acetylation may affect chromatin structure in a similar manner.

Discussion

ChIP on SNP: A Methodology for Analyzing Genome-Wide Histone Modifications

By combining chromatin immunoprecipitation using antibodies directed against modified histones with oligonucleotide microarray technology, we have developed a methodology to simultaneously assess histone modifications occurring at multiple chromosomal locations. In previous studies ChIP assays have been combined with dotted CpG island microarrays to globally assess both changes in chromatin structure and transcription factor binding (Fontemaggi et al., 2002; Liu et al., 2002; Robyr et al., 2002; Weinmann et al., 2002). The use of Affymetrix microarrays offers distinct advantages over dotted arrays. In particular with the elimination of the chip-to-chip variation often associated with dotted microarrays the reproducibility of the data is improved. In addition, since the Affymetrix SNP platform is expandable, the number of chromosomal locations assessed using this methodology will dramatically increase in the foreseeable future. Unlike studies using CpG island microarrays, our study of genome-wide changes to histone modifications is not biased towards the study of promoter regions, which in general are localized to CpG islands (Ioshikhes and Zhang, 2000). Although at present, the CpG island arrays are more amendable than SNP arrays for studies investigating transcription factor binding with the development of more comprehensive SNP arrays this advantage will be eliminated.

The temporally coordinated regulation of higher-ordered chromatin structure is essential for normal development and growth. Central to the regulation of chromatin

structure is both DNA and histone modifications. Disruption of these processes can lead to aberrant gene expression and inappropriate gene reprogramming during embryogenesis resulting in abnormal development and may in part account for the inefficiency of mammalian nuclear transfer (Li, 2002; Shi et al., 2003).

In our study we describe the genome-wide changes to histone H4 acetylation and histone H3-K9 dimethylation that occurred during a well-characterized tissue culture model of differentiation. In addition to furthering our understanding of the myoblast differentiation process, by highlighting the central role of histone modification to the differentiation process it will provide lessons that are generally applicable to other cell culture models of differentiation.

Regulation of Histone Acetylation in Myoblasts and Myotubes: Activity, Location and Targeting

The proportion of SNPs acetylated above background increased by 4% upon differentiation (28% compared to 32% of total SNPs assessed), however there was a genome-wide reduction in the level of acetylation by approximately 50% upon differentiation. The net acetylation of the histones is a result of an equilibrium reached between the opposing activities of HATs and HDACs. Shifts in this equilibrium induce the net change in the level of histone acetylation we observed during differentiation (for review (McKinsey et al., 2001)). Signal transduction pathways including the protein kinase C, mitogen-activated protein (MAP) kinase and CaMK pathways in part regulate this equilibrium by affecting the enzymatic activity of both HATs and HDACs and the intracellular localization of the HDACs. The regulation of histone-modifying enzymes by

various signal transduction cascades facilitates a rapid transcriptional response to changes in the extracellular milieu and may account for the previously described roles of these pathways in myoblast differentiation (Bergstrom et al., 2002; Gredinger et al., 1998; Li et al., 2000; McKinsey et al., 2000; Perry et al., 2001).

The pattern of histone H4 acetylation following differentiation was different from that observed in proliferating myoblasts (figure 3 and 4). This is consistent with the notion that distinct mechanisms (or proteins) are targeting histone-modifying enzymes in proliferating myoblasts and myotubes. This has been previously demonstrated for HDAC1 (Puri et al., 2001). Under growth conditions, HDAC1 is targeted to muscle specific promoters through a direct interaction with MyoD resulting in the repression of differentiation-specific genes. Following the receipt of pro-differentiation cues, the HDAC1/ MyoD complex disassociates allowing for the activation of differentiation-specific genes. Released from its association with MyoD, HDAC1 forms a complex with hypophosphorylated pRB, which in turn represses the transcription of genes required for cell cycle progression, including cyclin E and cyclin A, ultimately leading to cell cycle arrest (Puri et al., 2001; Zhang et al., 2000).

Histone H4 Hyperacetylation and Transcription

Whereas previous studies have clearly linked histone H4 hyperacetylation with transcriptional activation, in growing myoblasts we did not observe a strong correlation with increased acetylation and elevated levels of transcriptional activity or decreased acetylation and a reduction in transcriptional activity (figure 6). However there are numerous reports in the literature where histone H4 acetylation had no effect or a

repressive effect on transcription (for review (Ricci et al., 2002)). Moreover studies in yeast suggest that the pattern of histone acetylation is transcription activator-dependent and that hyperacetylation of the histones is not a prerequisite for transcriptional activation and is therefore a poor indicator of transcriptional activity (Deckert and Struhl, 2001). Consistent with this is the recent observation that MyoD targets are hypoacetylated when MyoD is absent suggesting MyoD is required to target HAT activity to these genes (Bergstrom et al., 2002). Further complicating the analysis of the acetylation / transcription association, is the changes in histone H4 acetylation that occur during the cell cycle and which correlate with cell-cycle dependent changes in chromosome structure (Jasencakova et al., 2000; Wako et al., 2002).

We did however observe a correlation between hyperacetylation of histone H4 and transcription in myotubes (figure 6). Generally in differentiated myotubes, in the genes where there was a significant change in transcriptional activity there was an accompanying change H4 acetylation. Since myotubes have exited the cell cycle analysis of the acetylation / transcription correlation is not complicated by the cell cycle-dependent changes chromosome structure discussed earlier. Our data argues that although in myotubes changes in the transcriptional activity of a gene are an indicator of an accompanying change in histone H4 acetylation, it is insufficient to predict the change in histone H4 acetylation.

It is interesting to speculate whether there would be a stronger correlation between acetylation and transcriptional status if as opposed to an unbiased study, one restricted analysis to promoter regions by using CpG islands microarrays. A quick survey of the

literature suggests this would be the case, numerous examples exist in which hyperacetylation of the promoter is correlated with increased transcription. There are however very few examples in the literature of promoters repressed by hyperacetylation and include the bi-directional promoter regulating the gene encoding kynarenine formamidase (Schuettengruber et al., 2003).

Regulation of Histone H3-K9 DiMethylation in Myoblasts and Myotubes: Activity, and Targeting

In response to pro-differentiation cues, the total number of SNPs dimethylated on histone H3-K9 above background doubled, however the genome-wide level of histone H3-K9 dimethylation as assessed by total signal did not change significantly. Presumably the net dimethylation of histone H3-K9 observed in a cell at any given time is a result of an equilibrium reached between the opposing activities of methylation and demethylation. Recently, there has been an explosion in the number of histone methyltransferase identified however a protein with histone demethylase activity has yet to be isolated. The enzymatic demethylation of histones was first described in 1973 (Bannister et al., 2002; Lachner and Jenuwein, 2002; Paik and Kim, 1973). Sequence analysis of the Elp3 histone acetyltransferase indicates it may contain a second catalytic domain capable of demethylating histones suggesting the demethylation of histone H3-K9 is linked to its acetylation however the role of Elp3 as a demethylase has yet to be confirmed experimentally (Chinenov, 2002). Regardless, there are a number of genes in which the active reversal of histone methylation is a prerequisite for transcriptional activation

suggesting mechanisms exist within the cell to remove methyl groups from histones (Bannister et al., 2002).

Significant changes in the pattern of histone H3-K9 dimethylation were observed following differentiation (Figure 7 and 8). This is consistent with the notion that histone methylation similar to histone acetylation is targeted to particular loci.

Hypophosphorylated pRb recruits in addition to HDAC1, SUV39H1 histone methylase to E2F target genes including cyclin E. As a result the promoter is hypermethylated creating binding sites for HP1 and transcription is inhibited (Nielsen et al., 2001; Vandel et al., 2001). Interestingly, a link between histone deacetylation and methylation occurs at MEF2 target genes in proliferating myoblasts. Via an association with HP1-HDAC, MEF2 recruits a histone methyltransferase to target gene promoter (Zhang et al., 2002). This association is responsive to CaMK signaling (Zhang et al., 2002).

Implications

In summary, we describe a novel methodology for assessing genome-wide histone modifications. With the involvement of histone modification in diseases as diverse as cancer, fragile X syndrome and Rubinstein-Taybi syndrome, using this methodology will likely lead to insights into the disease processes underlying these conditions (Timmermann et al., 2001). In addition, since the SNPs utilized in this study are highly informative (over 30% heterozygosity) allele specific chromatin modifications such as those occurring at imprinted loci can be readily assessed. Our results are consistent with the notion, that a combination of distinct histone and DNA modifications together and not a single modification defines the transcriptional competence of a particular gene. Our

data suggests that during myoblast differentiation histone modifying enzymes are targeted to particular loci.

Appendix: Preliminary ChIP and SNP Analysis Using Affymetrix HuSNP Arrays

Prior to undertaking the analysis of histone modifications using the WGA 10K microarray from Affymetrix, we tested the feasibility of the ChIP on SNP method using a microarray, which assesses a more limited number of SNPs. The HuSNP microarray allows for the examination of 1494 SNPs distributed over the 22 autosomes and the X chromosome. Similar to the WGA 10K microarray, the HuSNP microarray chip contains a series of oligonucleotides that specifically hybridize to defined single nucleotide polymorphisms (SNPs) within the human genome and therefore can be used to define a specific chromosome location. In this preliminary study we assessed the genome-wide changes to histone H4 acetylation that occur during myoblast differentiation and compare the pattern of histone H4 acetylation in myotubes to the pattern of histone H3 acetylation and histone H4-K9 dimethylation.

Methodology

Generation of Biotinylated Target

The HuSNP Mapping Assay steps (multiplex PCR, labeling PCR) in the Affymetrix protocol were modified to accommodate the chromatin IP procedure as follows (refer to figure). Briefly, precipitated DNA (from 2.5×10^6 cells) was amplified in 24 multiplex PCR reactions with each containing a distinct multiplex primer pair pooling. Each multiplex primer pair pool contained primer pairs that flanked a subset of the SNP being assessed. Each 12.5 μ L reaction contained 1 unique multiplex primer pair pool (Affymetrix), 1X Ampli Taq Buffer II (Perkin Elmer), 5 mM $MgCl_2$, 0.5mM dNTP

(Pharmacia Biotech), and 1.25 U AmpliTaq Gold (Perkin Elmer). The amplification conditions were as follows: 95°C, 5 min hot start; 30 cycles with temperature ramp 95°C, 30 s / 52°C + 0.2°C per cycle, 50 s / 72°C, 30 s; 5 cycles 95°C, 30 s / 58°C, 50 s / 72°C, 30; final extension 72°C, 7 min. Multiplex PCR reactions were diluted 1:1000 and 2.5 µL were re-amplified in the labeling reaction. Each 25 µL PCR reaction contained 1X AmpliTaq Buffer II (Perkin Elmer), 4 mM MgCl₂, 0.4 mM dNTP, 0.8 µM biotinylated T7 primer, 0.8 µM biotinylated T3 primer and 2.5 U AmpliTaq Gold. The amplification conditions were as follows: 95°C, 8 min hot start; 40 cycles with temperature ramp 95°C, 30 s / 55°C, 90 s / 72°C, 30 s; final extension 72°C, 7 min. PCR reactions were all preformed using a Perkin Elmer 9700 thermocycler. PCR reactions were pooled and concentrated using Microcon-10 spin columns (Millipore) as per manufactures instructions.

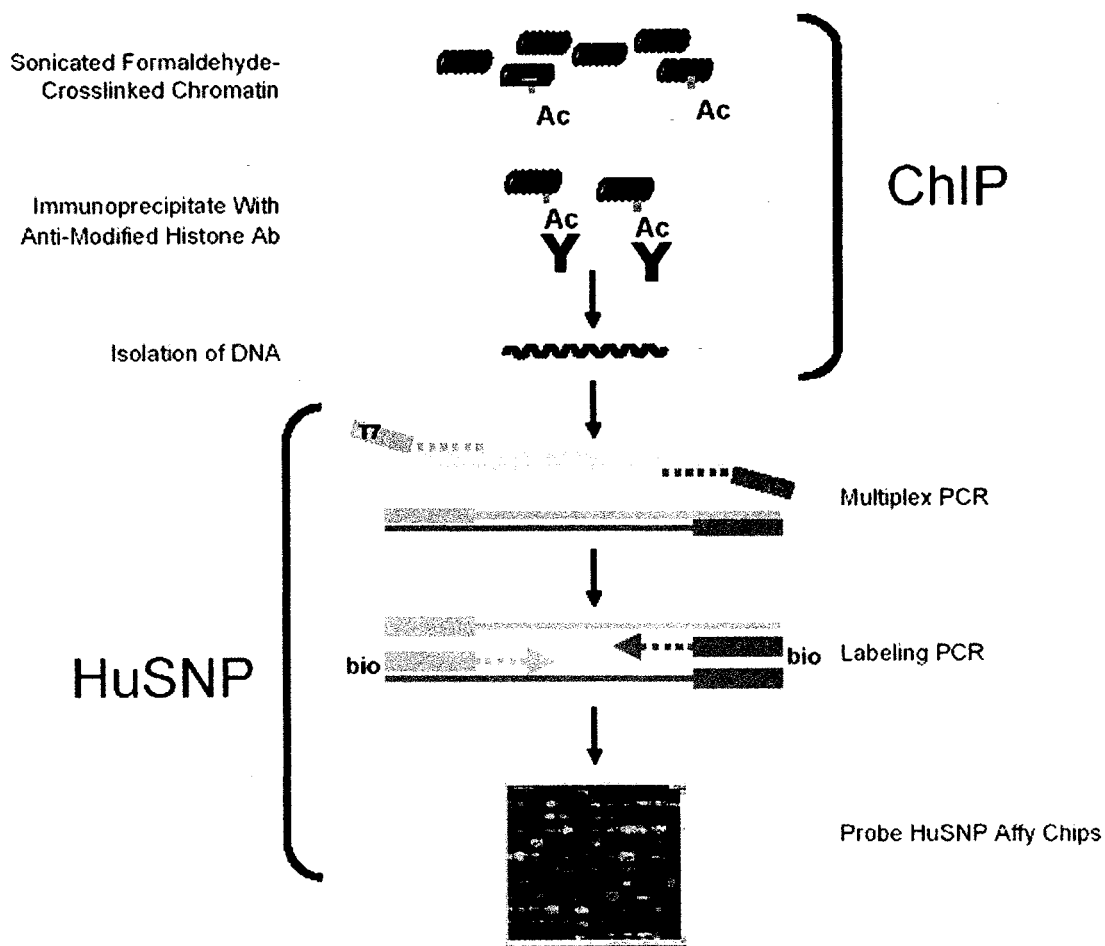
Target Hybridization, Washing, Scanning and Staining

Target hybridization, washing, scanning and staining were performed as recommended by Affymetrix.

Data Analysis

Data was analyzed using MAS 5.0 software from Affymetrix.

Figure 10. (A) In the schematic, the ChIP on Hu-SNP methodology is outlined. Briefly, human cells are crosslinked with formaldehyde to fix protein-DNA complexes. The crosslinked chromatin is isolated and sheared to an average length of 1000 bp by sonication. The sheared crosslinked chromatin is immunoprecipitated with antibodies directed against modified histones. The antibody-chromatin complexes are recovered by protein A-agarose and washed repeatedly. The chromatin is eluted and crosslinks reversed. The chromatin is deproteinated and the DNA is isolated. SNPs are amplified in a multiplex PCR reaction from which biotinylated targets are generated. Biotinylated target is hybridized overnight to the Affymetrix Hu- SNP array. The array is processed and relative signal determined. (B) Chromosomal distribution of SNPs assessed by the HuSNP array.



Results and Discussion

The chromatin from myoblasts or myotubes was precipitated with antibodies directed against modified histones. The DNA was isolated from the ChIP samples and further purified using Millipore spin columns. This DNA was used to generate biotinylated PCR products that were subsequently used to probe the HuSNP microarrays. A list of SNPs specifically immunoprecipitated with anti-acetyl histone H4, anti-acetyl histone H3 or anti-DiMethyl Histone H3-K9 and not control antibody was obtained. Using this data, chromosomal maps detailing histone modifications were generated.

Myoblasts and myotubes have distinct pattern of histone H4 acetylation.

Consistent with the previously described results from the WGA 10K study, the pattern of histone H4 acetylation in myoblasts is distinct from the pattern of histone H4 acetylation observed in myotubes (figure 10). Of the 1,494 SNPs assessed approximately 12% (188/1494) of SNPs were specifically hyperacetylated in myoblasts and not myotubes while approximately 6% (88/1494) of SNPs were specifically hyperacetylated in myotubes and not myoblasts. Unlike studies using this array where a reduction in the number of hyperacetylated SNPs with differentiation was observed, studies using the WGA 10K array clearly demonstrated a moderate increase in the number of specifically hyperacetylated SNPs following differentiation. However the change in genome-wide acetylation as assessed by total signal was consistent between the HuSNP and WGA 10K arrays. It should be noted that because of the limited genome coverage the HuSNP array compared to WGA 10K array, the differences in methodology including probe generation

and data analysis, and unique set of SNPs analyzed, a direct comparison between the data sets generated using these two approaches is difficult.

The validity of this method for examining myoblast chromatin structure was confirmed by identifying the SNPs precipitated by anti-acetyl histone H4. Several of the SNPs were within or closely linked to loci previously shown to be important in myoblast specification and growth or muscle development. In particular, precipitated SNPs included WIAF-1053, located within the 3'UTR of the myogenic regulatory factor MRF4 (also called Myf6), WIAF-3425 (cadherin 13), WIAF-2019 (syndecan 4), WIAF-4506 (MOX2) and WIAF-234 (LAMB3).

The pattern of Histone H4 acetylation in myotubes is similar to the pattern of Histone H3 acetylation but is distinct from the pattern of Histone H3-K9 dimethylation.

In myotubes, the chromosomal locations that are hyperacetylated on histones H3 and H4 are generally distinct from those dimethylated on histone H3-K9 (figure 11). Approximately 5% (69/1494), 6% (88/1494) and 5.5% (82/1494) of the SNPs assessed were specifically immunoprecipitated with antibodies directed against acetylated histone H3, acetylated histone H4 and dimethyl histone H3-K9 respectively. Of the 69 SNPs precipitated with anti-acetylated histone H3 antibody 22% (15/69) were also precipitated by anti-acetylated histone H4 antibody while only 4% (3/69) were also precipitated by anti-dimethyl histone H3-K9 antibody. 4.5% (4/88) of SNPs precipitated with anti-acetylated histone H4 antibody were also precipitated by anti-dimethyl histone H3-K9 antibody. The minimal overlap between SNPs precipitated with anti-acetyl histone H4

and anti-dimethy histone H3-K9 antibody is consistent with the results obtained using the WGA 10K arrays. These results are consistent with the notion that histone H3 and H4 acetylation affect chromatin condensation in a similar way that is distinct from the affect of histone H3-K9 dimethylation.

Figure 11. Using the ChIP on HuSNP methodology the genome-wide pattern of histone H4 acetylation was assessed. The pattern of histone H4 acetylation in myoblasts is distinct from the pattern of histone H4 acetylation observed in myotubes.

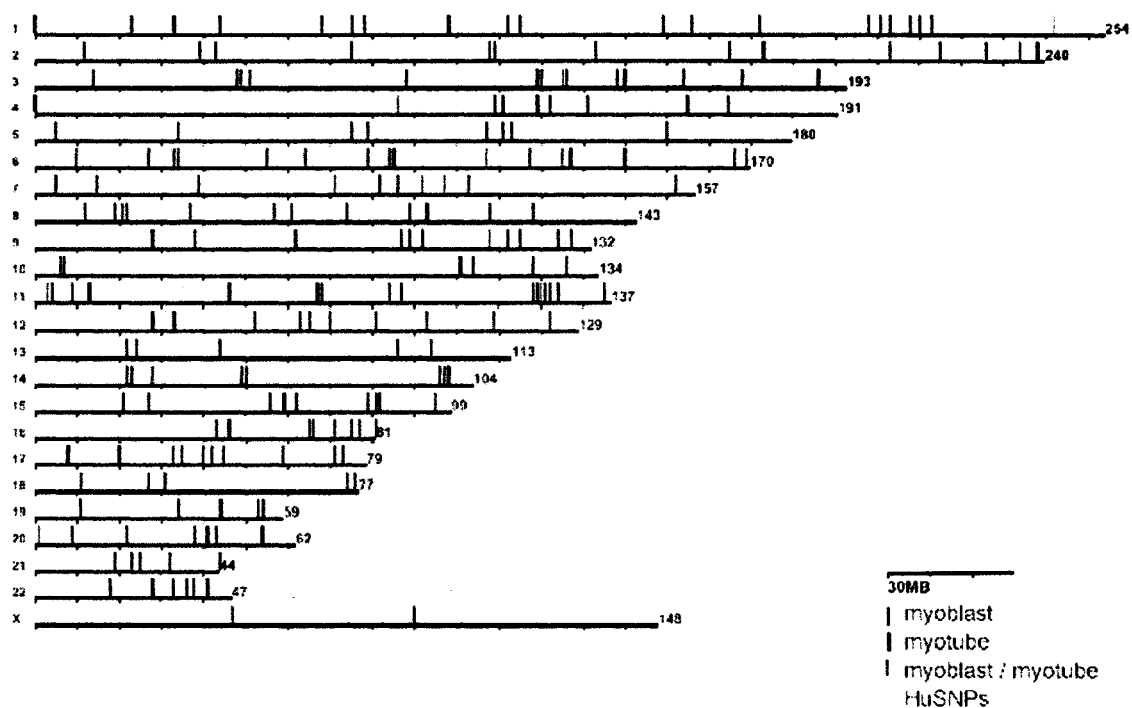
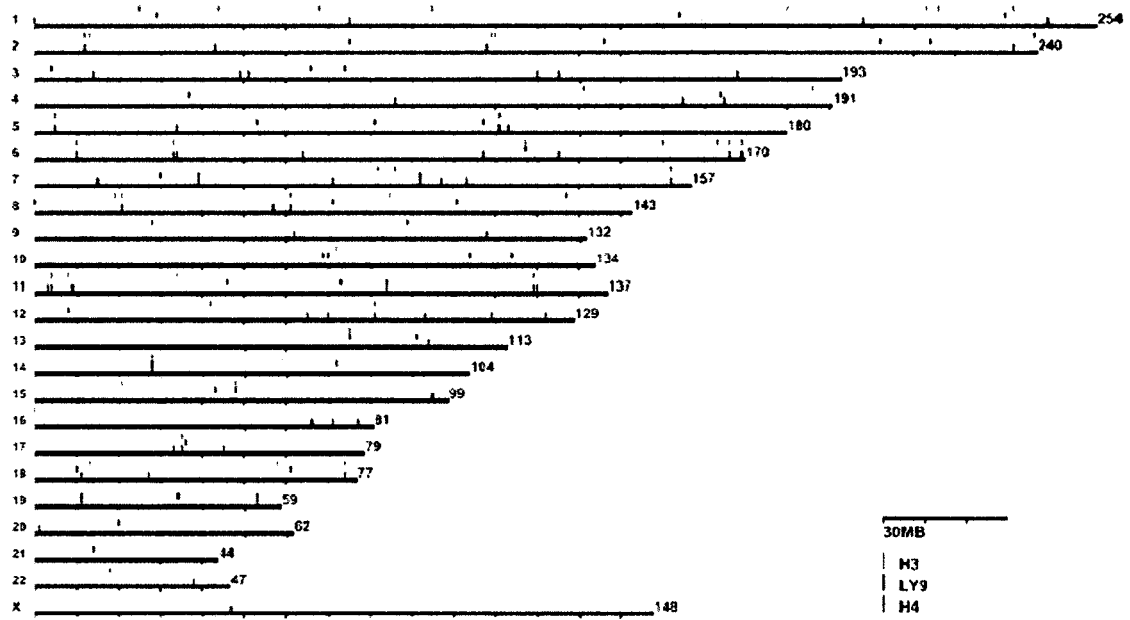


Figure 12. The pattern of Histone H4 acetylation in myotubes is similar to the pattern of Histone H3 acetylation but is distinct from the pattern of Histone H3-K9 dimethylation.



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CHAPTER 4: Inhibition of MEK1 with U0126 Promotes Differentiation by Increasing MyoD Associated HAT Activity

Foreword

The following chapter, entitled “Inhibition of MEK1 with U0126 Promotes Differentiation by Increasing MyoD Associated HAT Activity” examines the mechanism by which inhibition of MEK1 activity with the specific chemical inhibitor U0126 promotes myoblast differentiation. This work clearly demonstrates that U0126 increases the MyoD associated histone acetyltransferase activity. Claire Palmer completed the majority of the work and wrote the manuscript. A. Polesskaya completed the *in vitro* HAT assay. M.A. Rudnicki supervised this project.

Title Page

**Inhibition of MEK1 with U0126 Promotes Differentiation by Increasing MyoD
Associated HAT Activity**

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Abstract

To further elucidate the mechanism through which activated MEK1 inhibits MyoD dependent activation of muscle specific genes, we investigated the mode by which the specific MEK1 inhibitor U0126 accelerated the differentiation process. Inhibition of MEK1 with U0126 promoted myoblast differentiation. A 2.5 fold increase in the number of myosin heavy chain positive (a marker of differentiation) myoblasts was observed following treatment with U0126. This accelerated differentiation was accompanied by changes to the chromatin structure around muscle specific genes. In particular, the chromatin surrounding the myogenin promoter was more accessible to endonuclease digestion following treatment with U0126. In addition, inhibition of MEK1 increased the acetylation of histone H4 at muscle specific E boxes. These results are consistent with the notion that MEK1 regulates MyoD-associated histone acetyltransferase and deacetylase activity. Indeed, inhibition of MEK1 increased MyoD-associated HAT-activity suggesting U0126 either positively affects HATs or negatively affects HDACs. Together, these results demonstrate MEK1 modulates MyoD activity by regulating MyoD associated HAT and HDAC activities.

Introduction

The myogenic regulatory factors have a central role in myoblast specification and differentiation and include MyoD, Myf5, myogenin and MRF4 (Rudnicki and Jaenisch, 1995; Sabourin and Rudnicki, 2000)]. Members of this family of basic helix loop helix transcription factors all share a similar capacity to convert non-muscle cells to the myogenic lineage by inducing muscle specific gene expression. The induction of muscle specific genes by MyoD in both converted non-muscle cells and myoblasts is temporally regulated. In that, MyoD activates transcription from different promoters at different times during myogenesis (Bergstrom et al., 2002). Using gene targeting approaches the individual roles of the MRFs have in part been defined. MyoD and Myf5 are essential for myoblast specification and therefore categorized as primary MRFs, while myogenin is essential for myoblast differentiation and therefore is a secondary MRF (reviewed in (Megeney and Rudnicki, 1995)).

The primary MRFs through a direct interaction with either HATs or HDACs can modulate the chromatin structure around the promoters of muscle specific genes and therefore have the capacity to act as both transcriptional activators and repressors (Gerber et al., 1997; Mal and Harter, 2003; Polesskaya et al., 2001a; Puri et al., 2001; Sartorelli et al., 1997). For example, in proliferating myoblasts MyoD represses transcription from differentiation specific genes by targeting HDAC1 to these promoters (Mal and Harter, 2003). Subsequent to pro-differentiation cues, HDAC1 disassociates from MyoD and is replaced by the histone acetyltransferase CBP/p300 allowing for the activation of differentiation specific genes (Polesskaya et al., 2000; Polesskaya et al., 2001b; Puri et

al., 1997a; Puri et al., 2001; Puri et al., 1997b; Sartorelli et al., 1997; Sartorelli et al., 1999; Yuan et al., 1996).

The mitogen-activated protein kinase (MAPK) signaling pathway, in part, regulates the temporally appropriate activation of muscle specific genes by MyoD. Inhibition of MEK1 in C2C12 myoblasts with the specific chemical inhibitor U0126 stimulates the onset of differentiation (Perry et al., 2001). In addition, the alleviation of MEK1-dependent repression of MyoD transcriptional activity is a prerequisite to differentiation since the sustained activation of MEK1, after the receipt of pro-differentiation cues, is refractory to the activation of differentiation specific genes (Perry et al., 2001). The repression of MyoD by MEK1 is mediated by a directed interaction between activated MEK1 and the amino terminus transactivation domain of MyoD, however, is not dependent on the direct phosphorylation of MyoD by MEK (Perry et al., 2001). Interestingly, the amino terminus of MyoD interacts with CBP/p300, suggesting MEK1 inhibits MyoD dependent transactivation by interfering with the recruitment of essential co-factors (Sartorelli et al., 1999). Alternatively, various repressors of MyoD activity have previously been shown to bind the N terminus of MyoD. These repressors include COUP-TFII, which recruits histone deacetylases (Bailey et al., 1999; Bailey et al., 1998). Activated MEK1 may inhibit MyoD dependent transactivation by modulating the activity of these co-repressor complexes.

In this report, we examine the mechanism by which the specific inhibitor of MEK1 (U0126) enhances the differentiation process. Our data demonstrates that inhibition of MEK1, increases the MyoD associated HAT activity resulting in a

relaxation of the chromatin structure surrounding muscle specific genes and increase acetylation of histone H4 at these promoters and not globally.

Materials and Methods

Cell Culture

The cell cycle profiles were determined using anti-phosphorylated Histone H1 and H3 immunohistochemistry. Determination of cell cycle stages by immunohistochemistry was performed according to manufactures instructions (Upstate Biotechnology).

To assess the differentiation potential of the C2C12 following treatment with U0126, cells were seeded into growth media at a density of 10^4 cells / well of a 2-well chamber slide or 10^5 cells / 10 cm tissue culture dish and cultured until 75% confluence prior to the addition of differentiation media (DMEM supplemented with 2% horse serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin) containing either 10 μ M U0126 or equal volume of vehicle (DMSO). Differentiation media was changed every two days. Each day one chamber slide for control and treatment was fixed with 4% paraformaldehyde, cells were permeabilized by 0.2% Triton-X and immunostained for myogenin (M-225; Santa Cruz, sc-576) and myosin heavy chain (MF20 supernatant) at a dilution of 1/50 and 1/5 respectively.

Nuclease Access Assay

The nuclease access assay was performed as described elsewhere (Gerber et al., 1997). Briefly, cells were washed with PBS and trypsinized. Cell pellets were washed with ice-cold RSB buffer (10 mM Tris pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$). Cells were resuspended in ice-cold lysis buffer (RSB buffer + 0.1% NP40) to a density of 1.5×10^6 / mL and incubated on ice for 10 min. The nuclei were isolated by centrifugation at 2000

RPM for 5 min at 4°C and wash once with lysis buffer. 10^6 nuclei were resuspended in 200 μ L of 1 x NEB 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol) and digested with 40 U of Ban1 for 30 min at 37°C. The reaction was terminated by the addition of 200 μ L of stop buffer (0.6M NaCl, 20mM Tris Base at pH 7.4, 10mM EDTA, 1% SDS and 2mg/ml Proteinase K) and deproteinated overnight at 50°C. DNA was purified and digested to completion with indicated restriction enzymes. Digested DNA was subjected to Southern blot analysis. See figure legend for details regarding the probe.

Chromatin Immunoprecipitation

Chromatin immunoprecipitations were performed using the ChIP Assay kit from Upstate Biotech (Cat# 17-295) as per manufactures instructions. Briefly, 10^7 cells were crosslinked for 10 minutes at room temperature with formaldehyde (1% formaldehyde final concentration). The crosslinking reaction was terminated by the addition of 0.125 M glycine. Cells were washed twice with ice-cold PBS prior to being harvested. Cell pellets were resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH8.1) supplemented with protease inhibitors and sodium butyrate. Lysis was allowed to proceed for 10 minutes on ice prior to sonication. Lysates were sonicated until DNA was sheared to between 0.5 and 2 kb. Sonicated lysates were cleared by centrifugation for 10 minutes at 13,000 rpm at 4°C. Cleared lysates were diluted 10-fold in 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCl, pH8.1, 167 mM NaCl and incubated with Salmon Sperm DNA /Protein A Agarose-50% slurry to reduce non-specific background for 30 minutes at 4°C with rotation. Either 5 μ L of Anti-Acetylated Histone H4 (Upstate, Cat# 06-866) or Anti-dimethyl-Histone H3 (Lys9) (Upstate, Cat# 07-212)

was added to the lysates (per 10^6 cell equivalents). Immune complexes were recovered with Salmon Sperm DNA/Protein A Agarose and washed once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH8.1, 150 mM NaCl); once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH8.1, 500 mM NaCl); once with LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris HCl, pH 8.1) and twice with TE. Immune complexes were eluted with 1% SDS, 0.1 M NaHCO_3 for 15 min at room temperature. DNA-protein crosslinks were reversed for 4 hrs at 65°C with 0.2mM NaCl. Samples were deproteinated and DNA isolated using Qiaquick columns as described by the manufacture (Qiagen). DNA was eluted from columns with ddH_2O . PCR primers and conditions as described elsewhere (Bergstrom et al., 2002).

***In vitro* Acetylation Assay**

The *in vitro* acetylation assay was performed as described previously (Ait-Si-Ali et al., 1998). Briefly, C2C12 cells (1×10^8 per data point) were treated with U0126 or DMSO, harvested, and MyoD was immunoprecipitated using standard procedures with the sc-318 anti-MyoD antibody (Santa Cruz): beads were washed twice with RIPA buffer [50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and a cocktail of protease inhibitors (Boehringer) and three times with HAT buffer (50 mM Tris pH 7.5, 1 mM EDTA and protease inhibitors).

Immunoprecipitates were subsequently used in a HAT assay. The H4 biotinylated peptide (SGRGKGGKGLGKGGAKRHRKVLR-GSGSK-BIO)(Chiron) (30 mM final) was mixed with samples containing the immunoprecipitates immobilised on beads in 30 ml of HAT buffer supplemented with 100 nCi of [^{14}C]acetyl-CoA (63 mCi/mmol, ICN)

and incubated at 30°C for 45 min. After centrifugation, supernatants were incubated in HAT buffer with pre-washed streptavidin-agarose beads (Sigma) for 20 min at 4°C on a rotating wheel, beads were washed twice with RIPA buffer, mixed with 2 ml of scintillation liquid (Hionic, Packard) and counted using a β counter (LKB).

Results

Inhibiting MEK1 promotes myoblast differentiation.

To assess the involvement of the mitogen-activated protein kinase (MAPK) pathways in myogenesis, the ability of MyoD to induce the myogenic program in the presence of constitutively active MEK1 was assessed (Perry et al., 2001). Previously, we have shown that activated MEK1 binds to MyoD and represses its transcriptional activity. This interaction requires the N-terminus of MyoD and is not through a direct MyoD/MEK1 interaction (Perry *et al.*, (2001)). Consistent with these observations, inhibition of MEK1 activity by the chemical inhibitor U0126 promotes myoblast differentiation as indicated by early expression of myogenin mRNA and myosin heavy chain protein (figure 1, 2 and (Perry et al., 2001)). After 48 hrs of differentiation, the number of myosin heavy chain positive C2C12 myoblasts in cultures treated with U0126 was 2.5 fold greater than in cultures treated with vehicle alone. We sought to investigate the mechanism by which U0126 promotes myoblast differentiation and in particular MyoD activity.

Inhibition of MEK1 with U0126 promotes HEB binding to MCK enhancer.

MyoD forms transcriptionally active heterodimers with the widely expressed E proteins, including E12/E47, ITF-2 and HEB (Lassar et al., 1991; Murre et al., 1989). In myoblasts, evidence suggests HEB is the pro-differentiation heterodimerization partner of MyoD (M.H. Parker, R.L.S. Perry and M.A. Rudnicki, unpublished data).

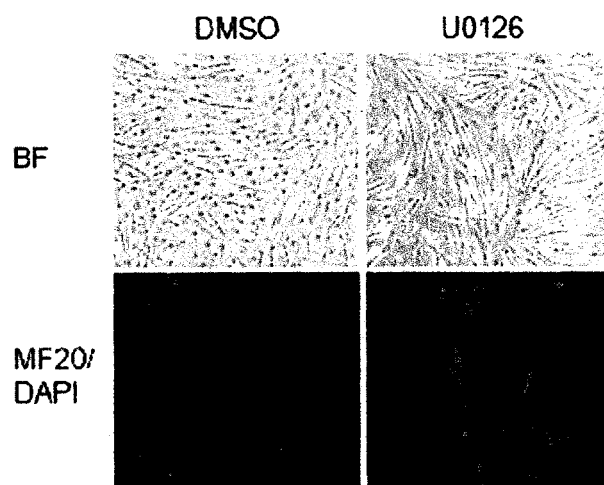


Figure 1. Inhibition of MEK1 by U0126 accelerates myoblast differentiation. MF20 is a mouse monoclonal antibody directed against myosin heavy chain, a marker of differentiation.

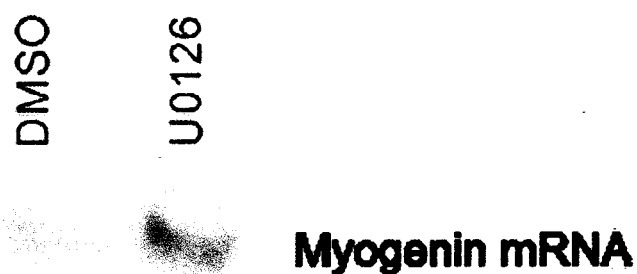


Figure 2. The expression of myogenin mRNA is increased in C2C12 myoblasts treated with U0126 compared to DMSO treated controls.

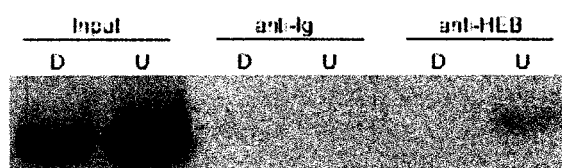


Figure 3. Inhibition of MEK1 with U0126 promotes HEB binding to MCK enhancer. The binding of HEB to the MCK enhancer was assessed using ChIP analysis.

Consistent with the pro-differentiation effects of U0126 on myoblasts, increased binding of HEB to the MCK enhancer is observed following treatment of C2C12 myoblasts with U0126 (figure 3).

The chromatin surrounding the myogenin promoter is more accessible following treatment with U0126.

MyoD, through a direct interaction with either HATs or HDACs, targets histone modifying activity directly to muscle specific promoters (Mal and Harter, 2003; Polesskaya et al., 2001a; Puri et al., 2001; Sartorelli et al., 1997). Various proteins that interact with the N terminus of MyoD either modify histones directly or recruit histone modifying enzymes and include the histone acetyltransferase PCAF (Sartorelli et al., 1999). To investigate the involvement of chromatin remodeling enzymes in U0126-induced accelerated differentiation the chromatin structure surrounding the myogenin promoter was assessed using a restriction endonuclease access assay. The BanI site within the myogenin promoter was more readily cleaved in C2C12 myoblasts treated with U0126 then DMSO treated control myoblasts. This result is consistent with the chromatin surrounding the myogenin promoter being more open following treatment and suggests that MEK1 may modulate MyoD-dependent chromatin remodeling (figure 4).

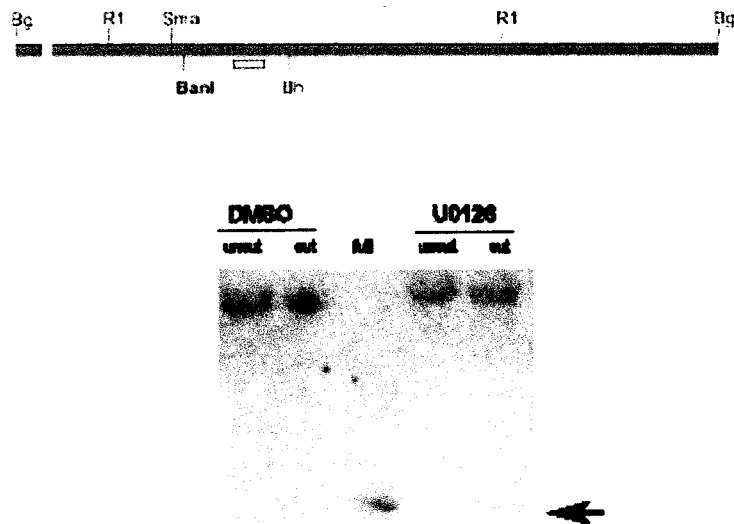


Figure 4. Increased Accessibility of the Myogenin Promoter Following Inhibition of MEK with U0126. The E box within the myogenin promoter is more accessible to cleavage with BanI in C2C12 myoblasts treated with U0126 compared to control DMSO treated C2C12 myoblasts. Southern blot analysis assessing the accessibility of a BanI site located within the myogenin promoter. Isolated nuclei were subjected to limited digestion with BanI as described previously (Gerber et al., 1997). DNA was purified and digested to completion with BamHI/EcoRI and probed with the fragment depicted in the schematic.

Inhibition of MEK1 increases the acetylation of histone H4 at muscle specific E boxes.

MyoD directly targets histone modifying activity to muscle specific promoters (Mal and Harter, 2003; Polesskaya et al., 2001a; Puri et al., 2001; Sartorelli et al., 1997). To test if inhibition of MEK1 effects MyoD-dependent hyperacetylation of muscle specific E boxes, we compared the histone acetylation in C2C12 myoblasts treated with U0126 and C2C12 myoblasts treated with vehicle alone. Inhibition of MEK1 increased histone H4 hyperacetylation at the MCK enhancer (figure 5a). This effect appears to be specific since there was no change in the overall level of histone acetylation as assessed by Western blot (figure 5b). Together these results suggest U0126 may have a direct effect on either MyoD-Associated HAT or HDAC activity.

Inhibition of MEK1 increases MyoD-Associated HAT activity.

To test if U0126 had a direct effect on MyoD associated histone acetyltransferase activity, we performed an *in vitro* histone acetylation assay using histone H4 peptide as a substrate and immunoprecipitated MyoD as a source of HAT activity. A modest increase in MyoD associated HAT activity was observed in proliferating C2C12 myoblasts treated with U0126 compared to those treated with DMSO. An approximately two-fold increase in MyoD associated HAT activity was observed in differentiating C2C12 myoblasts treated with U0126 compared to those treated with DMSO and likely accounts for the increased acetylation of the MCK enhancer observed in U0126 treated C2C12 myoblasts. These results suggest U0126 may either inhibit the activity of HDACs associated with MyoD or activate MyoD associated histone acetyltransferases (figure 5c).

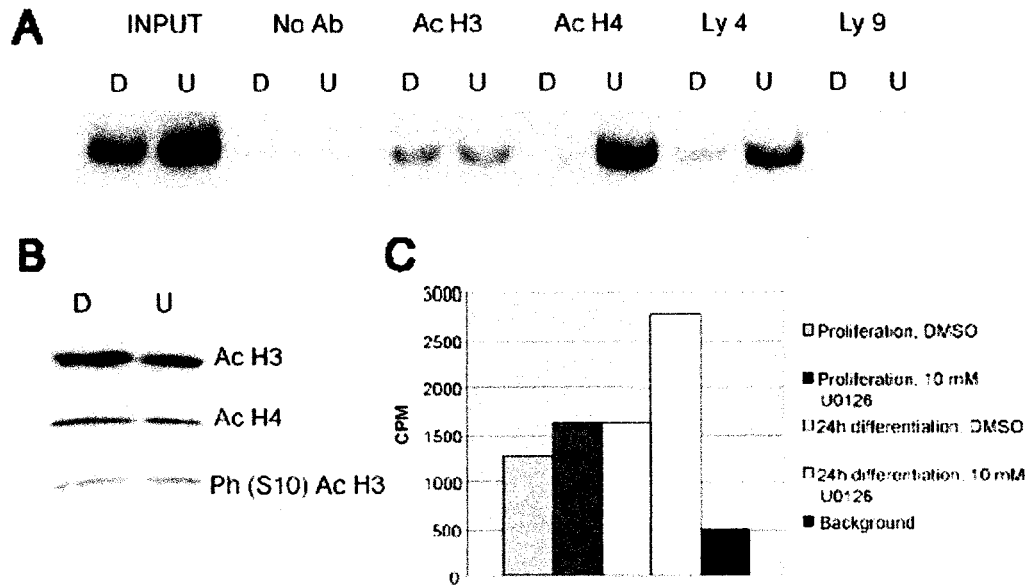


Figure 5. (A) The MCK enhancer is hyperacetylated following treatment with U0126. (B) This effect seems to be specific since no effect on overall levels of histone acetylation as assessed by Western blot was observed. (C) Consistent with these observations MyoD-associated histone acetyltransferase activity increased following U0126 treatment.

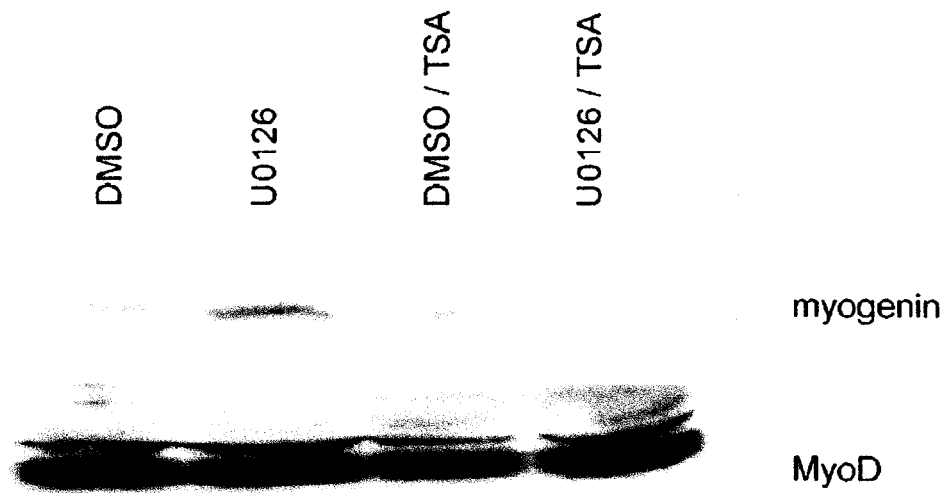


Figure 6. The class I and class II HDAC inhibitor trichostatin A (TSA) abrogates the differentiation inducing effect of U0126 as indicated by reduced myogenin expression. Myogenin and MyoD expression was assessed by Western blot.

Trichostatin A (TSA) abrogates the effects of U0126

TSA inhibits the activity of Class I and Class II HDACs. To investigate the role of HDACs in U0126 induced acceleration of myoblast differentiation, the combined effect of U0126 and TSA on myoblast differentiation was assessed. Simultaneous exposure of C2C12 myoblasts to either U0126 or DMSO and TSA mitigated the positive effect of U0126 on differentiation without effecting the differentiation of DMSO treated C2C12 myoblasts. This result is consistent with the notion that U0126 promotes myoblast differentiation by inhibiting HDAC activity (figure 6).

U0126 promotes cell cycle withdrawal

Treatment of C2C12 myoblasts with U0126 markedly increased the number of terminal differentiated cells suggesting U0126 promotes MyoD mediated cell cycle withdrawal (Perry et al., 2001). To test this directly, the cell profile of U0126 treated C2C12 myoblasts was assessed using phospho-histone staining and compared to that of DMSO treated cells. U0126 treatment increased the proportions of cells in G1 phase of the cell cycle consistent with it promoting MyoD mediated cell cycle withdrawal (figure 7).

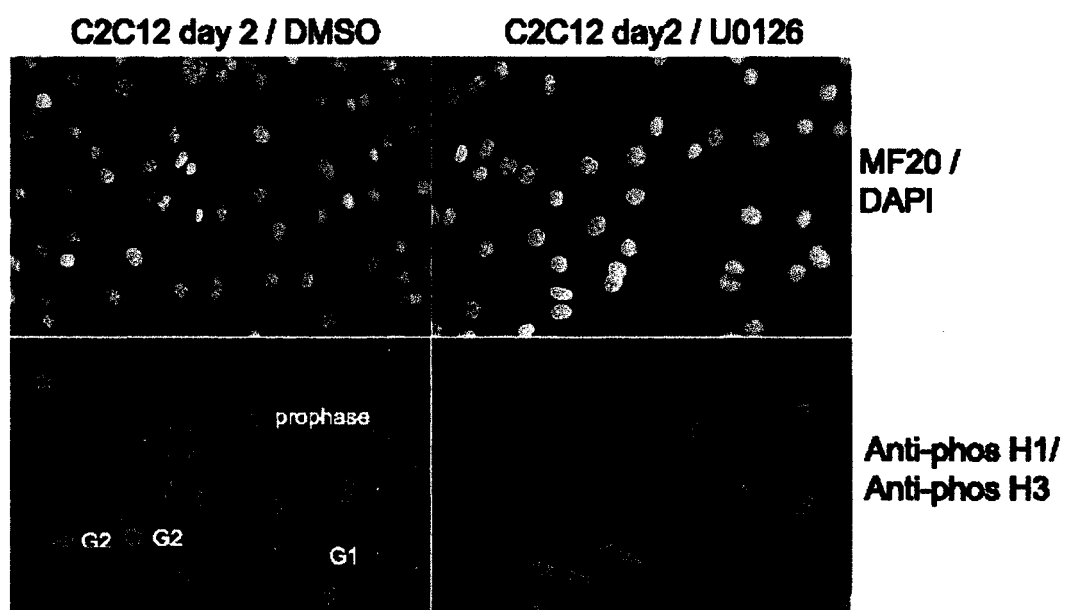


Figure 7. U0126 promotes cell cycle withdrawal. The phases of the cell cycle can be distinguished using antibodies directed against both phosphorylated histone H1 and H3.

Discussion

Activated MEK1 strongly inhibits MyoD dependent activation of muscle specific genes. This inhibition is not dependent on the direct phosphorylation of MyoD by MEK1 nor does it result from a change in MyoD stability or subcellular localization. It does however require the N terminus of MyoD (Perry et al., 2001). Consistent with these observations, inhibition of MEK1 activity by the chemical inhibitor U0126 accelerates myoblast differentiation as indicated by early expression of myogenin and MHC (figure 1 and 2). In this report, we investigate the mechanism by which U0126 promotes myoblast differentiation and from these results infer a mechanism by which activated MEK1 inhibits myoblast differentiation.

The alleviation of condensed chromatin structure surrounding muscle specific promoters is pivotal to the appropriate activation and execution of the myogenic differentiation program (Bailey et al., 1999; Bailey et al., 1998; Gerber et al., 1997; Puri et al., 1997b; Sartorelli et al., 1997; Sartorelli et al., 1999). Our data clearly indicates that MEK1 functions to modulate MyoD-associated histone modifying activity (figure 4, 5, 7). Inhibition of MEK1 increased MyoD-associated HAT activity therefore activated MEK1 likely functions to either repress this activity or increase MyoD-associated HDAC activity.

The activity of the MyoD co-factors CBP/p300 and HDAC1 are regulated by phosphorylation (Cai et al., 2001; Merienne et al., 2001; Pflum et al., 2001; Sang et al., 2003; Schwartz et al., 2003). In particular, recent studies have clearly demonstrated that CBP/p300 histone acetyltransferase activity is both positively and negatively regulated by phosphorylation (See et al., 2001; Yuan and Gambie, 2000). Phosphorylation of serine

89 of p300 by protein kinase C represses its activity (Yuan and Gambée, 2000). It is possible that activated MEK1 by a mechanism similar to that of protein kinase C regulates CBP/p300 histone acetyltransferase activity.

The enzymatic activity of HDAC1 is enhanced by phosphorylation suggesting activated MEK1 may repress MyoD activity by increasing its associated HDAC activity (Pflum et al., 2001). The mitigating effect of TSA on U0126-induced accelerated differentiation is consistent with this hypothesis (figure 8). However, to date HDAC1 has not been identified as a MAP kinase substrate (Cai et al., 2001; Pflum et al., 2001).

The MAP kinase-signalling pathway appears to regulate the ability of MyoD to induce cell cycle arrest in response to mitogen withdrawal (Perry et al., 2001). Consistent with this hypothesis, the number of myoblasts in the G1 phase of the cell cycle is markedly increased following treatment with U0126. This result is not inconsistent with the notion that activated MEK1 represses MyoD activity by increasing its associated HDAC activity. Activated MEK1 in proliferating myoblasts, may promote both the activity of HDAC1 and its association with MyoD. Inhibition of MEK1 activity with U0126 may thus accelerate differentiation by facilitating the disassociation of HDAC1 from MyoD and allowing it to form a complex with hypophosphorylated pRB. This sequentially interaction of HDAC1 with MyoD and pRB is essential for both the activation of differentiation specific genes and the repression of growth specific genes (Puri et al., 2001).

The regulation of chromatin remodeling enzymes by signal transduction cascades provides a link between the cell surface and the nucleosome and facilitates a rapid transcriptional response to changes in the extracellular milieu.

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CHAPTER 5: Synopsis

Chromatin Remodeling and Mammalian Development

Within a multi-cellular organism, the lineage identity of a particular cell is not defined by its genome but rather by its transcriptome or transcript repertoire. Changes to the transcriptome by either extracellular signals or experimental manipulation can either effect lineage choice or stage of differentiation (reviewed in (Fisher, 2002)). The effect of changes to the transcript repertoire on cell identity and differentiation are well illustrated in culture by myoblasts and the myogenic regulatory factors. For example, ectopic expression of MyoD initiates the cascade of molecular events necessary to form skeletal muscle from a variety of non-muscle cells including fibroblasts, chondroblasts, smooth muscle and retinal pigmented epithelial cells (Choi et al., 1990; Lassar et al., 1986).

The transcriptome of a cell is not solely defined by the complement of transcription factors within that cell but also the chromatin structure of its DNA. Chromatin is the biologically relevant target of transcription factors and signaling pathways. Since the structure of chromatin can either positively or negatively effect the transcriptional competence of a particular gene, thus effecting the cell's transcriptome, changes in chromatin structure are central to lineage choice, cellular identity and differentiation (reviewed in (Fisher, 2002)). The requirement of chromatin remodeling in cellular identity and differentiation is evident throughout myogenesis. The reprogramming of non-muscle cells to the myogenic lineage and the differentiation of

specified myoblasts to myotubes requires changes to the cell's chromatin structure (Pandey and Kanungo, 1984a; Pandey and Kanungo, 1984b; Wiid et al., 1988).

MyoD has unique activities during myoblast growth, and differentiation. This is, in part, due to the distinct repertoire of chromatin remodeling factors MyoD recruits to its transcriptional targets under mitogen-rich and differentiation-inducing conditions (figure 1). Under growth conditions, MyoD acts as a transcriptional repressor targeting HDAC1 and co-repressor complexes to muscle specific promoters resulting in the repression of these genes (Bailey et al., 1999; Mal and Harter, 2003; Mal et al., 2001; Puri et al., 2001). The targeting of co-repressor complexes or HDAC1 to these promoters by MyoD requires, in part, the activity of the mouse ISWI homolog Snf2h (Chapter 2). Following the receipt of pro-differentiation cues, MyoD disassociates from HDAC1 and forms a complex with histone acetyltransferases including CBP/p300 allowing for the activation of differentiation-specific genes (Mal and Harter, 2003; Mal et al., 2001; Polesskaya et al., 2000; Polesskaya and Harel-Bellan, 2001; Polesskaya et al., 2001a; Puri et al., 1997a; Puri et al., 2001; Puri et al., 1997b). The pattern of histone acetylation generated by MyoD-associated HATs at differentiation-specific promoters likely provides recruitment signals for other chromatin remodeling proteins including Brg1. Brg1 by remodeling tightly packed nucleosomes around the promoters of MyoD target genes would facilitate the initiation of transcription (de la Serna et al., 2001a; de la Serna et al., 2001b).

Central to cellular growth and differentiation is the translation of changes in the cell's environment into appropriate changes in gene expression. Signal transduction pathways, by directly regulating chromatin remodeling enzymes, connect the cell surface

with chromatin and thus facilitate a rapid transcriptional response to changes in the extracellular milieu. Signal transduction pathways, in particular MAPK pathway, regulate myoblast differentiation by modulating MyoD-associated chromatin remodeling activity (figure 1).

This thesis examines three distinct aspects of chromatin remodeling in relation to myogenesis. In particular, chapter 2 examines the role of the ISWI homology Snf2h in MyoD-mediated repression of target genes during myoblast proliferation. In chapter 3, the genome-wide changes to histone H4 acetylation and histone H3-K9 dimethylation that occur throughout myoblast differentiation are determined. In chapter 4, the role of chromatin remodeling factors in MEK1-dependent inhibition of MyoD activity was investigated. Although the results described in this thesis are specific to myoblast differentiation, lessons that are generally applicable to other cell culture models of differentiation can be gleaned from them.

Overexpression of an ATPase Defective SNF2H in C2C12 Myoblasts Accelerates Differentiation.

Transcription factor activity is often regulated by post-translational modifications or protein-protein interactions including the recruitment of essential co-factors. MyoD activity is regulated by both post-translational modifications and at the level of protein-protein interactions (Li et al., 1992; Liu et al., 1998; Polesskaya et al., 2001a; Polesskaya et al., 2001b; Puri and Sartorelli, 2000). To identify proteins, which bind to and potentially regulate MyoD function during early embryonic myogenesis, we screened a RTA yeast two-hybrid library generated from mRNA isolated from 10.5 day mouse

embryos with GAL4-DNA binding domain / MyoD fusion. From this screen, we identified the mouse ISWI homology Snf2h as a potential MyoD interacting protein.

The specific nature of the interaction was confirmed in yeast and using *in vitro* binding assays. The C-terminus of Snf2h interacts with the MyoD basic domain. The interaction between Snf2h and MyoD appears to be transient in nature since we failed to co-immunoprecipitate MyoD and Snf2h *in vivo*. Furthermore in reporter assays, Snf2h did not affect the ability of MyoD to transactivate four E boxes upstream of a luciferase reporter. From these results we hypothesized that MyoD targets Snf2h to particular promoters and that this does not require a sustained interaction.

To gain insight into the role of Snf2h in myoblast differentiation, the differentiation potential of C2C12 myoblasts ectopically expressing either hSNF2H or an ATPase dead form of hSNF2H was compared to control C2C12 myoblasts. Ectopic expression of either hSNF2H (WT) or hSNF2H (K211R) stimulated the onset of differentiation as indicated by the early expression of differentiation markers including myogenin and myosin heavy chain. These results suggest Snf2h activity is in part required to suppress differentiation specific genes and may be required for the maintenance of the myoblast proliferative state. Although differentiation was accelerated in C2C12 cells ectopically expressing either hSNF2H (WT) or hSNF2H (K211R), differentiation did not proceed normally. These cells exhibited a severe defect in the ability to form multinucleated myotubes suggesting Snf2h activity may be required later in myoblast differentiation. Alternatively, inhibition of Snf2h activity may perturb the

distinct subprograms of MyoD-mediate gene expression in different ways resulting in abnormal differentiation.

Analysis of the chromatin structure around the myogenin promoter during growth in C2C12 [hSNF2H (K211R)] and control C2C12 myoblasts suggest Snf2h activity is required to maintain the repressive chromatin structure. These results are consistent with the notion that MyoD in conjunction with Snf2h target either co-repressor complexes or HDAC1 to differentiation specific promoters. Both co-repressor complexes and HDAC1 are targeted to differentiation specific promoters by a direct interaction with MyoD and have been implicated in the repression of these genes prior to the receipt of appropriate pro-differentiation cues (Bailey et al., 1999; Bailey et al., 1998; Mal and Harter, 2003; Mal et al., 2001; Puri et al., 2001).

A role for SNF2H in transcriptional repression is not unique to myoblasts (Deuring et al., 2000) (Santoro et al., 2002; Strohner et al., 2001; Zhou et al., 2002). Previous studies have clearly indicated that SNF2H is required to repress rRNA transcription (Santoro et al., 2002; Strohner et al., 2001; Zhou et al., 2002). SNF2H by recruiting methyltransferases and the histone deacetylase containing Sin3 co-repressor complex to the promoter of rRNA genes establishes a heterochromatin structure around the promoter (Santoro et al., 2002; Zhou et al., 2002). Interestingly, the Sin3 co-repressor complex through its interaction with HDAC1 and N-CoR has previously been implicated in MyoD mediated repression consistent with the notion that Snf2h is required for MyoD-mediated repression of genes (Bailey et al., 1999; Mal et al., 2001; Puri et al., 2001).

The results presented here suggest a model in which Snf2h activity facilitates the repression of MyoD target genes by HDAC1 or co-repressor complexes. Using the model, we predict in growth the myogenin promoter in C2C12 myoblasts ectopically expressing hSNF2H (K211R) will be hyperacetylated compared to control C2C12 myoblasts. Furthermore, we predict that unlike in proliferating control C2C12 myoblasts, HDAC1 would not be targeted to the myogenin promoter in proliferating C2C12 [hSNF2H (K211R)] myoblasts. Using chromatin immunoprecipitation assays, both the status of histone acetylation at the myogenin promoter and the binding of HDAC1 and co-repressor complexes to MyoD target genes during proliferation can be readily assessed.

Histone Modifying Enzymes and Transcription Factors

Enzymes that covalently modify core histones, including acetyltransferases, deacetylases and methylases as well as ATP dependent chromatin remodeling motors are implicated in a wide range of cellular processes and are critical during embryonic development. In particular, ATP-dependent chromatin remodeling and histone modifications are essential for DNA replication and repair, tissue specific gene expression, genomic imprinting and X-inactivation (reviewed in (Li, 2002)). Central to myoblast differentiation is the coordinated activity of HATs and HDACs. In particular, the transactivating potential of MyoD is in part regulated through a direct association with either HATs or HDACs. The interaction of MyoD with histone modifying enzymes targets histone modifying activity directly to muscle specific promoters (Mal and Harter, 2003; Polesskaya et al., 2001a; Puri et al., 2001; Sartorelli et al., 1997). The ability of MyoD to act as both a transcriptional activator and repressor through its interaction with

distinct histone modifying enzymes is not unique. The tumor suppressor p53 shares a similar capacity to act as a transcriptional repressor and activator by recruiting either Sin3A co-repressor complex or CBP/p300 to target genes (Liu et al., 2003; Murphy et al., 1999).

Myoblasts and Myotubes Have Distinct Patterns of Histone Modifications

During myoblast differentiation the chromatin structure around muscle specific genes is decondensed while the chromatin associated with growth specific genes is condensed (Crowder and Merlie, 1988; Gerber et al., 1997; Pandey and Kanungo, 1984a; Pandey and Kanungo, 1984b; Wiid et al., 1988). The condensation of chromatin is, in part, modulated by covalent modifications to core histone proteins (for review see (Wu, 1997) and (Roth et al., 2001)). Previously, these modifications have been linked to transcriptional status. In general, hyperacetylation of histone H3 and histone H4 are associated with transcriptionally active chromatin, while dimethylation of histone H3-K9 is associated transcriptionally inactive chromatin. This correlation between histone acetylation and transcriptional status has been observed for the myoblast differentiation marker myogenin (Mal and Harter, 2003).

In chapter 3, we describe the ChIP on SNP methodology, which allowed us to extend the analysis of histone modifications that occur during myoblast differentiation genome-wide. Changes to the genome-wide pattern of both histone H4 acetylation and histone H3-K9 dimethylation occur during myoblast differentiation. Although there was a genome-wide reduction in the level of acetylation after differentiation, the percentage of SNPs acetylated above background increased. Change in the net acetylation of the

histones is a direct result of a shift in the equilibrium reached between the opposing activities of HATs and HDACs (for review (McKinsey et al., 2001)). This equilibrium is regulated by signal transduction pathways therefore there is a link between the cell's environment and the nucleus. This link allows the cell to mount a rapid transcriptional response to changes in the extracellular milieu (Bergstrom et al., 2002; Gredinger et al., 1998; Li et al., 2000; McKinsey et al., 2000; Perry et al., 2001). The modulation of chromatin remodeling activity by signal transduction pathways is clearly demonstrated by the effect of U0126 (MEK1 specific inhibitor) on MyoD-associated histone acetyltransferase activity.

Although there was no significant change in the genome-wide level of histone H3-K9 dimethylation, the number of SNPs dimethylated on histone H3-K9 above background doubled following differentiation. Similar to acetylation, the net dimethylation of histone H3-K9 observed in a cell at any given time is a result of an equilibrium reached between the opposing activities of methylation and demethylation. Although numerous histone methyltransferase have been identified, no histone demethylase has yet to be isolated (Bannister et al., 2002; Lachner and Jenuwein, 2002). Nevertheless since the active reversal of histone methylation is a prerequisite for the transcriptional activation of a number of genes, mechanisms must exist within the cell to remove methyl groups from histones (Bannister et al., 2002).

The significant changes to the genome-wide patterns of histone H4 acetylation and histone H3-K9 dimethylation following differentiation suggest distinct mechanisms are targeting histone-modifying enzymes to particular loci in proliferating myoblasts and

myotubes. In addition to the work described here, there is support for this hypothesis in the literature. In proliferating myoblasts, MyoD targets HDAC1 to muscle specific promoters. However following the onset of differentiation, HDAC1 is no longer recruited to these promoters by MyoD, rather it is recruited to growth specific genes through an association with hypophosphorylated pRB (Puri et al., 2001; Zhang et al., 2000). Furthermore, hypophosphorylated pRb in addition to HDAC1 recruits SUV39H1 histone methylase to E2F target genes (Nielsen et al., 2001; Vandel et al., 2001).

In general, we did not observe a correlation between hyperacetylation of histone H4 and hypo-dimethylation of histone H3-K9 and transcriptional activity. Our data argues that transcriptional activity of a gene is alone insufficient to predict the nature of the histone modifications of its associated nucleosomes. The corollary, histone modifications alone are insufficient to predict transcriptional status is also supported by our data. Although a more complete analysis of histone modifications and histone variants associated with transcriptional active genes is necessary, our data in conjunction with data from other groups lends support to the Histone Code Hypothesis in which a combination of histone tail modifications and histone variants acting either together or sequentially dictate the downstream effects (Strahl and Allis, 2000). Using the extensive repertoire of anti-modified histone and histone variant antibodies commercially available in the ChIP on SNP methodology outlined in Chapter 3 detailed maps of histone modifications could readily be generated. Using this technology, a temporal analysis of histone modifications through for example myoblast differentiation could be completed. This would on a genome-wide scale determine if pre-existing histone modifications

dictate subsequent modifications as suggested by the Histone Code Hypothesis (Strahl and Allis, 2000). Furthermore by using antibodies directed against methylated histone H3-K9 and MeCP2 (a DNA methyl binding protein) or methyl-cytosine the link between histone-K9 methylation and DNA methylation could be assessed more fully. Recent reports have suggested that the methylation of histone H3-K9 facilitates the recruitment of DNA methyltransferases (for review (Lachner and Jenuwein, 2002)).

The utility of the ChIP on SNP methodology is not limited to assessing histone modification throughout differentiation. Since perturbations in histone modifications have been implicated in a diverse array of diseases including cancer, this methodology could provide insights into the disease processes (Timmermann et al., 2001). Furthermore, since embryonic tissue can be utilized in a ChIP assay, the ChIP on SNP methodology would facilitate the genome-wide analysis of epigenetic modifications occurring during embryogenesis, in particular the dynamic demethylation and remethylation of the DNA that occurs early in development (Li, 2002). Moreover, since the ChIP on SNP methodology can distinguish between maternal and paternal alleles, allele specific histone modifications such as those occurring at imprinted loci and during X-inactivation can be readily assessed.

Inhibition of MEK1 with U0126 Promotes Differentiation by Increasing MyoD Associated HAT Activity

Activated MEK1 strongly inhibits MyoD dependent activation of muscle specific genes (Perry et al., 2001). Furthermore inhibition of MEK1 activity by the chemical inhibitor U0126 accelerates myoblast differentiation as indicated by the early expression

of myogenin and MHC. Since the repression of MyoD by MEK1 required a domain of MyoD previously implicated in the recruitment of histone modifying enzymes, it appeared likely that activated MEK1 repressed MyoD activity by inhibiting the recruitment of histone acetyltransferases or promoting the association between HDAC1 and MyoD (Perry et al., 2001; Sartorelli et al., 1999). Consistent with this the inhibition of MEK1 increased MyoD-associated histone acetyltransferase activity. Furthermore the inhibition of MEK1 increased the acetylation of histone H4 at muscle specific E boxes.

The results presented here suggest a model in which activated MEK1 represses MyoD activity by either promoting the recruitment HDAC1 or co-repressor complexes by MyoD or alternatively inhibiting the recruitment of HATs. Using both chromatin immunoprecipitation assays and co-immunoprecipitations, the MyoD dependent targeting of HATs and HDAC1 or co-repressor complexes to MyoD target genes during growth and differentiation in the presence of activated MEK1 can be readily assessed.

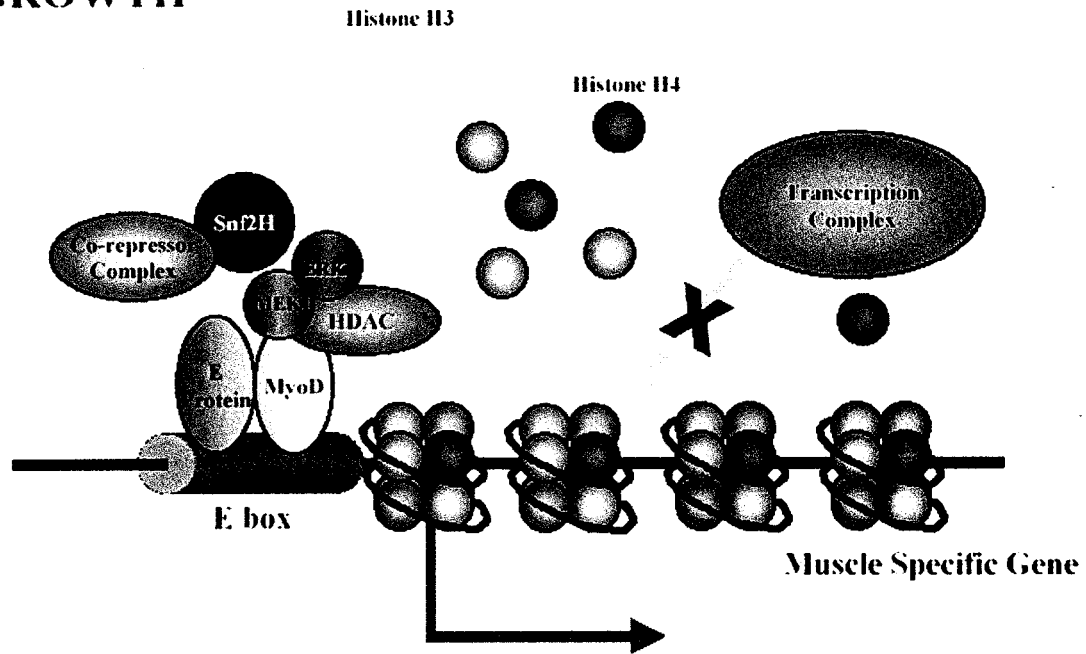
Figure 1. A Chromatin Based Model of Myoblast Differentiation. In proliferating myoblasts, MyoD, through a direct interaction with HDAC1, N-CoR and SNF2H, represses the transcription of differentiation specific genes such as myogenin and myosin heavy chain. SNF2H may act by facilitating the binding of either co-repressor complexes or HDACs.

Myoblast differentiation is regulated by extracellular signals. Mitogen-rich environments inhibit the differentiation process. Through an association with signal transduction pathways, MyoD rapidly responds to changes in the extracellular milieu. Our data suggests that in a mitogen-rich environment, MEK1 through a direct interaction with MyoD stimulates MyoD associated HDAC activity either by directly phosphorylating HDAC or phosphorylating HDAC via a downstream kinase.

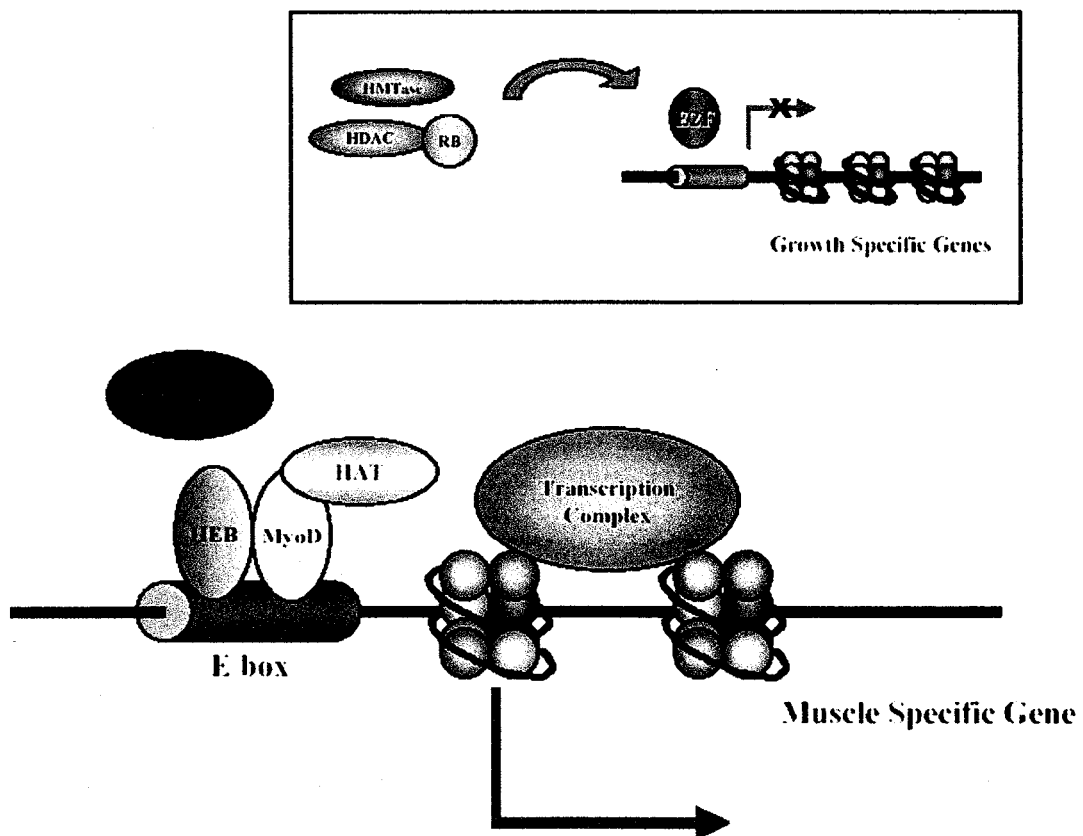
After the receipt of pro-differentiation cues, SNF2H, HDACs and co-repressor complexes disassociate from MyoD. Subsequently histone acetyltransferases associate with MyoD / HEB heterodimers bound to E boxes within the promoters of differentiation-specific genes and acetylate the histones locally. The acetylation of histones, in particular histone H4-K8 provides a recruitment signal for Brg1. Brg1 by remodeling tightly packed nucleosomes promotes MyoD-dependent transactivation of differentiation specific genes.

Growth specific genes are repressed during differentiation by the activities of HDACs and HMTases. These chromatin remodeling enzymes are recruited to E2F target genes after serum withdrawal via a direct association with hypophosphorylated pRB.

GROWTH



DIFFERENTIATION



Concluding Remarks

All three projects outlined in this thesis illustrate the integral role chromatin remodeling plays during myoblast differentiation. This remodeling requires the concerted action of both ATPase dependent chromatin remodeling motors and histone modifying enzymes. SNF2 motors are likely required to initiate the remodeling process by either winding or unwinding the DNA or moving histone octamers, while histone modifying enzymes may act to maintain the modified state be either acetylating, deacetylating and methylating histones. From my work and the work of others, it appears that both SNF2 motors and histone modifying enzymes utilize transcription factors as a mechanism for targeting their activity to particular promoters. The proposed mechanism by which MEK1 modulates MyoD dependent transcription highlights the link between cell signaling and chromatin remodeling, suggesting the cellular environment via the activity of signal transduction pathways directly modulate the activity of chromatin remodeling enzymes.

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