EPIGENETIC REGULATION
OF
SKELETAL MYOGENESIS

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Narendra Bharathy

10 January 2013

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"God hath bestowed us, flora and fauna, with the genetic codes such that we all live in harmony as we share most of them amongst us.
The more and more we, the highest order, delve into them the better and better we get to know Him and His creations. "- Gobal Pillai

"The significant problems we face cannot be solved at the same level of thinking we were at when we created them."- Albert Einstein

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Summary

Skeletal myogenesis is a tightly regulated process involving a series of steps which begins with the initial commitment of multi-potent mesodermal progenitors towards the myogenic lineage. Once committed, they proliferate as undifferentiated mononucleated myoblasts followed by irreversible withdrawal from cell cycle. Concomitantly, they differentiate and fuse into multinucleated myotubes. The terminal step of differentiation culminates with the maturation of differentiated myotubes into muscle fibers. This process of skeletal muscle development and differentiation is orchestrated by the combinatorial activity of two transcription factors families that include Myogenic Regulatory Factors (MRFs) and the Myocyte Enhancer Factor-2 (MEF2).

The expression and activities of MRFs and MEF2 are tightly controlled by both positive and negative regulatory factors. In addition there are several chromatin modifiers and remodelling complexes that regulate MRF and MEF2 activities and thereby regulating skeletal muscle differentiation. I have analyzed the role of one such chromatin modifier G9a, which mediates H3 lysine-9 di-methylation (H3K9me2) chromatin marks that are associated with transcriptional repression. G9a is expressed at high levels in undifferentiated myoblasts and is down-regulated upon differentiation. Over-expression of G9a in myoblasts retards skeletal muscle differentiation in a methyltransferase activity-dependent manner. RNAi-mediated knockdown of G9a in muscle precursor cells results in enhanced

differentiation. This is concomitant with early induction of differentiation genes myogenin and Troponin T. G9a-dependent impairment of differentiation is correlated with H3K9me2 marks on muscle-specific promoters. In addition, G9a interacts with and methylates MyoD, a MRF that is central to differentiation of skeletal muscle cells. G9a methylates MyoD at a single lysine (K) residue 104, suppressing its transcriptional activity. This modification blocks the ability of MyoD to activate its downstream target genes such as myogenin, resulting in impairment of differentiation. Interestingly, K104 in MyoD is also a site for acetylation by a histone acetyltransferase P/CAF. Consistent with this, G9a disrupts P/CAF-mediated MyoD acetylation which is critical for its transactivation function and for execution of the myogenic differentiation program. RNAi mediated reduction of G9a results in altered kinetics of MyoD acetylation, suggesting a possibility of cross-talk between G9a and P/CAF in controlling MyoD transcriptional activity and myogenesis. In addition, pharmacological inhibition of P/CAF activity enabled us to identify gene networks broadening the spectrum of its regulatory function in skeletal muscle differentiation. These findings provide insights into the mechanisms by which chromatin modifiers regulate MyoD activity via direct post-translational modifications to control skeletal muscle differentiation.

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List of Symbols and Abbreviations

Acetyl-CoA	Acetyl-CoenzymeA
ADAM12	A Disintegrin and Metalloproteinase Domain containing protein 12
Ala	Alanine
ANK	Ankyrin domain
ARMS	Alveolar Rhabdomyosarcoma
ATP	Adenosine Triphosphate
BAF	Brg1 and Brm-Associated Factor
BHLH	Basic Helix Loop Helix
BMP	Bone Morphogenic Proteins
BIX-01294	G9a\GLP methyltransferase inhibitor
BSA	Bovine Serum Albumin
C2C12	Mouse myoblast cell line
C3H 10T1/2	Mouse embryonic mesenchymal cell line
CARM1	Coactivator arginine methyltransferase1
CDKs	Cyclin Dependent Kinases
cDNA	Complementary DNA
CDP/cut	CCAAT displacement protein/ cut homolog
CDYL1	Chromodomain Y-like protein
CeMEF2	Caenorhabditis Myocyte Enhancer Factor 2
ChIP	Chromatin immunoprecipitation
CtBP	C-terminal binding proteins
DAPI	4', 6-diamidino-2-phenylindole
DIM-5	Defective in methylation-5
DM	Differentiation Medium
DMEM	Dulbecco's Modified Eagle Medium
DML	Dorsal-Medial Lip

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNMTs	DNA methyltransferases
E days	Embryonic days
E12/E47/HEB	E-proteins
E2F	Elongation Factor 2
EED	Embryonic Ectoderm Development
EGFP	Enhanced Green Fluorescent Protein
Embelin	P/CAF acetyltransferase inhibitor
ERMS	Embryonal rhabdomyosarcoma
ES	Embryonic stem cells
Ezh2	Enhancer of Zeste homolog 2
FBS	Fetal Bovine Serum
G1/S	G1 phase and the S phase
EHMT2	Euchromatic histone-lysine N-methyltransferase2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gfi 1	Growth factor independent 1 protein
GLP	G9a-like protein,
GM	Growth Medium
GRIP 1	Glucocarticoid Receptor Interacting Protein 1
H3K17	Histone 3 lysine 17
H3K27	Histone 3 lysine 27
H3K36	Histone 3 lysine 36
H3K4	Histone 3 lysine 4
H3K79	Histone 3 lysine 79
H3K9	Histone 3 lysine 9
H3R17	Histone 3 arginine 17
H3R8	Histone 3 arginine 8
H4K20	Histone 4 lysine 20
HATs	Histone acetyltransferases
HDACs	Histone deacetylases

HEK293T	Human Embryonic Kidney 293 cell line
HKMTs	Histone Lysine Methyltransferases
Id	Inhibitor of DNA binding/differentiation
IF	Immunofluorescence
IP	Immunoprecipitation
JMJD2A	JmjC domain containing demethylase
K	Lysine
LB	Luria-Bertani broth
LIF	Leukemia Inhibitory Factor
LP	Lateral Plate
LSD1	Lysine-Specific Demethylase 1
LSH	Lymphoid-Specific Helicase
MADS box	MCM, AGAMOUS, DEFECIENS, SRF
MCK	Muscle Creatine Kinase
MEF2	Myocyte Enhancer Factor-2
MHC	Myosin Heavy Chain
MRF4	Myogenic Regulatory Factor 4
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein
MyoR	Myogenic repressor
NaCl	Sodium chloride
NAD	Nicotinamide Adenine Dinucleotide
NAM	Nicotinamide
NC	Notochord
NT	Neural Tube
P/CAF	p300/CBP-associated factor
CDK1	Cyclin-dependent kinase inhibitor 1
Pax 3, 7	Paired box transcription factors 3, 7
PBS	Phosphate Buffered Saline
PcG	Polycomb group of family
PCR	Polymerase Chain Reaction

PKA	Protein Kinase A
PKC	Protein Kinase C
pRb	Retinoblastoma protein
PRC2	Polycomb Repressor Complex 2
PRMTs	Protein arginine N-methyltransferases
Q-PCR	Quantitative real-time polymerase chain reaction
R	Arginine
RA	Retinoic Acid
RCF	Relative Centrifugal Force
Rh30	Alveolar rhabdomyosarcoma cell line
RIPA	Radioimmunoprecipitation assay
RIZ	Retinoblastoma Interacting Zinc finger protein
RMS	Rhabdomyosarcoma
RPMI -1640	Roswell Park Memorial Institute-1640 medium
SAM	S-adenosyl-L-methionine
SC	Sclerotome
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SET	SU (VAR) 3-9 Enhancer of Zeste [E(Z)] Trithorax (TRX)
SETDB1	SET Domain, Bifurcated 1
SETDB2	SET Domain, Bifurcated 2
Sharp-1	Enhancer-of-split and hairy-related protein 1
Shh	Sonic hedgehog
Sir2	Silent information regulator 2
SirT1	Silent mating type information regulation 2 homolog 1
SMYD	SET and MYND domain containing proteins
SRF	Serum Responsive Factor
Suv39h1	Suppressor of variegation 3-9 homolog 1
SUZ12	Suppressor of Zeste 12
SWI/SNF	Switching (SWI) and/or Sucrose fermentation (SNF: Sucrose Non-Fermenter)
TAD	Transactivation Domain

Thr	Threonine
Tris-HCL	TRIS hydrochloride
UNC0638	G9a\GLP methyltransferase inhibitor
VLL	Ventral-Lateral Lip
WIZ	Widely Interspaced Zinc finger motifs protein
YY1	Ying and Yang 1

CHAPTER 1

INTRODUCTION

1. Introduction

Skeletal muscle is a complex and highly organized tissue which is involved in vital functions such as movement, maintaining posture, stabilizing joints and generating heat. It represents a large fraction of body mass accounting for approximately 40-45% (Lieber, 1992; Janssen *et al*., 2000). During development, skeletal muscles are established in an elaborately organized manner by the process referred to as 'myogenesis' which involves an array of distinct steps (Tajbakhsh, 2009). Any defect in skeletal myogenesis causes great disability in affected people as it might result in dystrophy, regeneration defects and other muscular myopathies (Amack and Mahadevan, 2004). Therefore, it is essential to understand the molecular mechanism behind skeletal muscle development which can form the basis of disease treatment.

1.1 Skeletal muscle development during embryogenesis

During embryogenesis, the majority of skeletal muscles of trunk and limbs originate from cells of the paraxial mesoderms which lie on either side of the neural tube (Wachtler and Christ, 1992). As development proceeds, the paraxial mesoderm condenses into segmental epithelial spheres called somites (Buckingham, 2001; Tajbakhsh and Cossu 1997; Summerbell and Rigby, 2000). These somites under the influence of a variety of signals arising from notochord and neural tube as well as from the overlying ectoderm and lateral mesoderm become compartmentalized into mesenchymal sclerotome and epithelial dermomyotome (**Figure 1.1a**). While the sclerotome gives rise to ribs and axial skeleton, the cells of the dermomyotome delaminate ventrally to

form the myotome (sheet of differentiating skeletal muscle cells) which gives rise to skeletal muscles of the trunk and limbs and the remaining structure is dermotome, which develops into skin (Braun *et al*., 1992; Lassar and Munsterberg, 1996; Christ and Ordahl, 1995; Ordahl and Le Douarin, 1992). The formation of skeletal muscle from the multipotent precursor cells involves a multi-step process that begins with the commitment of these cells to the muscle fate. The committed cells proliferate to give rise to large pools of myoblasts that migrate to their proper sites (Pourquie *et al*., 1995) and continue to divide. When appropriate environmental cues are received, proliferating myoblasts exit the cell cycle, differentiate and fuse into multi-nucleated myotubes (**Figure 1.3a**). Then the bundle of parallel myotubes forms a mature myofiber, the basic contractile unit of skeletal muscle (Lassar *et al*., 1994).

Studies have shown that myogenic regulatory factors (MRFs) namely Myf5 and MyoD establish two distinct populations of skeletal muscle precursor cells in the myotome that give rise to epaxial and hypaxial muscles respectively (Braun and Arnold, 1996). The muscle precursor cells from the dorsal-medial lip (DML) of the somite contribute to epaxial muscles of the back and body wall whereas muscle precursors from the ventral-lateral lip (VLL) of the somite migrate and form hypaxial muscles of the limbs, ventral body wall, diaphragm and tongue (**Figure 1.1a**) (Ordahl and Douarin, 1992; Christ and Ordahl, 1995).

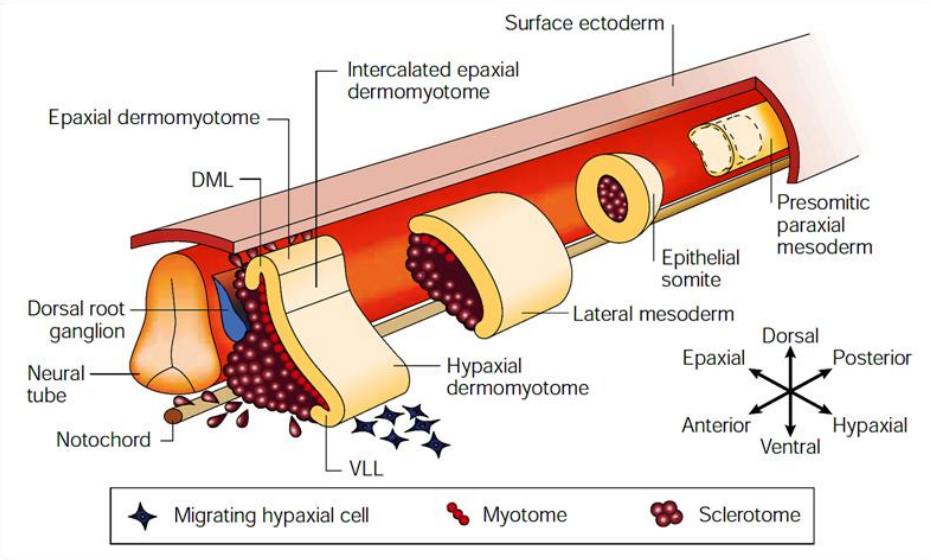


Figure 1.1a Schematic representation of the embryonic origin of limb and trunk skeletal muscle (Nat Rev Genet. 2003; 4:497-507). The paraxial mesoderm condenses into segmental epithelial spheres called somites following rostrocaudal gradient on either side of the axial structures. These somites under the influence of a variety of signals arising from notochord and neural tube as well as from the overlying ectoderm and lateral mesoderm become compartmentalized into mesenchymal sclerotome and epithelial dermomyotome.The myotome forms as a result of migration of cells from the dorsal-medial lips (DML) and ventral-lateral lips (VLL) of the dermomyotome. Cells from the dorsal-medial lip (DML) of the somite contribute to epaxial muscles of the back and body wall whereas muscle precursors from the ventral-lateral lip (VLL) of the somite migrate and form hypaxial muscles of the limbs, ventral body wall, diaphragm and tongue.

In addition, a sub-population of cells remains undifferentiated and mononuclear termed satellite cells located between the basal lamina and sarcolemma of myofibers (**Figure 1.1b**) (Mauro, 1961; Katz, 1961). It accounts for the remarkable property of adult skeletal muscles to adapt to physiological conditions such as postnatal muscle growth, hypertrophy and regeneration.

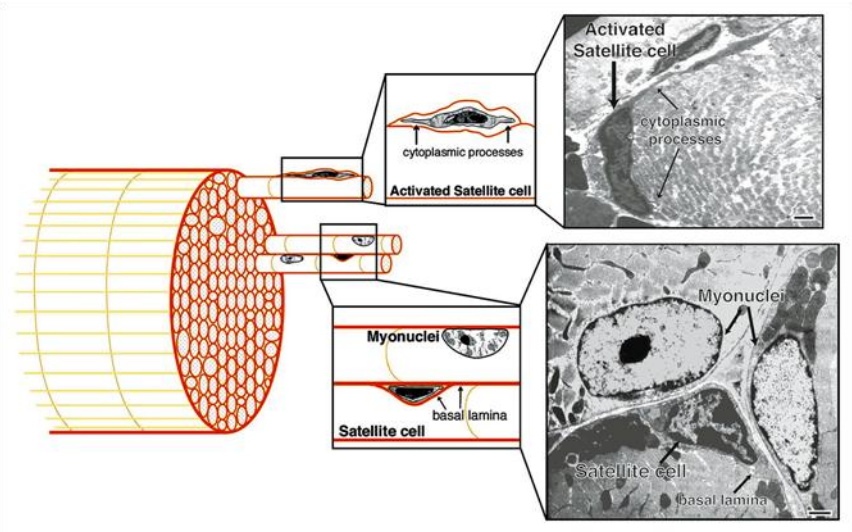


Figure 1.1b Satellite cells occupy sublaminar position in adult skeletal muscle (J Appl Physiol. 2001; 91(2):534-51). Satellite cells located between the basal lamina and sarcolemma of myofibers captured using ultrastructural techniques.They have distinctive morphological features such as high nuclear-to-cytoplasmic ratio with few organelle, a smaller nuclear size compared with the adjacent nuclei of myotube. In addition, they have more abundant heterochromatin compared to that of myonucleus.

1.2 Signaling molecules in the regulation of skeletal myogenesis

The myogenic specification and differentiation of the myotome is influenced by the complex signalling system provided by microenvironmental factors such as Sonic hedgehog (Shh), Wnts, BMP4 and noggin (**Figure 1.2**) (Tajbakhsh and Cossu, 1997). Sonic hedgehog, a signalling molecule expressed in the dorsal ventral neural tube and notochord, acts as one of the major players in skeletal muscle formation. It induces the expression of paired homeobox domain transcription factors Pax3 and Pax7 in presegmental plate

mesoderm and dermomyotome (Fan and Tessier-Lavigne, 1994, Fan *et al*., 1995, Johnson *et al*., 1994). These transcription factors induce the expression of their downstream target Myf5 and consequently MyoD, committing cells of the myotome to the myogenic lineage (**Figure 1.2**) (Munsterberg *et al*., 1995; Maroto *et al*., 1997). Gene disruption studies revealed that Pax3 and Pax7 have distinct functions with Pax3 being essential for the development of limb muscles (Bober *et al*., 1994), whereas Pax7 is important for satellite cell survival and maintenance (Relaix *et al*., 2006). Consistent with this, in Pax3 null mice (Splotch mice), dermomyotome is disorganized and the limb myogenic progenitors fail to migrate from somites into the growing limbs and the developing tongue (Goulding *et al*., 1994; Tremblay *et al*., 1998; Epstein *et al*., 1996) while loss of Pax7 results in the death of satellite cells (Relaix *et al*., 2006).

Studies have shown that loss of Shh in the neural tube and notochord leads to the disruption of epaxial muscles, while hypaxial muscles develop normally (Borycki *et al*., 1999). However, further studies showed that Shh is required even for the maintenance and expression of MRFs in hypaxial muscles and formation of limb muscle differentiated myotubes (Duprez *et al*., 1998; Kruger *et al*., 2001).

In addition to Shh, Wnt (wingless and integrated) family of growth factors, expressed in the dorsal-neural tube and members of BMPs (bone morphogenic proteins) and Noggin, an antagonist for BMP secreted from the dorsomedial

lip of dermomyotome play crucial roles in somite pattterning (Marcelle *et al*., 1997). While Wnt family members work in cooperation with Shh to induce myogenesis in the dorsal part of isolated somites *in vitro* (Munsteberg and Lassar, 1995), BMPs and Noggin are known to regulate Pax3 expression (**Figure 1.2**) in proliferating muscle precursor cells wherein they either promote embryonic muscle growth by increasing Pax3 expressing muscle precursor cells or restricts growth by inducing apoptosis (Amthor *et al*., 1998).

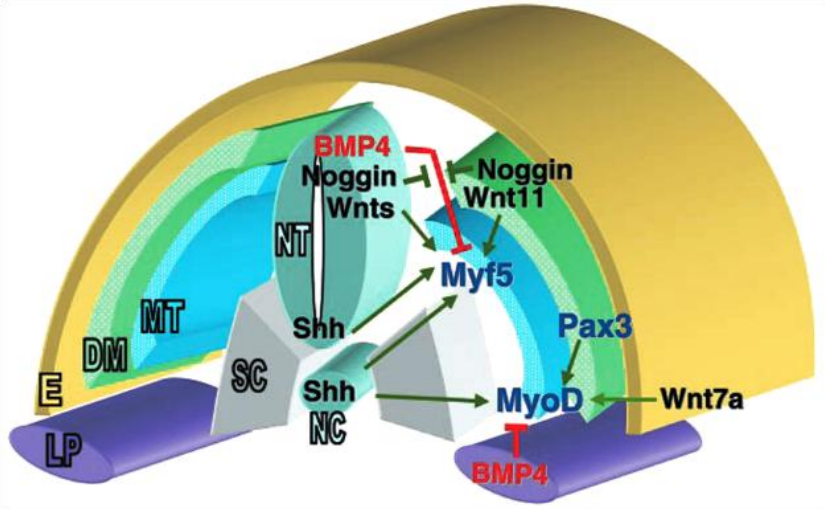


Figure 1.2 Signaling factors involved in embryonic skeletal muscle formation (Physiol Rev. 2004; 84:209-38). Mesodermal somatic cells from the dermomyotome (DM) receive signals from the surrounding tissues provided by signalling factors like Shh, Wnts, Noggin and BMP4 that induce or inhibit the expression of MRFs (Myf5 and MyoD) and commitment to the myogenic lineage. The committed myoblasts migrate laterally to form the myotome (MT), which eventually gives rise to skeletal muscles of the trunk and limbs. Pax3 promotes myogenesis in the lateral myotome. E, ectoderm; LP, lateral plate; SC, sclerotome; NC, notochord; NT, neural tube.

1.3 Myogenic regulatory factors (MRFs) and skeletal muscle development

In 1979, Taylor and Jones found that treating the mouse fibroblast cell line 10T1/2 with the demethylating agent 5-azacytidine resulted in generation of clones with skeletal muscle phenotypes. This suggested that DNA demethylation was sufficient to induce muscle gene expression in non-muscle cells. This was followed by another landmark study in which a single myoblast-specific cDNA was isolated and stably transfected into 10T1/2 cells which resulted in the conversion of fibroblast-like 10T1/2 cells to skeletal muscle cells (Lassar *et al*., 1986; Davis *et al*, 1987). The gene encoding this sequence was named as "MyoD1" (myoblast determination gene number 1). It is expressed exclusively in myogenic lineage *in vivo* and only in myogenic cell lines *in vitro.*

The identification of MyoD as an important regulatory gene of skeletal myogenesis paved the way for the identification of the roles of other tissue specific transcription factors in regulating cell differentiation (Tapscott, 2005). The discovery of MyoD (Davis *et al*., 1987) was soon followed by the discovery of myogenin (Edmonson and Olson 1989; Wright *et al*., 1989), Myf5 (Braun *et al*., 1989) and muscle-specific regulatory factor 4 (MRF4) (Rhodes and Konieczny, 1989; Miner and Wold 1990; Braun *et al*., 1990). Each of the four homologous muscle specific transcription factors is capable of converting cultured fibroblasts to a muscle phenotype (Braun *et al*., 1990; Wright *et al*., 1989) and broadly classified under the category 'MRF' (Myogenic regulatory factors). Together they play a central role in the

establishment of skeletal muscle lineages (Stockdale and Holtzer, 1961; Cooper and Konigsberg, 1961).

Analysis of MRF expression in cell lines have shown that MyoD and Myf5 are expressed first in proliferating undifferentiated muscle cells followed by myogenin whose expression is induced when cells begin to terminally differentiate (Smith *et al*., 1993; Andres and Walsh, 1996) followed by MRF4 expression (**Figure 1.3a**) during muscle development as well as in adult muscle tissue (Hinterberger *et al*., 1991).

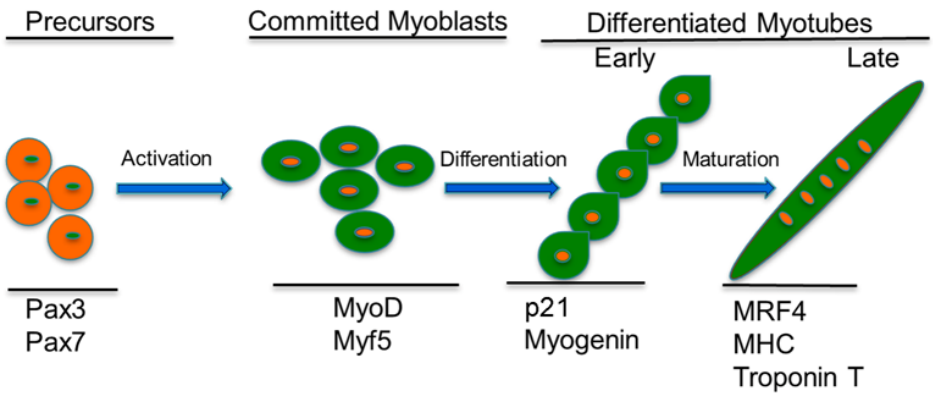


Figure 1.3a Schematic representation of skeletal muscle differentiation (Adapted from Subcell Biochem. 2012; 61:139-50). Pax3 and Pax7 positive precursor cells induce the expression of their downstream target Myf5 and consequently MyoD, committing cells to the myogenic lineage. The committed cells proliferate to give rise to large pools of myoblasts. When appropriate environmental cues are received, proliferating myoblasts irreversibly exit the cell cycle expressing p21 and early differentiation marker myogenin followed by terminal differentiation to form multinucleated myotubes characterized by the expression of terminal differentiation markers myosin heavy chain (MHC) and TroponinT.

MyoD and other MRFs contain basic (b) and helix-loop-helix (HLH) domain which are the two important domains. In addition, they have one or more transactivation domains (TADs) (**Figure 1.3b**). MRFs share significant homology throughout the highly conserved bHLH domain which accounts for their tissue-specific actions (Murre *et al*., 1989a; Dias *et al*., 1994). The basic domain mediates DNA binding at sequence-specific DNA elements known as E-boxes in order to activate transcription. These E-boxes have a consensus nucleotide sequence of 5'-CANNTG-3' that is present in the promoters of many muscle specific genes (Davis *et al*., 1990, Braun *et al*., 1990, Lassar *et al*., 1989). The HLH domain mediates heterodimerization with another family of non-myogenic bHLH proteins known as E-proteins (HEB/HTF4, E2-2/ITF-2 and E/12/E47) which increase affinity of MRFs for muscle specific genes (Murre *et al*., 1989b; Brennan and Olson, 1990; Lassar *et al*., 1991; Shirakata *et* al., 1993). TADs are the elements in the amino (NH2) and carboxy (COOH) terminal regions (**Figure 1.3b**) which account for the distinct activities of MRFs as the amino acid sequences of these regions are very different (Schwartz *et al*., 1992; Asakura *et al*., 1993).

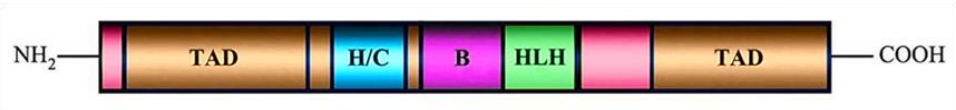


Figure 1.3b Diagrammatic representation of domain structure of MRFs (Adapted from Transcription. 2012; 3:1-6). The basic (B) domain; helix-loop-helix (HLH) motifs; NH2- and COOH-terminal transactivation domains (TADs); and the cysteine-histidine (H/C) rich region are shown.

Although each of the MRFs is capable of driving the entire myogenic differentiation program when expressed in non-muscle cells (Munsterberg *et al*., 1995), the distinct expression pattern and gene disruption studies suggested that all of them have different functions *in vivo*. Myf5 mRNA transcripts are the first to be expressed in mouse embryo at embryonic day 8 (E8.0) (Ott *et al*., 1991). Myogenin is transcribed 12 hours after Myf5 (E8.5), followed by MRF4 at E9.0 which exhibits a transient expression pattern. MyoD is expressed last at E10.5 and its expression persists throughout prenatal life of the mouse (Sassoon *et al*., 1989; Hinterberger *et al*., 1991).

Gene ablation experiments revealed the function of each MRF in myogenesis. Myf5 and MyoD have functional redundancy between them as mice deficient either in Myf5 or MyoD did not impair skeletal muscle formation (Rudnicki *et al*., 1992) while double mutants Myf5/MyoD fails to develop detectable amount of skeletal muscle cells as well as muscle fibers. Thus it was concluded from this observation that expression of at least one of them is essential for the commitment of cells to myogenic lineage (Rudnicki *et al*., 1993). Targeted inactivation of myogenin results in critical loss of skeletal muscles that tend to accumulate undifferentiated myoblasts, which results in prenatal death. Thus myogenin is crucial for the terminal differentiation of committed myoblasts by activation of muscle specific genes, such as muscle creatine kinase (MCK) and myosin heavy chain (MHC) (Hasty *et al*., 1993; Nabeshima *et al*., 1993). Interestingly, mice lacking either MRF4 or MyoD were viable and had no defect in muscle development. However,

MRF4/MyoD double mutants resulted in a critical muscle deficiency similar to that observed in myogenin mutants, which clearly revealed an unanticipated overlapping function for MyoD and MRF4 in the muscle differentiation pathway (Rawls *et al*., 1998).

Skeletal myogenesis proceeds in a strict temporal sequence from early to late gene expression (Andres and Walsh, 1996; Bergstrom *et al*., 2002). In committed moyblasts, MyoD or Myf5 expression is induced that promotes irreversible cell cycle exit by upregulation of CDK inhibitor p21 (Halevy *et al*., 1995) as well as transactivation of early differentiation marker myogenin. This results in terminal differentiation characterized by the expression of myosin heavy chain and Troponin T (**Figure 1.3a**) (Sabourin and Rudnicki, 2000; Tapscptt, 2005; Sartorelli and Caretti, 2005).

1.4 Myocyte Enhancer Factor 2 (MEF2)

In addition to MRFs, MEF2 family members play an important role in skeletal myogenesis (Olson *et al*., 1995). MEF2 family are nuclear phosphoproteins that were originally purified from muscle cells as myocyte-specific binding factor. It is also referred to as RSRF (Related to Serum Response Factor) and expressed in all developing muscle cell types (Gossett *et al*., 1989).

MEF-2 proteins can physically interact with MRF-E protein heterodimers. Like MRFs, they bind a conserved DNA sequence in a majority of muscle specific genes. These Mef2-dependent genes encode a large number of proteins which include various muscle specific enzymes and structural

proteins, as well as other transcription factors. MRF and Mef2 have been shown to amplify and regulate each other's expression (Cjerjesi and Olson 1991; Lassar *et al*., 1991). However, unlike MRF, MEF2 genes alone cannot activate transcription of muscle genes in vertebrates as they do not possess myogenic activity on their own. Instead, they co-operated with MRFs to activate muscle gene expression and increased the efficiency of conversion of non-muscle cells (Molkentin *et al*., 1995). There are several studies which suggest that MEF2 functions as an important myogenic co-regulator which is required by MRFs to initiate myogenesis. It has been shown by *in vivo* studies that MEF2 is essential for the proper regulation of myogenin gene expression (Cheng *et al*., 1993; Yee and Rigby 1993). This reflects the importance of MEF2 for the myogenic activity of MRFs and further evidence on this aspect came from the observation that in the presence of a dominant negative form of MEF2A that binds DNA but lacks a transcriptional activation domain, MyoD is incapable of initiating myogenesis in 10T1/2 fibroblasts (Ornatsky *et al*., 1997).

1.5 Negative regulators of myogenesis

Since MyoD and Mef2 are expressed in proliferating myoblasts, there is a need to tightly regulate their expression and activity, as lack of control can result in premature muscle differentiation. In addition to positive regulators of myogenesis such as MRFs and Mef2, muscle development is controlled by many negative regulators. It includes the inhibitor of differentiation (Id) family of proteins which contain a helix-loop-helix motif but lack a DNA

binding domain. In proliferating myoblasts, Id is expressed at high levels. Thus it forms a complex with E12/E47. This sequesters E12/E47 from forming a functionally active heterodimer with MyoD resulting in inhibition of terminal differentiation program. However, upon differentiation, Id level declines paving the way for E12 and/or E47 to form functional heterodimers with MyoD. Thus over-expression of Id in myoblasts results in the inhibition of MyoD dependent activation of MCK enhancer (Benezra *et al*., 1990).

In addition to Id, proliferating myoblasts express many other negative regulators of differentiation; they include Twist, Mist 1, MyoR and Sharp-1 which inhibit MyoD transcriptional activity, DNA binding and dimerization ability with E-proteins (Spicer *et al*., 1996; Lu *et al*., 1999; Lemercier *et al*., 1998; Azmi *et al*., 2004). Like Id, ectopic expression or activation of these negative regulators inhibits differentiation. Interestingly, some of them are regulators of cell cycle progression as well; one such regulator is c-myc which belongs to the myc family of proto-oncogenes that share structural similarity with the MyoD family of regulators. C-Myc has been strongly implicated in the control of cell proliferation in various cell lineages that include muscles (Kelly *et al*., 1983; Sejerson *et al*., 1985). It is expressed in proliferating myoblasts and declines substantially when these cells exit cell cycle and start differentiating (Endo and Nadal-Ginard, 1986). It has been shown that over-expression of c-myc affects MyoD dependent myoblast differentiation (Miner and Wold, 1991). Similarly, there are negative effects exerted by protein kinase A (PKA) (Li *et al*., 1992a), protein kinase C (PKC) (Li *et al*., 1992 b)

and elevated c-Jun activities (Bengal *et al*., 1992).

1.6 Epigenetic regulators of myogenesis

In addition to positive and negative regulatory factors, that tightly control the expression and activities of MRFs and MEF2, there are increasing evidence which point to the involvement of chromatin modifying enzymes and remodeling complexes that regulate MRF and MEF2 activities and thereby skeletal muscle differentiation. Epigenetic modifications play a central role by which muscle lineage-specific information encoded in chromatin merges with muscle regulatory factors to drive myoblasts through different transitional phases in myogenesis (Perdiguero *et al*., 2009).

1.7 Chromatin Structure

The packaging of eukaryotic DNA into an intricate and high-order structure known as chromatin is essential to fit it into the nucleus. This plays a vital role in the control of gene transcription. The basic structural unit of DNA packaging in eukaryotic organism is termed as "nucleosome" (Kornberg, 1974). It consists of 147 base pairs (bp) of DNA which are super-helically wrapped around each histone octomer which is comprised of a central tetramer of histones H3 and H4 and two dimers of histones H2A and H2B (Luger *et al*., 1997). This highly ordered structure is often compared to thread wrapped around a spool as visualization by electron microscopy revealed them as a series of nucleosomal beads on a DNA string (**Figure 1.7a**). "Histones" are the basic scaffolding proteins which are responsible for the compaction of the DNA. In the absence of histones, the unwound DNA will be very long. For

example, each eukaryotic cell has about 1.8 meters of linear DNA which when wrapped 1.7 times around an octomer of core histones is shrunken to 40,000 times to about 90µm, which when duplicated and condensed during mitosis, results in about 120µm of chromosomes (Redon *et al*., 2002). The DNA that extends between nucleosomal core particles is called linker DNA which is approximately 10-80 bp in length. It connects each nuceosome to its neighbors resulting in the formation of 30nm of compact chromatin fiber of packed nucleosome (**Figure 1.7a**).

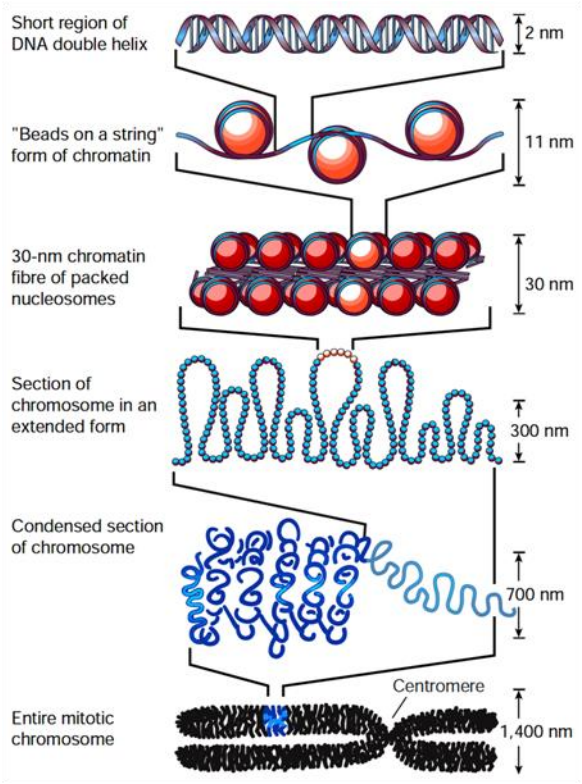


Figure 1.7a Packaging of eukaryotic DNA within the chromatin structure (Nature. 2003; 421:448-53).

Chromatin within the eukaryotic nucleus can be distinguished into euchromatin and heterochromatin based on cytological studies. They were initially identified based on how intensely they are stained. The heterochromatin is intensely stained, whereas euchromatin is stained less intensely. The difference in the intensity of staining is attributed to their cytological appearance as heterochromatin has dense packing of DNA as the cell returns from metaphase to interphase compared to euchromatin which has less compact structure.

Interestingly, heterochromatin and euchromatin represent two varied structural environments that have significant effects on gene expression (Elgin, 1996). For example, if a gene is located in a domain where the DNA is less densely packed (euchromatin), it has the potential to be transcriptionally active; however, when a gene is within or adjacent to a more densely compact domain (heterochromatin), it is transcriptionally repressed. The dense packing makes heterochromatin less accessible to protein factors which usually bind DNA or its associated factors.

The modulation of chromatin structure is brought about by a broad spectrum of enzymes which can have an overall effect over nucleosome stability by either disrupting histone-DNA contacts or by covalently modifying histones and/or DNA (Sif, 2004). Chromatin modifying complexes are structurally classified into two distinct categories which are involved in activating chromatin modifications and thereby regulating chromatin access.

Chromatin modifying enzymes contain the catalytic subunits of different histone modifying enzymes that are involved in catalyzing the post-translational modification of histone tails as well as other chromatin factors. Such modifications include acetylation, methylation, phosphorylation, poly-ADP-ribosylation and ubiquitination (**Figure 1.7b**) (Fischle *et al.*, 2003) which have vital roles in regulating gene transcription, heterochromatin formation, X chromosome inactivation, DNA replication, DNA repair and cellular memory (Zhang and Reinberg, 2001; Jenuwein and Allis, 2001; Kouzarides, 2002; Lachner and Jenuwein, 2002; Strahl and Allis, 2000; Turner, 2002).

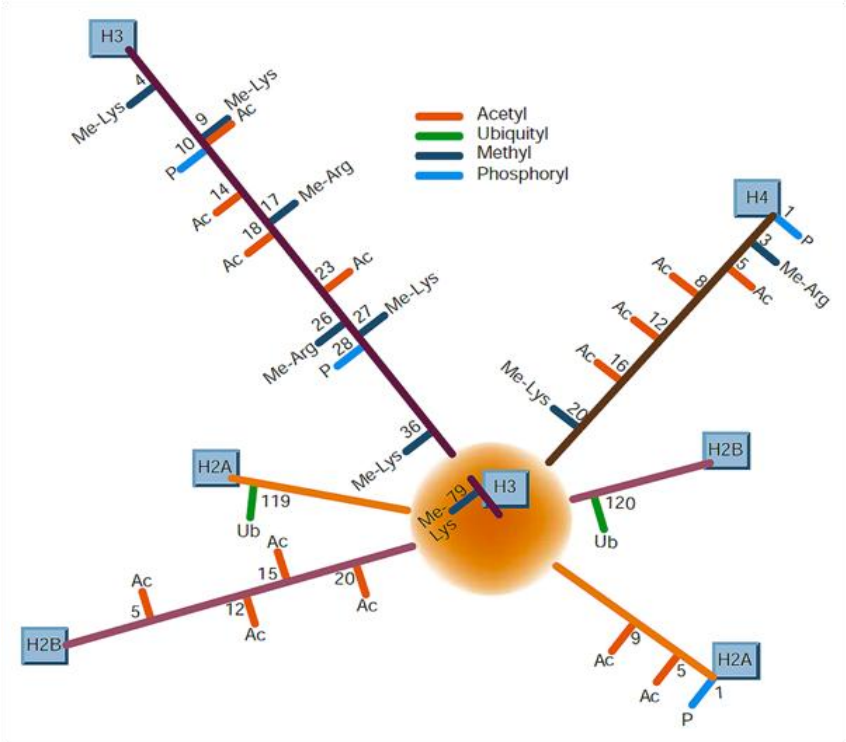


Figure 1.7b Various histone tails and their modifications. (Nature. 2003; 421:448-53).

The second major class is, ATP-dependent chromatin remodeling complexes which can move nucleosome positions, thereby playing a pivotal role in altering chromatin structure and increasing accessibility to DNA on the surface of histone octomer (Narlikar *et al*., 2002; Kadam, S. and Emerson, B.M. *et al*.. 2003).

In addition to histone modifying enzymes and chromatin remodeling complexes, there are DNA modifying enzymes which are involved in methylating CpG rich sequences which constitute an important epigenetic mechanism to repress specific set of genes (Lande-Diner *et al*., 2007).

1.8 Histone lysine acetylation

Of all the histone modifications studied so far, histone acetylation has been the most well studied and appreciated (Grunstein *et al*., 1997). The acetylation of histones decreases the positive charges of the histone tails which results in reduction of its affinity for the negatively charged DNA, thereby allowing greater accessibility for the binding of proteins that regulate transcription to chromatin templates (Hong *et al*., 1993; Workman and Kingston, 1998).

The most common post-translational modification of histone is acetylation of lysines (K) which is controlled through the actions of two families of antagonistic enzymes, histone acetyltransferases (HATs) and deacetylases (HDACs). They are involved in catalyzing the acetylation/deacetylation of histones, thereby acting as transcriptional activators and repressors respectively (McKinsey *et al*., 2001). HATs catalyze the transfer of an acetyl

group from acetyl-coenzyme A (acetyl-CoA) to ε-amino group of certain lysine side chains within a histone's basic N-terminal tail region (Loidl, 1994).

Acetylation of particular lysine residues in histone 3 and 4 (acetyl H3, acetyl H4) by acetyltransferases facilitates a more relaxed chromatin structure paving the way for gene activation. However, hypoacetylation of histone by deacetylases results in closed heterochromatin, which is often associated with gene repression. Thus the acetylation status of histones is maintained by the counteracting action of HATs and HDACs.

1.9 Histone Methylation: Arginine and Lysine methylation

Histone methylation plays an important role in a wide variety of biological processes which include transcriptional regulation, heterochromatin formation, X-chromosome inactivation and DNA methylation (Martin and Zhang, 2005). Unlike histone acetylation, histone methylation does not affect the overall charge of the histone tails. However, increase in the number of methyl group added increases its basicity and hydrophobicity. This in turn enhances the affinity of histone tails for anionic DNA which results in increased resistance to tryptic digestion (Baxter and Byvoet, 1975; Byvoet *et al*., 1972).

Histones can be methylated on either arginine (R) or lysine (K) residues. Histone lysine methylation occurs predominantly on histones H3 and H4 and to a lesser extent in histone H1 (DeLange *et al*., 1970; Sarnow *et al*., 1981; Byvoet *et al*., 1986). Reported so far are six lysine residues, located on these two histones which have been identified as sites of methylation; those residues

are H3K9, H3K4, H3K27, H3K36, H3K79 and H4K20. Each of these lysine residues can be subjected to either mono-, di- or tri-methylation, inducing different biological responses unlike lysine acetylation. In addition to methylation of lysine residues, the other amino-acid residue which can be methylated is arginine. Methylation of arginine results in mono- or di-methylation (symmetric or asymmetric).

All of these residues are located within the unstructured N-terminal tail of the histone protein with the exception of H3K79 which resides in the globular domain (Feng *et al*., 2002). Depending on the sites of methylation it can signal either activation or repression. For example, methylation of H3K9, H3K27 and H4K20 has been correlated with transcriptional repression whereas H3K79, H3K4 and H3K36 are associated with active transcription. On the other hand methylation of arginine-8 and -17 (H3R8me and H3R17me) is associated with transcriptional activation (Strahl *et al*., 2001).

1.10 PRMTs and HKMTs

Methylation of histone tails is one of the key events in epigenetic silencing (Martin and Zhang, 2005). Methylation of histone proteins is carried out by a group of conserved enzymes called histone methyltransferases (HMTs) which catalyze the transfer of one, two or three methyl groups to lysine and arginine residues of histone proteins. They are divided into two main groups: protein arginine N-methyltransferases (PRMTs) and histone lysine methyltransferases (HKMTs).

PRMTs catalyze the transfer of methyl groups from the co-factor S-adenosyl-L-methionine (SAM), a methyl donor to the guanidino nitrogens of arginine residues (Gary and Clarke, 1998) whereas HKMTs catalyze the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to a substrate lysine (Dillon *et al*., 2005; Qian and Zhou, 2006).

Several histone lysine methyltransferases (HKMTs) have been recently identified (Strahl *et al*., 1999; Jenuwein *et al*., 1998; Kennison, 1995). Their catalytic activities are dependent on the evolutionarily conserved SET domain which occurs in most proteins that are known to possess histone lysine methyltransferase activities with the exception of Dot1/Dot1L. Biochemical and genetic analyses had revealed that methylation of different lysine residues, with the exception of H3K79 is catalyzed by different SET domain containing proteins (Zhang and Reinberg, 2001). It was originally identified as a 130-residue protein present in the Drosophila PEV-modifier SU (VAR) 3-9 (Tschiersch *et al*., 1994), the Polycomb-group protein Enhancer of Zeste [E (Z)] (Jones and Gelbart, 1993) and in the trithorax-group protein Trithorax (TRX) (Jenuwein *et al*., 1998).

The SET domain has unique structure made up of a collection of variable length β-strands, a small amount of α-helical structure and an assorted collection of extended loops (Xiao *et al*., 2003). It is flanked by two distinctive domains at its N and C termini. There are totally seven main families of SET domain proteins which are known, namely, the SUV39, SET1, SET2, EZ,

RIZ, SMYD and SUV4-20 families. The SUV39 family molecules were the first SET domain protein methyltransferases to be identified and characterized in most detail. SUV39h1 (Suppressor of variegation 39h1) was the first to be cloned and expressed (Rea *et al*., 2000). It was followed by its close relative, Suv39h2, which was also cloned and characterized (O'Carroll, 2000). Since then many others have been characterized and almost all of them contain a SET domain which includes G9a, GLP/Eu-HMT1; SETDB1, SETDB2 and DIM5 (Tachibana *et al*., 2001; Rea *et al*., 2000; O'Carroll, 2000; Tamaru and Selker 2001; Zhang *et al*., 2002). Since the discovery of first histone-lysine methyltransferases in the year 2000, sixty of them have so far been identified to be containing the SET domain.

1.10.1 H3K9 methyltransferases: Suv39h1/h2

The well-known SET domain containing H3K9 methyltransferases includes Suv39h1/h2, G9a/GLP, SETDB1, SETDB2 and DIM-5 (Tachibana *et al*., 2001; Rea *et al*., 2000; O'Carroll, 2000; Zhang *et al*., 2002; Tamaru and Selker 2001).

Suv39h1 and Suv39h2 belong to a family of peri-centromeric proteins. They share 59% identity over the entire length of the aminoacids and display an H3K9 selective HMTase activity (O'Carroll *et al*., 2000). Suv39h1/h2 act predominantly in the pericentric heterochromatin, where they play a crucial role in the maintenance of genome stability, and in turn, the development of the embryos. Studies have shown that combined disruption of Suv39h1 and

Suv39h2 results in severe impairment of H3K9 trimethylation at the pericentric heterochromatic regions. As a consequence, defects such as impaired embryonic viability after day E12.5, perturbed chromosome segregation and non-homologous recombination between chromatids were observed (Peters *et al*., 2001). These results suggest that Suv39h1 and Suv39h2 are responsible for selective regulation of H3K9 trimethylation,

1.10.2 H3K9 methyltransferase: G9a

The predominant euchromatic H3K9 methyltransferase described in higher eukaryotes is G9a, also known as Euchromatic histone lysine N-methyltransferase 2 (EHMT2), Bat 8, KMT1C, GAT 8, C6orf30 and NG36. It was originally mapped as a gene in the class III region of the human major histocompatibility complex locus on human chromosome 6 in an attempt to identify genes related to auto immune disorders (Dunham *et al*., 1990; Spies *et al*., 1989). It was later characterized by Tachibana and others (2001) as HKMTase whose catalytic activity was dependent on its SET domain. They found structural similarities between G9a and the Suv39h families of HKMTases.

G9a is a large protein with very distinct domains. The human G9a is located on chromosome 6 p21.31 and the Mus musculus G9a is located on chromosome 17. It exists in two different isoforms generated by alternative splicing namely the short and long form. The human G9a, which was initially thought to contain 1001 amino acids, was later found to lack NG36 transcripts

(**Figure 1.10.2a**) (Milner and Campbell, 1993). It was revealed by further analysis that the full length G9a (isoform a) is a product of NG36 and G9a transcripts (Figure 1.10.2a). In addition, shorter isoform which lacks exon 10 (isoform b) has been characterized. In mouse, while the short form (G9a S) corresponds to human homolog, the long isoform (G9a L) (**Figure 1.10.2a**) differs with additional 53 residues repeats at the N-terminus compared to human full length G9a (isoform a). These additional amino acids have eight GR (Gly-Arg) repeats (Tachibana *et al*., 2002; Esteve *et al*., 2005).

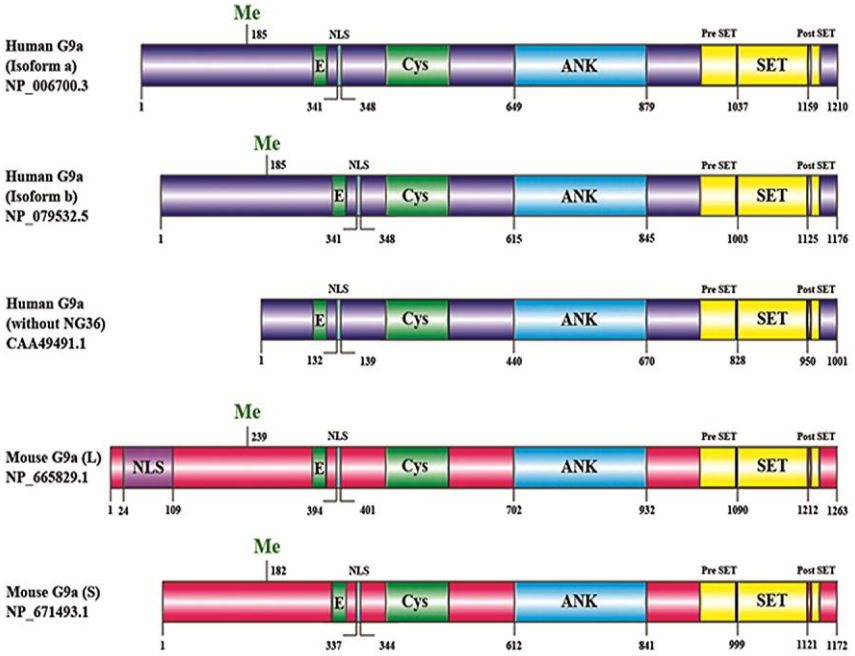


Figure 1.10.2a Diagrammatic representation of G9a domain structure (Adapted from Shankar *et al*., 2012. Epigenetics). The isoforms of human and mouse G9a are shown here. The Cysteine (CS) rich region, ankyrin repeats (ANK) and the SET domain with flanking preSET and postSET regions are shown. The site for methylation (Me), nuclear localization signals (NLS) and the glutamic acid (E) region are indicated. Numbers indicate aminoacid residues.

The amino half of G9a has very little similarity with any conserved protein domain and it comprises of polyglutamic acid stretch, a cysteine rich region. However, the carboxyl half has homology with ANK (ankyrin) repeats comprising a series of 6 ANK repeats which are implicated in protein-protein interactions in diverse protein families (Davis *et al*., 1991). It is followed by a SET domain flanked by cysteine-rich (Cys) regions (**Figure 1.10.2a**).

Study by Ogawa and others (2002) found out that G9a has closely related paralog, G9a-like-protein (GLP/EHMT1). GLP is 45% identical with human G9a and most of the difference lies in the amino-terminal third of the protein (Dillion *et al*., 2005).

Biochemical studies have revealed that G9a and GLP possess the same substrate specificities on histones. Both enzymes have H3K9 methyltransferase activity *in vitro* (Ogawa *et al*., 2002; Tachibana *et al*., 2001). They form a homomeric and heteromeric complex via their SET domain when they are transiently expressed; however, endogenously they function exclusively as a heteromeric complex in a wide variety of human and mouse cells and exert their enzymatic activity *in vivo* (Tachibana *et al*., 2005).

G9a mediates both mono and di-methylation of H3K9 which is associated with transcriptional repression of many euchromatic genes (Tachibana *et al*., 2001; Gyory *et al*., 2004; Nishio and Walsh, 2004; Duan *et al*., 2005). Studies have shown that in addition to mediating H3K9 mono- and di-methylation, G9a is also involved in H3K9 tri-methylation *in vivo* (Yokochi *et al*., 2009).

Further studies have shown that G9a is also involved in H1 and H3K27 methylation *in vivo* (Trojer *et al*., 2009; Weiss *et al*., 2010; Wu *et al*., 2011).

In addition to histones, increasing number of evidences point out that non-histone proteins too are direct targets for G9a mediated methylation with its recognition motif RK found in several non-histone proteins. Its non-histone target includes p53, CDYL1 (Chromodomain Y-like protein), WIZ (Widely interspaced zinc finger motifs protein), ACINUS and Reptin (**Figure 1.10.2b**) (Ueda *et al*., 2006; Sampath *et al*., 2007; Pless *et al*., 2008; Rathert *et al*., 2008; Lee *et al*., 2010; Huang *et al*., 2010). While the biological consequence of such methylation of non-histone proteins has not been completely explored in each case, many transcription factors that are methylated by G9a result in negative regulation of their activity. For example, methylation of p53 at K373 by G9a/GLP inhibits its transcriptional activity (Huang *et al*., 2010), whereas methylation of Reptin at K67 under hypoxic conditions enables reptin-dependent inhibition of hypoxia responsive genes thereby creating a mechanism to adjust the cellular response to hypoxia (Lee *et al*., 2010).

Interestingly G9a can automethylate itself at lysine residue K239 resulting in the creation of ARKT motif which is very similar to methylated H3 tail, making it a perfect candidate for interaction with HP1 thereby generating a platform for recruitment of other repressors DNMT1 and HDAC1 (Chin *et al*., 2007).

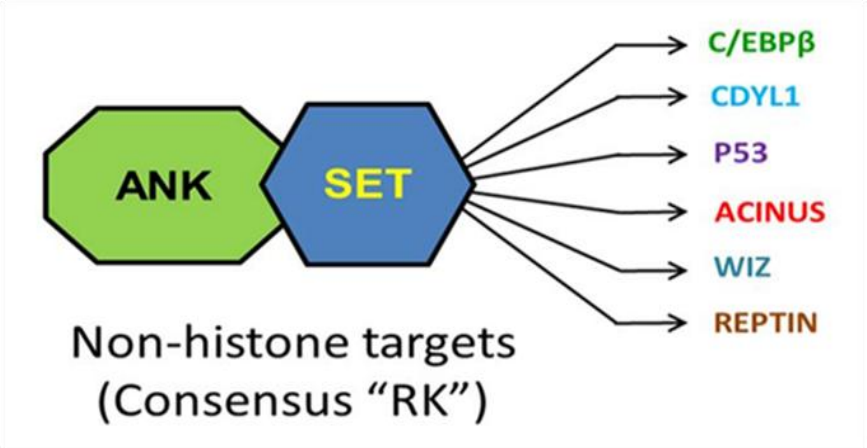


Figure 1.10.2b G9a methylates non-histone proteins (Adapted from Nucleic Acids Res. 2010; 38:3503-11).

1.10.2.1 Functions of G9a

G9a plays an essential role in cellular differentiation, development and cell cycle progression. It is ubiquitously expressed in most tissues including fetal liver, bone morrow, thymus, spleen and peripheral blood leukocytes (Brown *et al*., 2001). G9a mediated H3K9 methylation in euchromatic regions is essential for early embryonic development which cannot be compensated by other H3K9 methyltransferases (Tachibana, *et al*., 2002). G9a deficient mouse embryos die between embryonic (E) days E9.5-E12.5 with severe growth retardation and accumulation of apoptotic cells as H3K9 methylation is drastically reduced in euchromatic regions.

G9a plays a key role in early development by targeting key pluripotency genes for post-implantation repression. Oct-3/4, Nanog and Dnmt3L are early embryonic genes required for maintenance of pluripotency in embryonic stem (ES) cells. They are inactivated by G9a through the establishment of methylated H3K9 heterochromatinization via its SET domain as well as

through *de novo* methylation by recruiting DNA methyltransferase Dnmt3a/3b via its ANK domain. The establishment of these two separate epigenetic marks prevents reprogramming of ES cells to an undifferentiated state. Consistent with this, G9a-/- ES cells differentiated with retinoic acid (RA) revert back to a pluripotent state by expressing Oct3/4 and Nanog when re-cultured in leukemia inhibitory factor (LIF) containing medium (Feldman *et al*., 2006; Epsztejn-Litman *et al.*, 2008*).* In addition, a recent study shows that Oct3/4 and Nanog expressions are sustained until E7.5 in G9a null embryos (Yamamizu *et al*., 2012). G9a has also been implicated in lineage specification and differentiation of distinct cell types. For example, study by Lehnertz and others (2010) shows that $CD4^+$ T from conditional knockout mice in which G9a is specifically deleted in T-cells fails to differentiate into Th2 cells impairing cytokine induction.

There are evidences which show that G9a is recruited as a co-repressor by various transcriptional factors.

Study by Nishio and Walsh have shown that CCAAT displacement protein/ cut homolog (CDP/cut), a transcriptional factor involved in most cellular processes including differentiation, development and proliferation, recruits G9a to the promoter p21 which results in the repression of the p21 gene (Nishio and Walsh, 2004). Another study has shown that growth factor independent 1 (Gfi 1), a transcriptional regulator oncoprotein which plays a crucial role in regulating diverse cellular processes such as self-renewal of

hematopoietic stem cells, proliferation, apoptosis and differentiation, recruits G9a on the promoter of p21 resulting in an increase in H3K9 dimethylation thereby represses the expression of p21 (Duan *et al*., 2005).

1.11 Chromatin modifying enzymes in undifferentiated myoblasts

The myogenic activities of MRFs and MEF2 are both positively and negatively regulated by different chromatin modifying enzymes (McKinsey *et al*., 2000b).

1.11.1 HDACs in the control of MyoD and MEF2 activities

There are three distinct families of HDACs (Thiagalingam *et al*., 2003) that play a crucial role in maintaining the inactive state of muscle regulatory region in proliferating myoblasts. They are divided into three categories based on their homology to yeast protein: Rpd3p (class I), Hda1p (class II), and Sir2p (class III) (Ruijter *et al*., 2003; North and Verdin, 2004). Class I HDACs are expressed ubiquitously, whereas Class II HDACs are expressed in heart, skeletal muscle and brain. It was shown that HDAC1, which belongs to Class I HDAC subfamily, associates with MyoD through its bHLH domain in proliferating myoblasts (Figure 1.11) (Mal *et al*., 2001; Puri *et al*., 2001). This interaction mediates repression of muscle specific gene expression by deacetylation of histones on late muscle promoters MCK and MHC. In addition, HDAC1 can deacetylate MyoD *in vitro*, which may additionally contribute to keeping MyoD inactive (Mal and Harter, 2003; Mal *et al*., 2001).

However, upon induction of differentiation, this association between MyoD

and HDAC1 is being disrupted by both the decline in the level of HDAC1 protein and the hypophosphorylation of the tumor suppressor retinoblastoma protein, pRb. Dephosphorylation of pRb facilitates the formation of pRb-HDAC1 complex which coincides with the disassociation of MyoD-HDAC1 complex. The formation of this complex represses E2F target genes, which are responsible for the G1/S transition (Harbour and Dean 2000). This paves the way for transcriptional activation of muscle restricted genes and cellular differentiation of skeletal myoblasts (Puri *et al*., 2001). Conversely, ectopic expression of HDAC1 inhibits muscle differentiation.

Furthermore, class II HDACs namely HDAC 4 and 5 which are expressed in proliferating myoblasts interact directly with MEF2 factors resulting in repression of MEF2-dependent transcription (**Figure 1.11**) (Lu *et al*., 2000; McKinsey *et al*., 2000a; McKinsey *et al*., 2000b). Consistently, over-expression of HDAC4/5 inhibits differentiation by inhibiting both early and late muscle differentiation genes. They can efficiently block MyoD-dependent conversion of fibroblasts into muscle. This effect on MyoD function was mediated indirectly through MEF2.

A third class of HDACs playing a critical role in regulating muscle differentiation program is SirT1 (Silent mating type information regulation 2 homolog 1). It belongs to the family of sirtuins whose activity is NAD (+) dependent. It is recruited to muscle regulatory region on chromatin through its ability to form complexes with histone acetyltransferase, p300/CBP associated

factor (P/CAF) and MyoD. This association facilitates the deacetylation of histones on muscle specific promoter and repression of muscle specific gene expression (**Figure 1.11**). However, upon differentiation, SirT1 expression and NAD+/NADH ratio declines resulting in reduced SirT1 activity, thus facilitating P/CAF to acetylate histone and MyoD. Over-expression of SirT1 in myoblasts inhibits MyoD activity and thereby retards muscle differentiation. Conversely, reduction in SirT1 levels by RNA interference results in premature differentiation (Fulco *et al*., 2003). Thus all the three classes of HDACs are involved in preventing premature activation of muscle differentiation program. The repression of muscle specific genes by all the three classes of HDACs depends on their catalytic activities.

In addition to HDACs, which mediate deacetylation of histones in proliferating myoblasts, the SET domain containing HKMTs are critical mediators of muscle gene repression. Interestingly, the recruitment of the HDAC complexes which results in the removal of acetate from histone on the muscle promoter, paves the way for subsequent H3K9 methylation by HKMTs on the same promoter (Zhang *et al*., 2002).

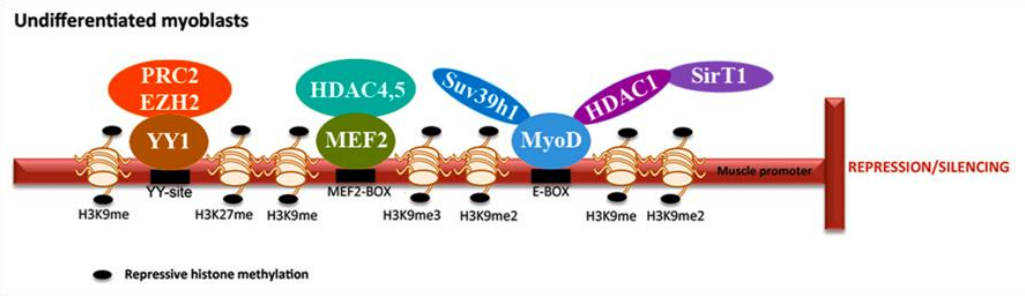


Figure 1.11 Model for epigenetic regulation of promoters in undifferentiated muscle cells. (Adapted from Transcription. 2012; 3:16). In undifferentiated muscle cells, transcriptional repression of muscle gene expression is facilitated through recruitment of chromatin modifiers such as HDACs (HDAC1, HDAC4/5 and SirT1) and HKMTs (Suv39h1 and Ezh2), that interact with MyoD, MEF2 and YY1 as shown. Suv39h1 and Ezh2 mediate two signature repressive chromatin marks H3K9me2/me3 and H3K27me3 respectively that restrain MyoD and MEF2 activities resulting in closed chromatin configuration.

1.11.2 Role of HKMTs in myogenesis

Lysine methyltransferases: Suv39h1, which is associated with H3K9 trimethylation at the pericentric heterochromatic regions, has been reported to be involved in skeletal muscle gene repression. It interacts with MyoD in proliferating myoblasts (**Figure 1.11**) and represses MyoD dependent skeletal muscle gene expression such as myogenin, MHC and non-muscle genes such as p21. This is by maintaining H3K9 trimethylation at the myogenin promoter through its HKMTase activity. However, upon differentiation, the level of Suv39h1 declines at protein level and thereby its association with MyoD paving the way for the activation of myogenin. When Suv39h1 is over-expressed in C2C12 cells, it results in severe impairment of myotube formation with the repression of myogenin, p21 and MHC under

differentiation permissible condition (Mal, 2006). The requirement of Suv39h1 for muscle gene repression in proliferating myoblasts was verified by siRNA mediated knockdown of Suv39h1 in muscle cell which resulted in activation of MyoD mediated muscle gene expression, confirming its role as checkpoint between proliferation and differentiation.

The Polycomb group (PcG) represents an important and broadly conserved group of transcriptional repressors that are involved in maintaining the silent state of Drosophila homeotic (HOX) genes, which are essential for proper embryonic development. They remodel chromatin through epigenetic modifications which dynamically define cellular identity by maintaining transcriptional patterns, throughout development and adulthood (Shuettengrubber and Cavalli, 2009; Simon and Kingston, 2009).

Enhancer of Zeste [E (Z)] which is the SET domain containing proteins with HKMTase activity belongs to the PcG family (Kuzmichev *et al*., 2002). Ezh1 and Ezh2 are the two related genes that have been isolated and characterized (Laible *et al*., 1997). The Polycomb Repressor Complex (PRC2) contains three core subunits--Enhancer of zeste homolog 2 (Ezh2), Embryonic Ectoderm Development (EED), and Suppressor of Zeste 12 (SUZ12). Ezh2, an H3K27 methyltransferase is the catalytic subunit of PRC2. It transfers a methyl moiety from S-adenosyl methionine to H3K27 resulting in H3K27 trimethylation and thereby repressing gene expression. It is expressed during embryonic development and plays an important role in various biological

processes which include multi-cellular development, cancer development and stem cell biology.

Ezh2 also plays a crucial role in skeletal muscle differentiation which is associated with transcriptional repression. Ezh2 is expressed in proliferating myoblasts (**Figure 1.11**) and declines upon differentiation and becomes undetectable in differentiated myotubes which coincides with the activation of terminal myogenic program (Caretti *et al*., 2004; Juan *et al*., 2009). Over-expression of Ezh2 inhibits muscle differentiation and this differentiation block is mediated by the SET domain as the deletion of this domain renders Ezh2 incapable of blocking myogenic differentiation and transcription.

Van der vlag and Otte (1999) showed that Ezh2 interacts with the histone deacetylase HDAC1 suggesting that both histone deacetylation and methylation are in sync to ensure transcriptional repression. This work was further expanded by Caretti and others (2004) who, based on their data on a two-step activation model of muscle gene expression, suggested that in proliferating myoblasts, the DNA-binding protein YY1 recruits a complex containing both Ezh2 and HDAC1 on the regulatory regions of transcriptionally inactive muscle specific genes. This transcriptional silencing is being mediated via histone methylation (H3K27) and histone deacetylation. However, upon transcriptional activation, chromatin repressive complexes of Ezh2, YY1 and HDAC1 disassociate and H3K27 becomes hypo-methylated. This repressive complex is being replaced by the recruitment of post

transcriptional regulators namely the serum responsive factor (SRF), whose interaction with the CarG-box is required for muscle specific transcription to proceed and the MyoD family of transcription factors and associated acetyltransferases.

1.12 Chromatin modifying enzymes and remodeling complexes during differentiation

1.12.1 P/CAF and p300

P/CAF (p300/CBP associated factor) is one of the most important members of the HATs family. It is a transcriptional co-activator involved in various biological functions and part of the large protein complexes that include p300, another important acetyltransferase. They are both involved in acetylating H3K9 under diverse physiological conditions. In skeletal myogenesis, these transcriptional co-activators are essential for the myogenic factor MyoD to initiate the expression of skeletal muscle genes. Although it is difficult to delineate the distinct roles played by P/CAF and p300 as both of these factors can acetylate MyoD in addition to mediating histone acetylation, studies have revealed that p300 first acetylates H3 and H4 within the promoter region and then recruits P/CAF which then interacts (**Figure 1.12**) and directly acetylates MyoD (**Figure 3.1.12**) at lysine residues 99, 102 and 104 which is conserved across species and in every member of MRF family resulting in its transactivation (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010). This increases the affinity of MyoD for its transcriptional DNA target, switching on genes for differentiation thereby controlling myogenic

transcription and differentiation. Thus, the acetylation of MyoD by P/CAF is functionally very relevant, as the study with mutant MyoD (RRR), wherein lysine residues at 99, 102 and 104 were substituted with arginine had clearly shown that non-acetylable MyoD loses its transcriptional and myogenic ability (Sarorelli *et al*., 1999).

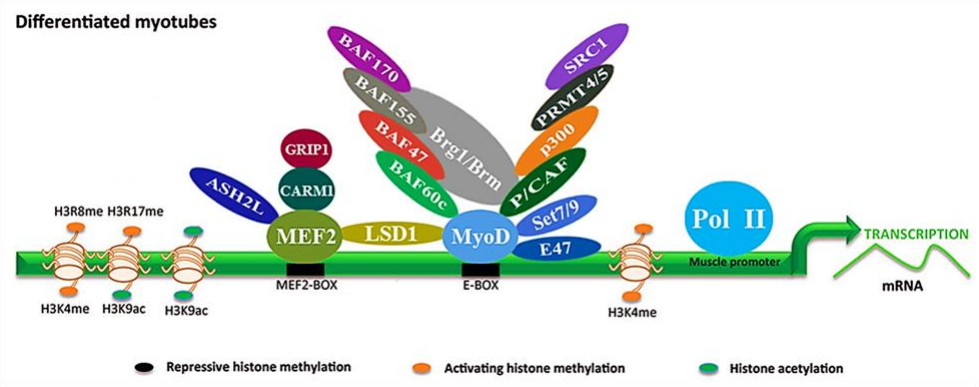


Figure 1.12 Model for epigenetic regulation of promoters in differentiated muscle cells. (Adapted from Transcription. 2012; 3:16). In differentiated muscle cells, the expression of co-repressors such as HDACs and HKMTs declines and also their association with MyoD. These paves way for subsequent recruitment of other chromatin modifiers such as HATs (PCAFs and P300), PRMTs (CARM1, PRMT5) and chromatin remodelling complexes (SWI/SNF, Brg1/Brm), mediating active chromatin marks H3K9ac, H3K14ac, H3R8me, H3R17me and extensive reprogramming of muscle promoters that facilitates open chromatin configuration and activation of MyoD and Mef2 dependent gene expression triggering muscle differentiation.

In addition to p300 and P/CAF, SWI/SNF remodeling complexes play an essential role in triggering myogenesis by reading the combinations of histone marks generated by muscle specific transcription factors and shaping the DNA of target muscle genes during myoblasts transition from muscle precursors to myotubes (Albini and Puri, 2010).

1.12.2 ATP-dependent chromatin remodeling enzymes: SWI/SNF complex

On the basis of similarities of their ATPase sub-units, the ATP-dependent chromatin remodeling complexes are divided into three classes, namely, Swi2/Snf2, ISW1, and Mi-2 proteins. These ATPases are the catalytic subunits, the motors of the complexes. They remodel chromatin structure by hydrolyzing ATP to enhance the accessibility of chromosomal DNA which is a prerequisite for various steps in transcription (Eisen *et al*., 1995; Lusser and Kadonaga, 2003).

There is a variety of chromatin remodeling enzymes and one of the important and best studied classes among these enzymes is SWI/SNF. It is a 2MDa multi-subunit DNA dependent ATPase which plays an important role in the regulation of gene transcription by altering chromatin structure (Cairns *et al*., 1994; Cote, 1994). It comprises of the products of several genes originally identified in yeast by defects in mating type switching (SWI) and/or sucrose fermentation (SNF: sucrose non-fermenter) and is highly conserved among eukaryotes. The unique feature of this family is the presence of a bromodomain motif in the ATPase subunit either brahma (BRM) or brahma

related gene 1 (BRG1) which facilitates recognition and binding with acetylated lysine residues within histone N-terminal tails *in vitro* (Horn and Peterson, 2001; Martens and Winston, 2003). In addition to these ATPase sub-units which provide the enzymatic activity necessary for chromatin remodeling, there are 4-12 sub units referred to as Brg1 and Brm-associated factor (BAF) which regulate the ATPase activity (Simone *et al*., 2006). They mediate interaction with specific transcription factors thereby controlling cell type-specific and signal regulated SWI/SNF distribution along the genome (Chi *et al*., 2004).

Studies have shown that the interaction between Brg1, Brm and acetylated histones accounts for the stable binding of SWI/SNF to hyperacetylated chromatin within the regulatory regions of muscle genes. SWI/SNF chromatin remodelers facilitate muscle-specific gene expression by altering chromatin structure at the regulatory regions of both early and late myogenic genes (de la sarna *et al*., 2001; de la sarna *et al*., 2005; Ohkawa *et al*., 2007; Simone *et al*., 2004). SWI/SNF interacts with both MyoD and Mef2 proteins and this interaction is necessary for MyoD to activate muscle gene transcription (**Figure 1.12**). Recently, the findings of Forcales and others (2012) revealed a critical role for BAF60c during skeletal myogenesis. They identified BAF60c as a MyoD binding partner (**Figure 1.12**). Importantly, their finding revealed that BAF60c co-localizes with MyoD at the myogenic promoter prior to the activation of transcription without other SWI/SNF subunits such as Brg1. Furthermore, knockdown of BAF60c disrupted the recruitment of MyoD to

most of its target genes resulting in the impairment of their transcription. This absence of MyoD also prevented the binding of BAF60c to the chromatin of myogenin suggesting BAF60c and MyoD facilitate each other's bindings to muscle specific loci.

1.12.3 PRMT4 and PRMT5

Prmt4 also known as coactivator arginine methyltransferase1 (CARM1) belongs to type I methyltransferase which dimethylates histones H3 arginine R17. It was originally discovered in a yeast two-hybrid screen to identify proteins that interact strongly with the glucocarticoid receptor interacting protein 1 (GRIP). It interacts with and methylates transcriptional co-activators such as p300 and GRIP1. CARM1 mediated methylation of R17 of histone H3 enhances GRIP1 mediated transcriptional activation (Chen *et al*., 1999; Bauer et *al*., 2002).

PRMT5, which is a type II methyltransferase, was first discovered in a yeast two-hybrid screen to identify proteins interacting with JAK2. Hence it is also known as Janus kinase binding protein 1 (Branscombe *et al*., 2001). It symmetrically dimethylates histone H3 arginine 8 (H3R8) and plays a crucial role in transcriptional repression.

Studies by various groups have shown that these two members of the PRMT family, CARM1 and PRMT5 play a crucial role in skeletal myogenesis.

CARM1 functions as a secondary co-factor for Mef2 mediated transcription in an SRC/GRIP1 (a co-factor which recruits CARM1/PRMT4) dependent

manner in the regulation of muscle specific gene expression. It is expressed in proliferating myoblasts and increases upon differentiation and reaches peak in multinucleated myotubes. *In vitro* studies show that CARM1 interacts with GRIP1 as well as MEF2 proteins directly. However, it doesn't interact with either MyoD or myogenin. In addition, it has been demonstrated that MEF2-GRIP1 complex interacts with CARM1 in the cellular context as well, and thus accounts for the co-activation of MEF2C mediated transcription (**Figure 1.12**). Hence, it positively regulates skeletal myogenesis, as inhibition of CARM1 inhibits differentiation and disrupts the expression of myogenin and MEF2 that are involved in initiating the differentiation cascade.

Studies have shown that PRMT5 mediated methylation of H3R8 is essential for the interaction of the SWI/SNF ATPase Brg1, for chromatin remodeling of myogenin locus and for all subsequent events leading to gene activation (**Figure 1.12**). The loss of PRMT5 results in the substantial reduction in the recruitment of Brg1 at the myogenin promoter and this affects the subsequent events such as histone methylation, ATP-dependent chromatin remodeling, and MyoD binding resulting in abrogation of myogenin expression. Thus the arginine methyltransferase PRMT5 is important for MyoD mediated skeletal muscle differentiation as it facilitates ATP-dependent remodeling (Dacwag et *al*., 2006).

1.13 Role of HKMTs in skeletal muscle pathology

It is clear from all the above discussions that a crucial role is played by epigenetic chromatin modifiers such as HATs, HDACs and HKMTs in modifying chromatin to epigenetically promote or repress transcription. Thus the impact of these chromatin modifying enzymes as well as the chromatin remodelers on transcription factors such as MRFs and MEF2 translates into a vital role in shaping almost all aspects of skeletal muscle development positively and negatively. However, any aberrant form of either transcription factors or their regulators and their deregulation may lead to numerous muscle diseases such as congenital myasthenias, myotonic dystrophy, rhabdomyosarcoma and defects in skeletal muscle regeneration (Martin *et al*., 2003).

There are growing evidences which point out that transcription factors such as MyoD associate with histone modifying enzymes namely HDACs and HKMTs even in differentiation permissible condition that results in abrogation of their transcriptional activity leading to defect in skeletal muscle differentiation and tumorigenicity such as Rhabdomyosarcoma (Lee *et al*., 2011; Ciarapica *et al.*, 2009).

1.13.1 HKMTs in Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is a highly malignant and most common soft tissue sarcoma of childhood and adolescence which accounts for approximately 5-8 % of pediatric cancer. The annual incidence of RMS is

stated to be 4 to 7 cases per million children (Kramer *et al*., 1983). It continues to pose a major challenge in clinical management in spite of advances in its treatment, due to recurrence and metastasis. It originates as a consequence of disruption of growth and differentiation pathways of myogenic precursor cells. As a result RMS has poorly differentiated mesenchymal stem cells with morphological and biochemical similarities to primitive skeletal muscle cells.

RMS is classified into two major morphological subtypes, embryonal (ERMS) and alveolar (ARMS), and several minor sub-types based on their histopathologic features. ERMS is the most common (60%) and treatable sub-type occurring usually in infants and children less than ten years of age, most frequently in the head and neck, genitourinary tract, and the retroperitoneum. ARMS accounts for approximately 25-30% of RMS and represents a highly aggressive tumor in adolescents and young children. It occurs typically in the trunk and extremities and has a poorer prognosis (Tsokos *et al*., 1992). These clinical and pathologic differences between ERMS and ARMS occur as a result of distinct genetic alterations and clinical prognosis (Merlino and Helman, 1999; Anderson *et al*., 1999).

ARMS carry a distinct genetic signature: Pax3-FKHR and Pax7-FKHR fusion products generated as a result of 2;13 or 1;13 of pathogenetic chromosomal translocations, the consequence of these specific translocations being altered expression, function and sub-cellular localization of the fusion products

compared to wild-type proteins. This paves the way for harboring oncogenic behavior by altering growth, differentiation and apoptosis pathways. However, ERMS is less genetically defined (Mercado *et al*., 2008) and has allelic loss at chromosome 11p15.5.

In spite of the morphologic differences and the fact that they affect different patient populations, ERMS and ARMS share the common characteristic of defects in both differentiation program and cell cycle arrest which result in uncontrolled proliferation and incomplete myogenesis. This is despite the fact that RMS cells generally express one or both of the key early myogenic regulators of differentiation namely Myf5 and MyoD as well as its target gene, myogenin (Scrable *et al.*, 1989; Sebire *et al*., 2003; Ren *et al*., 2008; Tapscott *et al*., 1993). Although it is well known that MyoD has the capability to drive the entire process of skeletal muscle terminal differentiation and when stably transfected in non-muscle cells can convert it to skeletal muscle cells, yet the ability of MyoD in bringing about such differentiation in RMS is impaired. One of the main reasons put forth for such defectiveness is the loss of MyoD's transactivation ability. Although it is able to bind to its cognate DNA sequences, MyoD fails to induce muscle gene transcription in the RMS cells. Moreover, ectopic expression of MyoD is not sufficient to induce myogenesis. It was concluded by Tapscott and others (1993) based on their experimental results that the inactivity of MyoD in RMS may be due to the presence of inhibitor that may suppress its function or the absence of a factor that enables MyoD to function as a transcriptional activator.

It has been shown by other studies that RMS might arise as a result of disruption of mutually exclusive yet tightly coupled pathways of muscle differentiation and growth arrest. There are several evidences which point to the involvement of p21 in the process of normal muscle differentiation by inhibiting CDK's thereby inducing cell cycle arrest. MyoD which is involved in the induction of muscle-differentiation specific genes, promotes cell cycle exit by inducing the expression of p21 (Halevy *et al*., 1995). However, in RMS, the expression of MyoD does not lead to cell cycle arrest or terminal differentiation, implicating that MyoD pathway is functionally defective. Study by Weintraub and others in 1997 revealed that the expression of p21 and MyoD has an inverse relationship in these tumors. For example, those cell lines and tumors that express high levels of MyoD were found to express low levels of p21 and vice-versa. This result suggested that endogenous MyoD/p21 pathway is highly compromised and hence unable to induce cell cycle arrest and terminal differentiation. However, forced expression of wild-type p21 resulted in marked inhibition of cell proliferation. Thus they concluded from their findings that concurrent expression of MyoD and p21 is critical for the integrity of the differentiation pathway in skeletal muscle cells and such cooperative expression is defective in RMS pathogenesis.

There are growing evidences which confirm the relevance of epigenetic alterations such as post translational modifications in the progression of cancer (Jones *et al.,* 2002; Ting *et al*., 2006; Sparmann *et al*., 2006). The one in particular associated with various cancer types is altered acetylation and

methylation patterns of histones H3 and H4.

A recent study by (Lee *et al*., 2011) had shown that histone methyltransferase Suv39h1 (KMT1A) aberrantly mediates H3K9 methylation in alveolar rhabdomyosarcoma (ARMS). Their study shows that Suv39h1 is over-expressed in differentiation permissible condition thereby preventing ARMS growth arrest which is a pre-requisite to myogenic differentiation.

The previous study from the same lab (Mal *et al*., 2006) had shown that Suv39h1 is expressed in proliferating myoblasts in C2C12 cells and associates with MyoD and represses MyoD dependent muscle gene expression through its HKMTase activity thereby maintaining the undifferentiated state in myoblasts. However, its expression declines upon myogenic differentiation and thereby its association with MyoD. Similarly in human skeletal myoblasts (HSMM), Suv39h1 is expressed in proliferating myoblasts and declines upon differentiation. Conversely, its expression is deregulated in ARMS cells as it is induced in differentiation permissible condition resulting in increased association between MyoD and Suv39h1causing a substantial increase in the occupancy and activity of Suv39h1 on the myogenin promoter. However, knock-down of Suv39h1 restores MyoD transcriptional activity, arresting cell proliferation and induces terminal differentiation of ARMS cells. Thus their study demonstrated that down-regulation of Suv39h1 ablates its ability to maintain the cells continuously at proliferative state resulting in shifting of the cell to a differentiated state. It was accompanied by the loss of tumorigenicity

as seen by the compromised ability of rhabdomyosarcoma cell to form colonies on soft agar and tumors in mice.

Additional findings point to a role of other HKMTs in rhabdomyosarcomas. Ezh2, a H3K27 methyltransferase which is another well-known negative regulator of skeletal myogenesis, is over-expressed in various tumors particularly in aggressive and poorly differentiated breasts and prostrate carcinomas and is associated with poor prognosis (Simon *et al*., 2008; Caretti *et al*., 2004). Consistent with this, studies have shown that Ezh2 is over-expressed in RMS compared with normal myoblasts (Ciarapica *et al.*, 2009). This work was further expanded by another group recently (Marchesi *et al*., 2012) to analyze the functional role of Ezh2 in RMS. They further confirmed that Ezh2 is over expressed in RMS cell lines and in addition, siRNA mediated knockdown of Ezh2 in rhabdomyosarcoma cells resulted in the re-activation of muscle-specific genes and partial recovery of the differentiated phenotype with the formation of myotube-like cells. Moreover, down-regulation of Ezh2 restores MyoD binding to its target muscle-specific genes in RMS cells.

These studies shed light on the altered expression of HKMTs in pathological conditions and their impact on the normal functions of major transcription factors like MyoD that lead to defect in the execution of terminal differentiation programs. Thus targeting HKMTs through pharmacological intervention may lead to treatment of aggressive pediatric muscle cancer and many other muscle related diseases.

1.14 Perspectives and aims of study

The major aim of this thesis is to analyze the epigenetic regulation of skeletal myogenesis.

Skeletal myogenesis is a multi-step process, which involves commitment, proliferation and differentiation of skeletal muscle precursor cells and culminates in the formation of muscle. This process requires the coordinated expression of various transcription factors like MyoD, Myogenin, Myf5 and MRF4 that control different stages of myogenesis.

There is growing evidence that recruitment of chromatin modifying enzymes and remodeling complexes epigenetically reprogram muscle promoters that either permit or block MRF and Mef2 activities.

Although MyoD and Mef2 factors are expressed in committed myogenic precursor cells and have the ability to trigger the differentiation program, their transcriptional activity is prevented by recruitment of HDACs and HKMTs. For instance, in undifferentiated myoblasts, muscle promoters exhibits a key epigenetic modification on H3K9 characterized by reduced acetylation, a mark of active transcription, as well as increased di-methylation (H3K9me2) which is a transcriptional repression mark (Zhang *et al*., 2002; Mal and Harter, 2003). This occurs as a result of association of MyoD with co-repressors such as HDAC1, and the SET domain containing histone methyltransferase Suv39h1 that mediates H3K9 deacetylation and tri-methylation (H3K9me3) respectively (Puri *et al*., 2001; Mal *et al*., 2001; Mal, 2006). In addition to H3K9me3, another key modification is H3K27me3, which is mediated by

Ezh2 (Caretti *et al*., 2004). Thus reduced acetylation and increased methylation results in a closed chromatin configuration that restrains MyoD activity. However upon differentiation, the expression of HDACs and HKMTs declines and also their association with MyoD. Subsequently other chromatin modifiers such as p300 and P/CAF (HATs) are recruited. p300 mediates acetylation of H3 and H4 at muscle promoters followed by P/CAF which interacts and directly acetylates MyoD. This results in transactivation of MyoD which in turn activates genes important in muscle differentiation (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010).

G9a is a predominant H3K9 methyltransferase in euchromatin and is associated with transcriptional silencing. However, it has not been implicated in regulation of skeletal myogenesis, although the role of other histone lysine methyltransferases like Suv39h1 and Ezh2 are well established in skeletal muscle development and differentiation.

The first part of my thesis work demonstrates that G9a is a negative regulator of skeletal muscle differentiation that plays a dominant role in MyoD activation through chromatin modifications as well as its methylation. It inhibits myogenesis in a methyltransferase activity dependent manner. G9a overexpressing cells exhibit increased H3K9me2 on myogenic promoters. In addition, G9a interacts with MyoD and methylates at K104 restraining MyoD transcriptional activity. Interestingly, K104 is an overlapping site for G9a mediated methylation and P/CAF mediated acetylation, suggesting the possibility that G9a might disrupt P/CAF-mediated MyoD acetylation. Thus

the second part of my thesis work demonstrates the cross-talk between G9a, and P/CAF to control MyoD activity. Protein interaction studies revealed that G9a alters the ability of P/CAF to interact with MyoD and correspondingly MyoD acetylation. Moreover, depletion of G9a in proliferating myoblasts by siRNA mediated knockdown alters the kinetics of acetylation. In addition to its impact on MyoD acetylation, the use of a selective P/CAF inhibitor in muscle cells followed by microarray analysis has allowed us to identify novel P/CAF target genes that suggest a much broader role in regulating skeletal muscle differentiation.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Preparation of Basal Media

Basal media was prepared with Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Sigma Aldrich) supplemented with 3.7g/L of Sodium bicarbonate (Sigma Aldrich) and 1% penicillin-streptomycin (Sigma Aldrich), which was then filtered using a filtration unit.

2.2 Cell culture

2.2.1 C2C12 Cell Culture

C2C12 cells (mouse myoblasts cell line) were cultured at 50-60% density in growth medium (GM) containing DMEM supplemented with 20% fetal bovine serum (FBS) (Hyclone) and 1X penicillin-streptomycin. For differentiation assays, C2C12 cells were cultured at high density (80-90%) in GM and induced to differentiate in differentiation medium (DM) made in DMEM supplemented with 2% horse serum (Gibco).

2.2.2 HEK293T and C3H10T1/2 Cell Culture

HEK293T and C3H10T/2 (10T1/2) cells were cultured in DMEM supplemented with 10% FBS. 10T1/2 cells were switched to DM (DMEM supplemented with 2% horse serum) for myogenic conversion assays.

2.2.3 Rh30 Cell culture

Rh30 (ARMS cell line) cells were cultured at 50-60% density in RPMI 1640 basal medium supplemented with 10% FBS and switched to DM (RPMI 1640 supplemented with 2% HS) at a density of 80-90%.

All cultures were incubated at 37˚C, in a humidified 5% CO_2 incubator (Sanyo).

2.3 DNA constructs

Plasmids EGFP-G9a, EGFP-G9aΔSET (1-830 aa), Flag-G9a (1001 aa), Flag-G9aΔANK (814 aa) were kindly provided by Dr. Martin J. Walsh, (Mt Sinai School of Medicine, New York, NY 10029). Myc-MyoD, Flag-MyoD, Flag-MyoD (K104R), Flag-MyoD (RRR) were kindly provided by Dr.Vittorio Sartorelli (National Institute of Arthritis, Musculoskeletal and Skin diseases, Bethesda, MD20892) and Flag-P/CAF was kindly provided by Dr.Yoshihiro Nakatani (National Institutes of Health). For luciferase reporter assays, a firefly luciferase reporter construct (4R-tk-Luc) containing four tandem E boxes from the Muscle Creatine Kinase (MCK) enhancer upstream of the thymidine basal promoter was used (kindly provided by Dr.Robert S. Krauss, Mt Sinai School of Medicine, New York, NY 10029).

2.4 Treatment of cells with G9a and PCAF inhibitors

2.4.1 Treatment of cells with BIX-01294

BIX-01294 (a diazepin-quinazolin-amine derivative), a small molecule inhibitor of G9a and GLP methyltransferase activity (Kubicek *et al*., 2007, Gazzar *et al*., 2008, Chang *et al*., 2009) was used. To determine the optimal BIX-01294 concentration, 0 to 6μM was tested in C2C12 cells and C3H10T1/2 cells. Treatment with 2.5μM resulted in enhanced differentiation and a concentration higher than 4μM resulted in cell death. Thus 2.5μM was used for subsequent experiments.

C3H10T1/2 cells cultured in GM were incubated with 2.5µM BIX-01294 for 8 hours and cells in DM were incubated with 2.5µM BIX-01294 for another four days. As controls, cells were treated with 2.5µM DMSO (vehicle). Cell lysates were collected at the end of respective time points and analyzed by western blot. Differentiated cells were immunostained with anti-MHC antibody.

2.4.2 Treatment of cells with UNC0638

UNC0638, a chemical probe which selectively inhibits G9a and GLP methyltransferase activity with higher potency and lower toxicity (Vedadi *et al*., 2011) compared to BIX-01294 was used. After trying different titrations (0-3µM) in Rh30 cells, 1.5µM was found to be the optimal concentration and was used for subsequent experiments.

Rhabdomyosarcoma cells (Rh 30) were seeded in a six-well plate at a density of 0.1 million cells for day 0 time point and 0.2 million cells were seeded for differentiation. Cells were treated with 1.5µM of UNC0638; and control cells were treated with 1.5µM of DMSO. The following day, cell lysates were collected for Day 0 time point. For differentiation, medium was changed from GM to DM along with 1.5µM of UNC0638. Cell lysates were collected at different time points as indicated in the figures (results section).

2.4.3 Treatment of cells with embelin

Embelin (a hydroxy benzoquinone class of natural compound), a small molecule inhibitor of PCAF was used. To determine the optimal embelin concentration, 0 to 20µM was tried in C2C12 cells. As treatment with 10µM resulted in impairment of myotube formation and a concentration higher than

10µM resulted in cell death; 10µM was used for subsequent experiments. An equivalent concentration of its analogue derivative, MJTK-4 (an inactive compound) was used as control, in addition to DMSO treated cells.

2.5 Transformation

100ng of DNA was added to 25-50µL of DH5α competent cells, mixed by tapping gently and incubated on ice for thirty minutes. Cells were subjected to heat shock at 42ºC for 45 seconds and quickly cooled on ice for 2 minutes. The transformed cells were grown in 1mL of pre-warmed LB (Luria-Bertani) broth in an orbital shaker at 37ºC at 200 rpm for 1 hour. The LB broth containing transformed cells were centrifuged at 13,000 rpm for 1 minute; the supernatant was decanted, and 100µL of fresh LB broth was added to the pellet for resuspension. 70µL of the cultured medium were plated into LB agar plates containing ampicillin (100µg/mL) and incubated overnight at 37˚C. The next day, colonies were picked and inoculated in a starter culture of 2mL of LB medium containing ampicillin and incubated in an orbital shaker at 37˚C overnight with vigorous shaking (200 rpm). After overnight incubation, the starter culture was diluted by transferring 2mL of the starter culture into1L conical flask containing 300mL of LB-ampicillin and grown overnight in an orbital shaker at 37˚C with vigorous shaking (200 rpm).

On the next day, the bacterial culture was transferred into centrifuge (GSA) bottles (Nalgene-Thermo Scientific) and harvested by centrifugation in GSA rotor at 5000 rcf for 15 minutes at 4˚C. The supernatant was decanted and plasmid was extracted from the bacterial pellet. The bacterial pellet was

resuspended completely in 4mL of buffer P1 (with RNaseA added) by vortexing and pipetting. The resuspended bacterial pellet was transferred to 50mL tube. The bacterial cells were lysed using 4mL of buffer P2 by vigorously inverting the sealed tubes 4-6 times and incubated at room temperature for 5 minutes. Pre-chilled buffer P3 was then added to the lysate, mixed thoroughly and was incubated on ice for 15 minutes.

After 15 minutes incubation on ice, the lysate was centrifuged at 4000 rpm for 30 minutes at 4˚C; the clear supernatant was then collected in another 50mL eppendorf tube. The supernatant was again centrifuged at 4000 rpm for 15 minutes at 4˚C to avoid the presence of any suspended material which could clog Quiagen-tip. Qiagen midi column was equilibrated with 4mL of buffer QBT. The cleared lysate was filtered into the pre-equilibrated column. The column was washed with 10mL of buffer QC. The wash was repeated one more time with 10mL of buffer QC to ensure removal of all contaminants. The DNA was then eluted using 5mL of buffer QF, precipitated by adding isopropanol, and centrifuged at 13,000 rpm for 30 minutes at 4˚C. Then the supernatant was carefully decanted and the DNA pellet was washed with 2mL of 70% ethanol; centrifuged at 13,000 rpm for 10 minutes at 4˚C. The supernatant was again carefully decanted and the pellet was air-dried for 10 minutes. The air-dried DNA pellet was redissolved in TE buffer (pH 8.0). The concentration of DNA was measured by nano-spectrophotometry.

2.6 Transient Transfection

2.6.1 Transient Transfection of plasmids

A day before transfection, one million cells were seeded on a 100 mm-diameter plate. The plasmid was transfected into cells using Lipofectamine Plus (Invitrogen) according to manufacturer's protocols.

Briefly, on the day of transfection, 4µg plasmid was diluted in 300µL of basal DMEM (without serum) in a 1.5mL eppendorf tube. To this, 15µL of Plus reagent was added, mixed and incubated for 15 minutes at room temperature. In another tube, 20µL of lipofectamine reagent was diluted in 300µL of basal DMEM (without serum) and mixed.

The pre-complexed DNA (with Plus reagent) was mixed with diluted lipofectamine reagent and incubated for 15 minutes at room temperature. The medium for cells plated for transfection was replaced from serum-containing to serum-free medium.

After 15 minutes of incubation, the transfection mixture in the tube was added in a drop-wise manner to the cells. The cells were incubated at 37ºC in 5% CO_2 incubator for 4-5 hours. At the end of incubation time period, the basal DMEM (without serum) was replaced with complete medium.

2.6.2 Transient Transfection for dual-luciferase reporter assay

10T1/2 cells were transfected with 50ng of 4R-tk-Luc and MyoD in the absence and presence of 50ng of EGFP-G9a or EGFP-G9aΔSET in a 24-well plate. For another reporter assay, C2C12 cells were transfected with 50ng of

4R-tk-Luc in the absence and presence of 50ng of EGFP-G9a or EGFP-G9aΔSET.

Transfection was carried out using the Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. 5ng of renilla luciferase was used as an internal control. pCS2-empty expression was added to normalize the amount of DNA.

After 48 hours of transfection, media was removed from the plate and rinsed with 1XPBS. Cells were harvested by lysing with 1X Passive Lysis Buffer and gently rocking the plate for 15 minutes at room temperature. The luciferase activities were measured using Tecan microplate reader and Magellan 6 software.100µL of LAR II was added to 20µL of the lysates dispensed in a 96-well plate and measured for firefly luciferase signal. This was followed by the addition of 100µL of Stop and Glo reagent to stop the firefly luciferase activity and measure the renilla luciferase activity. The firefly luciferase reading was normalized to that of renilla luciferase signal for each individual well. All the transfections were carried out in triplicates and repeated at least two times.

2.6.3 Transient transfection of siRNAs

A day before transfection, 0.1 million cells were seeded in a six-well plate. On the day of transfection, each well was transfected with 100nM of siRNA specific for G9a (Dharmacon; on-target plus smart pool) or control scrambled siRNA (Dharmacon; on-target plus control pool; non-targeting pool).

Sequences for human and mouse siG9a are shown in Table II and Table III respectively.

The siRNAs were transfected into the cells using Lipofectamine RNAi Max (Invitrogen) according to manufacturer's protocol. Six microliters of siRNAs were added to 100µL of basal DMEM and 3µL of RNAi Max Lipofectamine was added to 100µL of basal DMEM. The two reaction mix tubes were combined, mixed well and incubated for 15 minutes at room temperature. The mixture was added drop-wise to each well containing 1mL of basal DMEM. After three hours, transfection medium (serum free basal DMEM) was aspirated and replaced with fresh complete medium. 48 or 72 hours after transfection, C2C12 myoblasts or Rh30 cells were switched from GM to DM for differentiation time points.

2.7 Western Blotting

2.7.1 Protein Extraction

At appropriate time points, the medium was aspirated from the 100 mm-diameter plate or six-well plates using pasteur pipette and washed with 3mL of 1X PBS to remove any residual medium. Total protein extraction was carried out using Radioimmunoprecipitation assay (RIPA) lysis buffer containing 50mM NaCl (Numi), 1mM EDTA (Sigma-Aldrich), 50mM Tris-HCl (1^{st} Base), 1% Triton X-100 (USB), 0.05% SDS (Numi), 1X Protease inhibitor (Roche) and 0.1% Sodium deoxycholate (Sigma-Aldrich). Lysates were collected using cell scraper (SPL Life Sciences) and transferred to pre-chilled 1.5mL eppendorf tubes. The tubes containing lysates were incubated on

rotator (Barnstead Thermolyne) at 4ºC for 30 minutes before being spun (Eppendorf Centrifuge 5417R) at 12000 rpm for 15 minutes at 4ºC. The supernatant was transferred to another empty 1.5mL eppendorf tube and stored at -20ºC, while the pellet was discarded.

2.7.2 Bradford Protein Assay

Protein concentration was measured using Bio-Rad Protein Assay Kit (Bio-Rad). One part of the Bradford reagent was diluted with four parts of MilliQ water. 1μL of protein sample was added to 1mL of diluted reagent in a cuvette, vortexed to ensure homogeneity and incubated at room temperature for 5 minutes (cuvette containing 1mL of diluted reagent was used as a blank). Protein concentration was measured by absorbance (ABS) at 595 nm using spectrophotometer.

2.7.3 SDS-PAGE

A 10% resolving gel and 4% stacking gel were casted using 30% Acrylamide-Bis solution 37.5:1 (2.6% C). Protein lysate was mixed with 1X loading dye in 1.5mL eppendorf tube and heated at 95ºC for 5 minutes. Protein samples were spun at 12,000 rpm for 1 minute and loaded into wells of SDS-PAGE gel. Gel containing protein lysate samples were electrophoresed into 1X running buffer (3.03g Tris base, 14.4g glycine, 10ml of 10% SDS/L) at 80V for fifteen minutes, followed by 95V for two hours until the dye front reached the end of the plate. After electrophoresis, the gel was removed and proteins were transferred onto a nitrocellulose membrane (AmershamHybond ECL, GE

Healthcare) in 1X transfer buffer (3.03g Tris base, 14.4g glycine, 20% ethanol/L) for 1.5 hours at 100V.

2.7.4 Immunodetection

The nitrocellulose membrane with protein was blocked with 5% w/v skimmed milk in 1X PBST with 0.1% Tween-20 (Bio-Rad) for one hour at room temperature. The membrane was incubated with diluted primary antibody in 5% milk in PBST for one hour at room temperature or overnight in 4°C. Unbound primary antibody was removed by washing thrice with 1X PBST. Following this, the membranes were incubated with horse radish peroxidase conjugated secondary antibodies (goat anti-mouse or rabbit IgG (Sigma) at 1:5,000 dilution for one hour at room temperature.

The following primary antibodies required overnight incubation at 4ºC : rabbit polyclonal anti-MyoD (Santa Cruz Biotechnology) at 1:500 dilution, rabbit polyclonal anti-Myogenin (Santa Cruz Biotechnology) at 1:400 dilution, mouse monoclonal anti-GFP (Santa Cruz Biotechnology) at 1:400 dilution, rabbit polyclonal anti-G9a (Cell signaling technology) at 1:300 dilution, rabbit polyclonal anti-acetyl-lysine (Ac lysine) (Abcam) at 1:1000 dilution, rabbit polyclonal anti-methyl-lysine (Me lysine) (Abcam) at 1:1000 dilution.

The following primary antibodies were all incubated at room temperature for one hour: monoclonal anti-Myc (Sigma) at 1:2000 dilution, monoclonal anti-Flag (Sigma) at 1:1000 dilution, monoclonal anti-Troponin T (Sigma) at 1:3000 dilution, monoclonal anti-β-actin (Sigma) at 1:10,000 dilution and

monoclonal anti-EF1-α (Upstate Biotechnology) at 1:1000 dilution; (the last two were used as internal controls).

2.7.5 Visualization

The bound antibodies were detected using the detection reagent (Amersham ECL Western Blotting System, GE Healthcare) for one minute. The blot was exposed to X-ray film (Thermo Scientific) for different exposure times.

2.8 Immunoprecipitation

The following steps were used for co-immunoprecipitation:

20µL of anti-mycagarose beads (Sigma Aldrich) or anti-flag agarose beads (Sigma Aldrich) were added to 1.5mL eppendorf tubes and washed twice with 1x PBS. The supernatant was decanted and the beads were retained by centrifuging at 12,000 rcf. To the beads, 500µg of cell lysates were added and topped up to 500µL with RIPA lysis buffer containing 1X protease inhibitor. The tubes were incubated overnight on a rotator at 4ºC. The next day, the samples were spun at 10,000 rcf for 5 minutes at 4ºC. The supernatant was discarded carefully without disturbing the beads. The beads were washed with RIPA lysis buffer (without 1X protease inhibitor) four times for five minutes in rotator at 4ºC. At the end of the last wash, the supernatant was removed by centrifugation at 10,000 rcf for three minutes and the beads were retained. To the beads, 2X sample buffer was added, mixed gently by tapping. Samples were boiled on the heat block (for denaturation of proteins) at 95ºC for 10 minutes. Samples were spun at 10,000 rcf for 2 minutes and then loaded into SDS-PAGE.

The following steps were carried out for endogenous immunoprecipitation:

15µL of A/G plus agarose beads (Santa Cruz Biotechnology) were added to 1.5mL eppendorf tubes. To the beads, 1mg of cell lysates were added and the tube was incubated on ice and tapped every ten minutes thrice, for a total of thirtyminutes. At the end of thirty minutes, samples were spun at 10,000 rcf for five minutes, the supernatant was collected in another tube and the beads were discarded. This step is meant for pre-clearing the lysates. To this pre-cleared lysates, 2µg of antibody was added and topped up to 500µL with RIPA lysis buffer containing 1X protease inhibitor and incubated overnight at 4ºC on a rotator. The next day, 20µL of A/G plus agarose beads were added to each sample containing tubes and again incubated at 4ºC on a rocker for 2 hours. The samples were spun at 10,000 rcf for five minutes at 4ºC. The supernatant was removed and the beads retained. The beads were washed with RIPA lysis buffer (without 1X protease inhibitor) three times for five minutes on rotator at 4ºC. At the end of the last wash, supernatant was removed by spinning at 10,000 rcf for three minutes and the beads were retained. To the beads, 2X sample buffer was added, mixed gently by tapping gently before heating the sample at 95˚C for 10 minutes. Samples were spun at 10,000 rcf for two minutes and then loaded into SDS-PAGE.

2.9 In vitro methylation assay

In vitro methylation assay was carried out as described (Nishio *et al*., 2004). Briefly, 3µg of purifiedpoly-histidine–tagged recombinant proteins, full length human G9a (hG9a), the SET mutant (hG9aΔSET) was incubated with 2µg of

purified full length human MyoD (hMyoD) fused to a GST affinity tag in a reaction buffer containing 50mM Tris–HCl, pH 8.5, 1mM DTT, 5mM $MgCl_2$, 1µM [^{14}C]-labeled S-adenosyl-L-methionine [^{14}C] SAM (Perkin Elmer), 50mM SAM (Sigma). GST-histone H3 tail (1-57) was used as a control. In addition to G9a, human Set7/9 (H3K4 methyltransferase) was also used. Proteins were separated using 15% SDS-Polyacrylamide gel and visualized on the gel by coomassie blue staining and fluorography.

2.10 Liquid chromatography-mass spectrometry (LC-MS) analysis

LC-MS analysis (Thermo Electron, Finnigan LTQ) was carried out using the tryptic digestion of recombinant MyoD polypeptide treated with wild-type G9a or its mutant G9aΔSET.

2.11 Quantitative real-time polymerase chain reaction (Q-PCR)

Total mRNA was isolated using TRIZOL (Invitrogen) according to the manufacturer's instructions. First strand cDNA was synthesized using AMV Reverse transcriptase (Promega) according to the manufacturer's instructions with 2µg of RNA. QPCR was performed using Lightcycler 480 SYBR Green 1 Master Kit (Roche). GAPDH was used as an internal control. Gene specific primer sequences for G9a, MCK, MRF, MYH1 and GAPDH are provided in Table I. QPCR reaction was performed in triplicates to obtain CT values. CT values of the samples were normalized to internal control GAPDH to obtain delta CT (ΔCT). Relative expression was calculated by 2^{Λ}- ΔCT method.

2.12 Cell culture for differentiation assay

C2C12 or Rh30 cells were seeded in six well plates (Greiner bio-one). For day 0 time point, 0.1 million cells (50% - 60% confluent) were seeded per well in GM and for differentiation time points, 0.27 million cells (80% - 90% confluent) were seeded and then switched to DM for various differentiation time points.

2.13 MyoD dependent myogenic conversion assay

2.7×10^6 C3H10T1/2cells were seeded on six-well plate in GM, a day before transfection. On the day of transfection, each well was transfected with 1.5µg of MyoD alone or co-transfected with either 0.5µg of EGFP-G9a or EGFP-G9aΔSET in basal DMEM using Lipofectamine Plus reagent (Invitrogen) according to manufacturer's protocols. pCS2-empty expression was added to normalize the amount of DNA. The cells were incubated at 37ºC in 5% CO_2 incubator for 3 hours. At the end of incubation, medium was replaced from basal DMEM (without serum) to serum containing DMEM. At the end of 24 hours of transfection, cells were switched to DM for 96 hours (Day 4).

2.14 Immunofluorescence

C2C12 or 10T1/2 cells were cultured on Thermonax plastic cover slips (Rochester NY, USA) with GM in a six-well plate and switched to DM. At the end of three to four days of differentiation, media was aspirated from differentiating cells and a sufficient volume of 1X PBS (Phosphate Buffered Saline) was added to wash the cells. Washing was done thrice by gently swirling the plate to remove any traces of media. Cells were fixed with 4%

paraformaldehyde for 10 minutes. Fixed cells were washed three times with 1X PBS for 5 minutes each. Cells were then permeabilized and blocked with 0.5% Triton-X-100 in 1X PBS with 10% horse serum for one hour at room temperature.

After one hour of blocking, cells were incubated for one hour at room temperature with the following antibodies:

Myosin Heavy Chain, anti-MHC (MY32) antibody (Sigma) at 1:400; anti-MyoD (5.8A) antibody (Santacruz Biotechnology) and anti-G9a (C6H3) antibody (Cell Signaling) at 1:300 concentrations were used. Cells were washed with 1X PBS thrice, for five minutes each. Secondary antibody coupled with Alexafluor (Alexafluor 508 and Alexafluor 488) at 1:250 was added for one hour. Cells were again washed with 1X PBS thrice, for five minutes each. Nuclei were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA).

2.15 Myogenic index

Myogenic index was calculated by quantifying the ratio of nuclei in MHC^{+}myotubes/total nuclei. At least 400 nuclei were counted.

2.16 Immunofluoroscence microscopy

Immunofluorescence images were taken using the fluorescence microscopy (Nikon Eclipse TE-2000U) with Metamorph software version 7.0r3 with 10X objective lens.

2.17 Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out using ChIP assay kit as described earlier (Gulbagci *et al.* 2009). Briefly, C2C12 cells were cultured in GM (Day 0) and switched to DM (Day 2). Cells were then fixed in 1% formaldehyde for 10 minutes at 37ºC. Fixed cells were washed with 1X PBS and scraped from culture plate. Cells were resuspended in SDS lysis buffer (50mM Tris-Cl PH 8.1, 1% SDS, 10mM EDTA, 1µg/mL aprotinin, 1mM PMSF, 1µg/mL pepstatin A). Cells were sheared by sonication to obtain DNA fragments and the chromatin was immunoprecipitated with 2µg antibodies directed against H3K9ac (histone 3 acetyl lysine 9, Abcam), H3K9K14ac (histone 3 acetylated lysine 9 and lysine 14, Upstate), H3K9me2 (histone 3 dimethyl Lysine 9, Upstate). The cross links were then heat reversed at 65ºC and DNA was purified using phenol chloroform. DNA was quantified by Q-PCR using primers which are specific to myogenin and MCK promoter sequence and β-actin as described in Table I. Each sample was performed in triplicates and the values obtained were normalized to β-actin.

2.18 Global H3K9 acetylation

C2C12 cells were cultured in GM (Day 0) and then switched to DM with 10µM of embelin or MJTK-4 (control) and induced to differentiate for two days (Day 2). Total protein extraction was carried out using 1X Laemmli buffer containing 62.5mM Tris, pH 6.8, 10% glycerol and 2% SDS. Samples were heated for 10 minutes at 90ºC and allowed to cool down to room temperature. Samples were then briefly sonicated (5 pulses for 2 seconds each)

and heated for 5 minutes at 90ºC after addition of 5X loading buffer. It was then analyzed by western blot for H3K9ac using anti-H3K9ac antibody (Abcam). HistoneH3 levels were assayed as a control using anti-histone H3 antibody (Abcam).

2.19 Microarray on C2C12 cells

C2C12 cells were cultured in GM for 24hours (Day 0) in the presence of 10µM embelin or DMSO (control) and induced to differentiate in DM in presence of 10µM embelin or DMSO for another 24 hours (Day1) and 48hours (Day2) respectively. Total RNA was isolated from proliferating and differentiated C2C12 cells with Trizol (Invitrogen). The experiment was carried out with two biological replicates obtained from DMSO or embelin treated cells. RNA was cleaned using RNeasyMinElute Cleanup Kit (Qiagen) and reverse transcribed, labeled and subsequently hybridized to Illumina mouse WG-6 v2.0 array.

Normalization of gene expression data in Genespring GX 12.0(Agilent) was carried out by shifting the intensities to 75th percentile and baseline transformation was done to median of all samples (treated and control). Statistical significance was determined by the two-tailed, un-paired ($p<0.05$) student's t test and all significant changes above 1.5-fold were selected (Bionivid Technology, Bangalore). Gene ontology (GO) and significantly altered signaling pathway identification was carried out using GeneSpring GX v10.0.2 (Bionivid Technology, Bangalore).

Biological network analysis was carried out using Genespring GX v.12.0. A total of 171 Genes that are annotated to be involved in myogenic differentiation was obtained from Entrez Gene database. Biological networks were produced by comparing the input list to a reference list containing >1.4 million reactions which was generated by natural language processing algorithm and from different interaction databases. Expanded networks were constructed by setting an advanced filter that included the categories of binding, expression, metabolism, promoter binding, protein modification and regulation.

2.20 Statistical analyses

All graphs shown indicate mean values and error bar indicate standard deviation (SD). Statistical significance was determined by two-tailed, un-paired Student's t test and *p* values of <0.05 were considered to be statistically significant. Different degrees of statistical significances were indicated by asterisks as follows: * is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$.

CHAPTER 3

RESULTS

3. Results

3.1 Role of G9a in skeletal muscle differentiation

3.1.1 Analysis of G9a expression during skeletal muscle differentiation

To examine whether G9a has a role in skeletal myogenesis the expression of G9a was analyzed in skeletal muscle cells. Total mRNA and protein levels were examined in undifferentiated and differentiated C2C12 cells by quantitative real time PCR (Q-PCR) and western blot. G9a mRNA was expressed in undifferentiated cells (Day 0) and was rapidly down-regulated after culture of cells in differentiation medium for three days (Day 3) (**Fig 3.1.1.A**). Similarly, G9a protein also declined upon induction of differentiation, which inversely correlated with the expression of MyoD and its target gene Troponin T (a terminal marker for differentiation) which were up-regulated upon differentiation (**Fig 3.1.1.B**). From these data, it is clear that G9a is expressed at high levels in proliferating myoblasts and its expression declines upon differentiation. This suggests that the decline of G9a may be essential for differentiation of myoblast into myotubes.

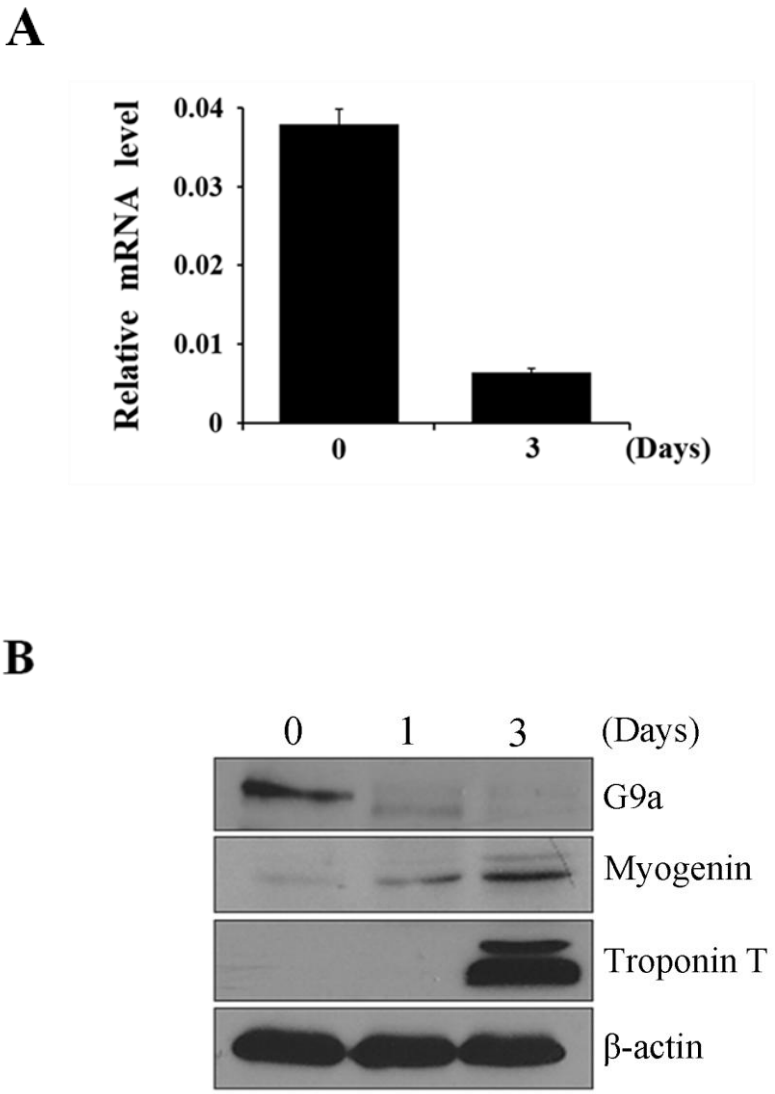


Figure 3.1.1 The expression of G9a decreases during skeletal muscle differentiation: (A) Q-PCR analyses of G9a expression using RNA isolated from undifferentiated (Day 0) and differentiated (Day3) C2C12 cells. GAPDH was used as a loading control. Raw data provided in Table IV. (B) G9a, MyoD and Troponin T proteins were analyzed using cell extracts from C2C12 cells at the given time points (Day 0, 1 and 3) by western blot. β-actin was used as internal control.

3.1.2 Overexpression of G9a inhibits skeletal muscle differentiation

To test whether G9a regulates myogenesis, gain of function studies were carried out. Since G9a is down-regulated during myogenic differentiation in C2C12 cells, the consequences of ectopic expression of G9a in C2C12 cells under conditions that normally promote differentiation was analyzed. C2C12 cells were transfected with full length G9a (FLAG-G9a). Empty vector (pCS2) transfected cells were used as controls (**Fig 3.1.2.A**). Cells were switched to DM and induced to differentiate for four days. C2C12 cells expressing full length G9a (FLAG-G9a) were examined for their ability to differentiate, compared to control vector (pCS2) cells. In contrast to control cells, FLAG-G9a cells exhibited a morphological defect in the formation of elongated multinucleated myotubes suggesting a defect in myogenic differentiation (**Fig 3.1.2.B**). To further validate these findings, control and G9a overexpressing cells were fixed with paraformaldehyde and immunostained with anti-myosin heavy chain (MHC^+) antibody, which is a cellular marker of terminal differentiation. As shown in (**Fig 3.1.2.C**) the percentage of MHC^+ cells was reduced upon G9a overexpression. In addition, cell lysates were collected at Day 0, 1 and 3 time points and analyzed for expression of myogenin, which was down-regulated (**Fig 3.1.2.D**). These data show that G9a causes an overall defect in myogenic differentiation.

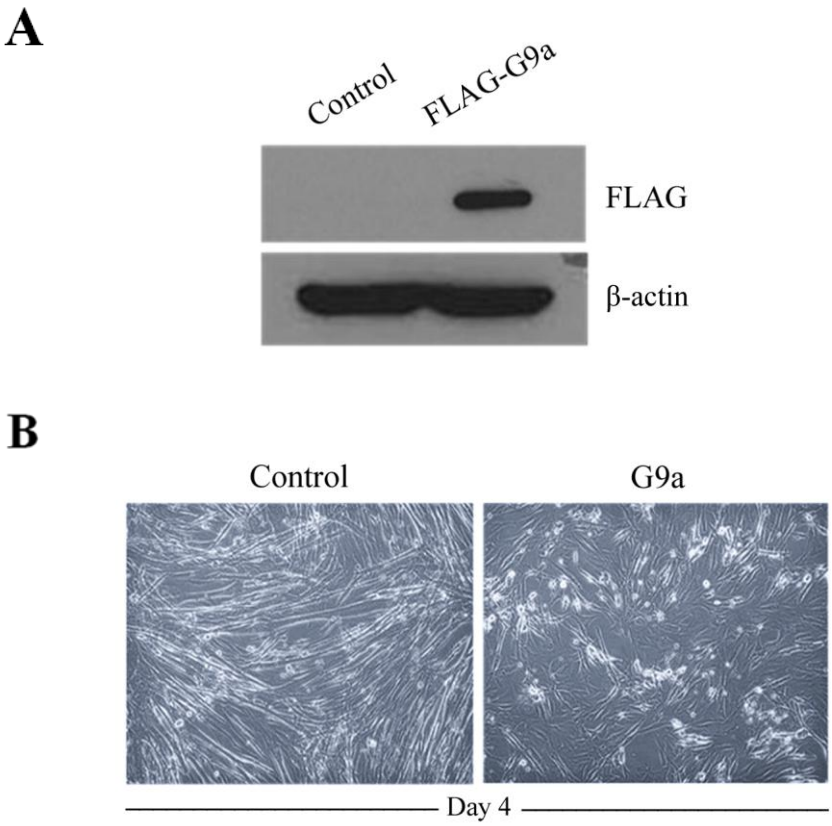


Figure 3.1.2 A&B Over-expression of G9a inhibits skeletal muscle differentiation: (A) C2C12 myoblasts were transfected with full-length FLAG-G9a or pCS2 (vector control) and analyzed for G9a expression by Western blot analyses. β-actin was used as internal control. (B) Control and Flag-G9a expressing cells were then switched to differentiation medium for four days and the myoblast differentiation defect was observed in FLAG-G9a over-expressing cells compared to control cells by capturing the image at 10X magnification using phase contrast microscopy.

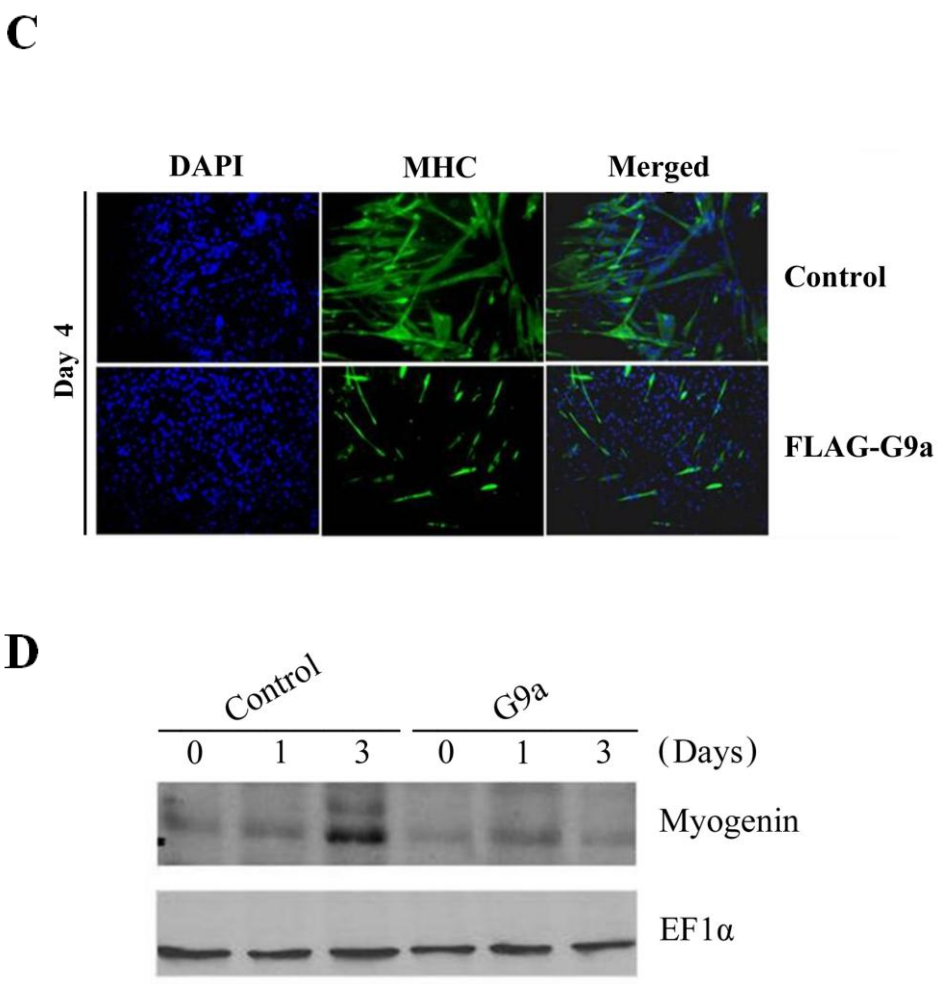


Figure 3.1.2 C&D Over-expression of G9a inhibits skeletal muscle differentiation. (C) Differentiated cells (Day 4) were also immunostained with anti-MHC (green) antibody. Nuclei were stained with DAPI (blue). Images were captured under fluorescent microscope using 10X magnification. (D) Cell lysates were analyzed by western blot for myogenin expression at days 0, 1 and 3. β-actin was used as internal control.

3.1.3 G9a represses myogenesis in a methyltransferase activity dependent manner

To examine whether G9a represses myogenic differentiation in a SET domain dependent manner, we over-expressed a deletion mutant lacking the SET domain (EGFP-G9aΔSET) in addition to expressing full length G9a (EGFP-G9a) in C2C12 cells (**Fig 3.1.3.A**). Control cells were transfected with empty vector (pCS2). Western blot analysis showed that both proteins were expressed at similar levels (**Fig 3.1.3.B**). Cells expressing EGFP-G9a and EGFP-G9aΔSET were compared to control vector (EGFP) cells for their ability to differentiate. Morphological analysis of cells indicated that EGFP-G9a overexpression caused a defect in myotube formation (**Fig 3.1.3.C**). However, EGFP-G9aΔSET overexpressing cells differentiated normally similar to control (EGFP) cells (**Fig 3.1.3.C**). This suggests that G9a requires its methyltransferase activity to repress muscle differentiation.

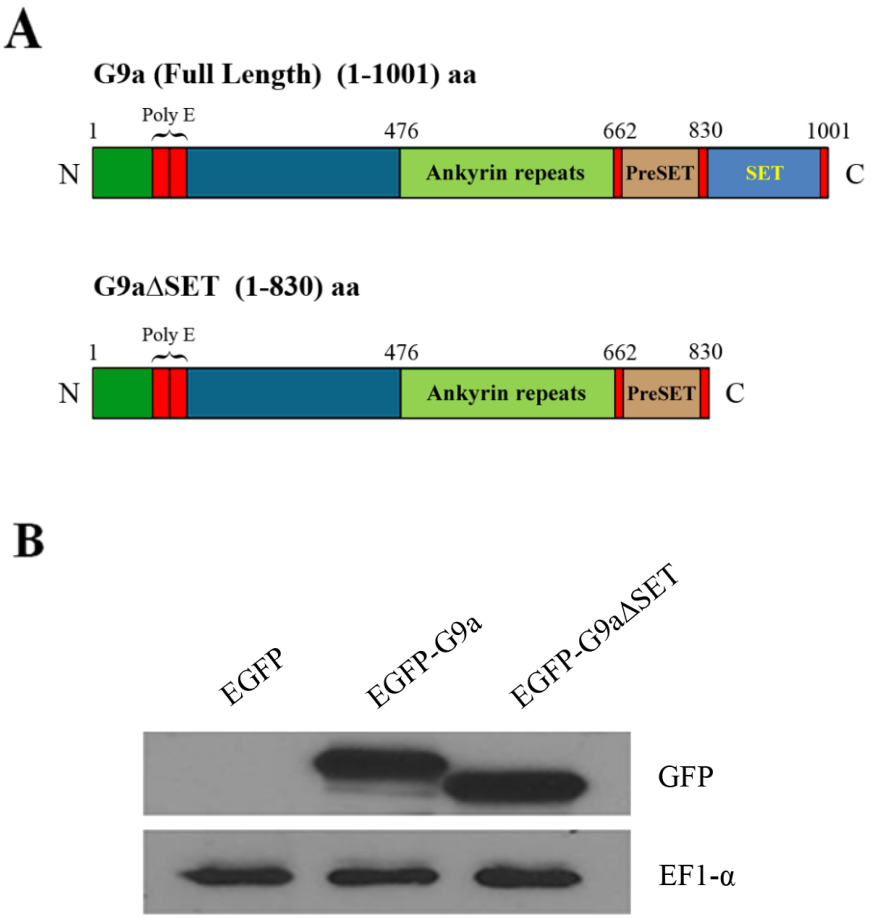


Figure 3.1.3A&B G9a represses myogenic differentiation in a methyltransferase activity dependent manner: (A) Diagrammatic representation of full length G9a (1-1001 aa) and a deletion construct G9AΔSET (1-830 aa) which lacks catalytic activity. (B) C212 myoblasts were transfected with full length EGFP-G9a or EGFP-G9aΔSET and analyzed for expression by western blot. EGFP-G9a and EGFP-G9aΔSET were expressed at equivalent amount. EF1α was used as internal control.

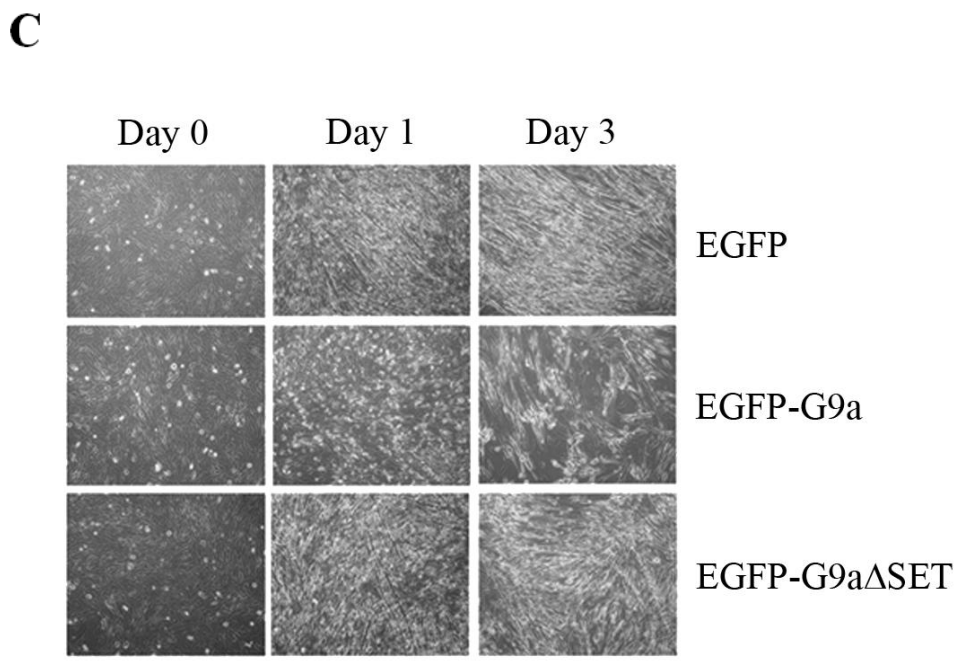


Fig 3.1.3C G9a represses myogenic differentiation in a methyltransferase activity dependent manner. (C) Cells were switched to differentiation medium for indicated time points (Day 1 and Day 3) and differentiation was observed by phase-contrast microscopy.

3.1.4 Myogenic differentiation is enhanced by knockdown of G9a in muscle cells

Since ectopic expression of G9a results in impairment of myotube formation with the repression of differentiation associated genes, we examined whether G9a silencing has an effect on the overall differentiation program. Proliferating C2C12 cells were transfected for 72 hrs with 100nM scrambled siRNA or G9a specific siG9a. As shown in **Fig 3.1.4.A**, siG9a reduced endogenous G9a levels in C2C12 cells. Densitometric quantification of western blots revealed a 71% down-regulation of G9a expression at protein level compared to control cells that were untransfected, or those expressing siRNA. To assess the effect of G9a knockdown on myogenesis, control and siG9a cells were induced to differentiate into myotubes after 72 hrs of transfection. As expected, siRNA transfected cells had no effect on the overall morphological differentiation compared to control cells. In contrast, C2C12 cells transfected with siG9a exhibited enhanced muscle differentiation within two days of culture in differentiation medium as seen by the increase in the number of myotubes compared to untransfected C2C12 cells or those expressing siRNA (**Fig 3.1.4.B**). This was also reflected by increased number of MHC^+ cells (**Fig 3.1.4.C**) as well as a significant increase in the myogenic index (**Fig 3.1.4.D**) compared to controls (untransfected and siRNA cells). Western blot analyses was performed for Myogenin as well as Troponin T on cell extracts obtained from G9a depleted cells (siG9a) or control (untransfected and siRNA) C2C12 cells. The expressions of both proteins, were induced with an earlier kinetics in siG9a cells (**Fig 3.1.4.E**).

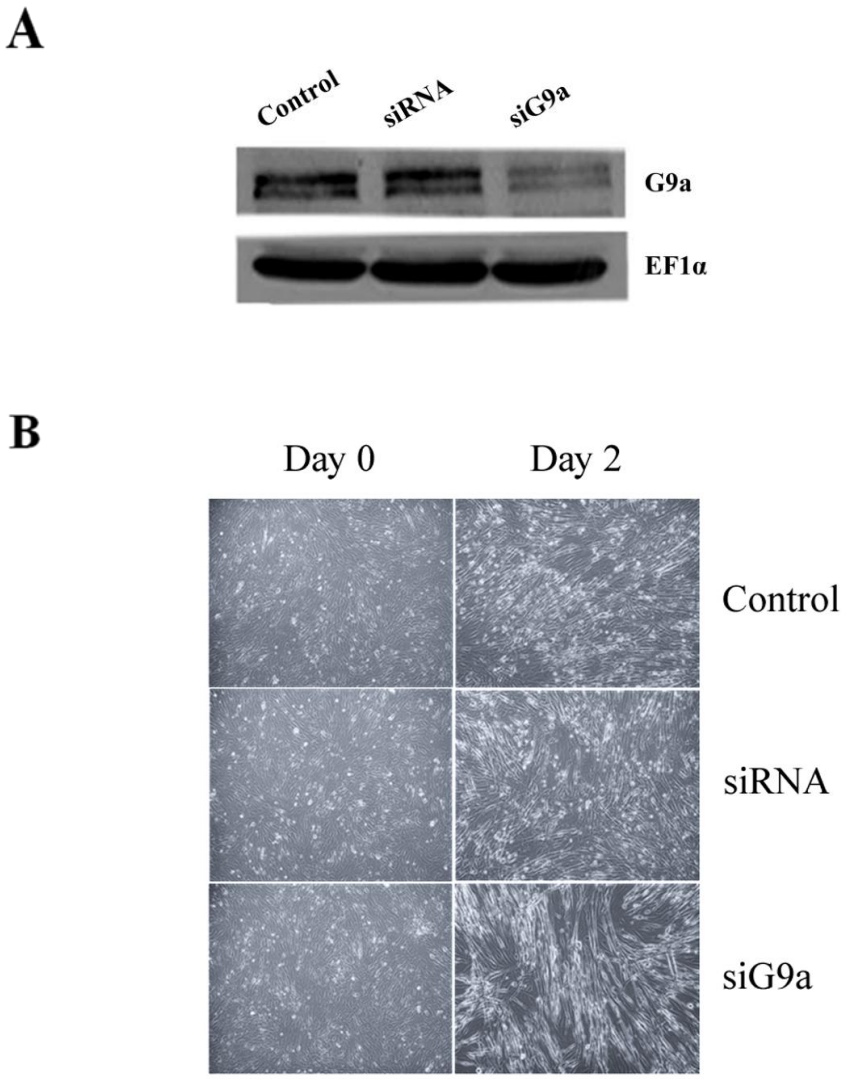
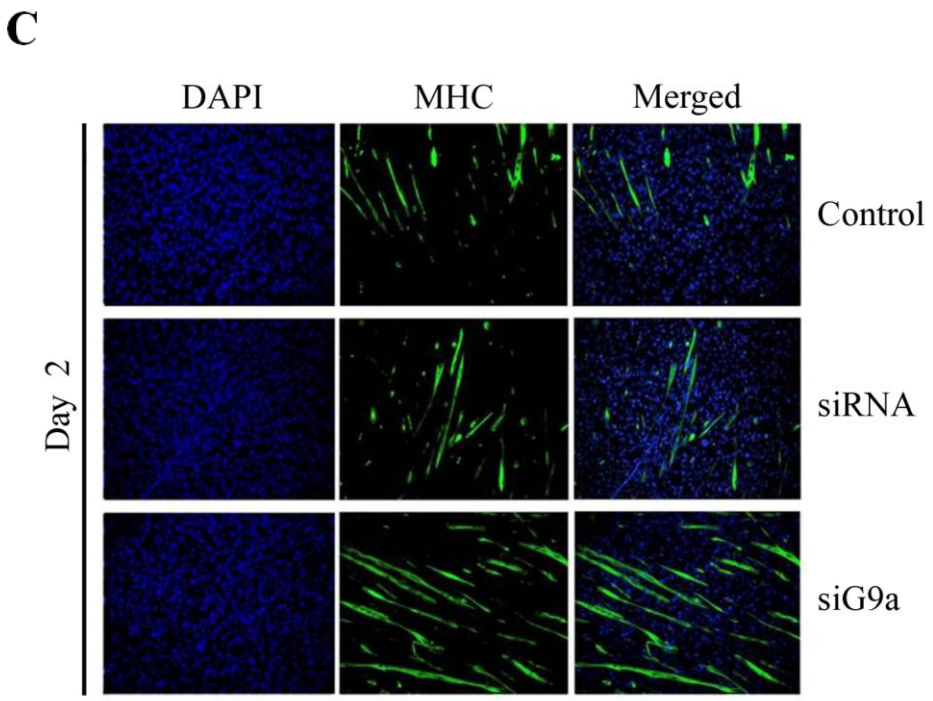


Fig 3.1.4A&B Knockdown of G9a enhances skeletal muscle differentiation

(A) C2C12 myoblasts were transfected with siRNA against G9a (siG9a), scrambled siRNA (siRNA) or left untransfected (control). Lysates were collected for day 0 and analyzed for the knockdown of endogenous G9a at protein level by Western blot. (B) Cells were subjected to differentiation by transferring to differentiation medium for 48 hours (Day 2) and the extent of myoblast differentiation was observed in siG9a cells compared to siRNA and untransfected control cells by capturing the image at 10X magnification using phase contrast microscopy. A significant increase in the number of myotubes was observed in siG9a cells compared to control cells.



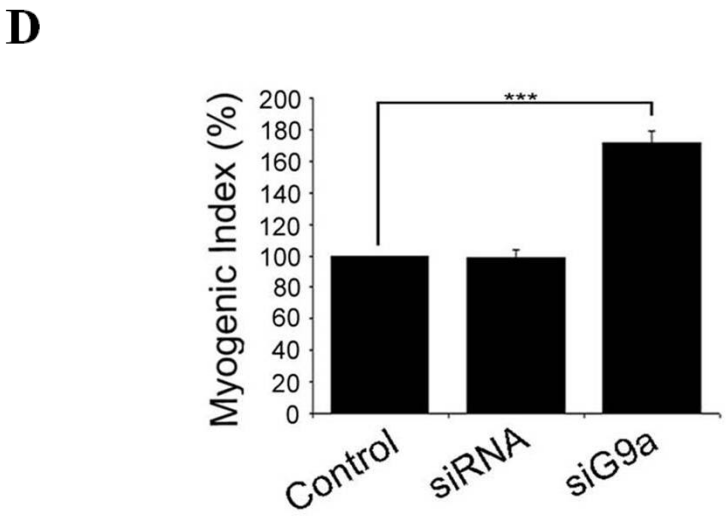


Fig 3.1.4C&D Knockdown of G9a enhances skeletal muscle differentiation. (C) Differentiated cells (Day 2) were also immunostained with anti-MHC (green) antibody. Nuclei were stained with DAPI (blue). Images were captured under fluorescent microscope using 10X magnification. (D) Quantification of myogenic index from 4 randomly chosen microscopic fields (calculated as the ratio of MHC-positive stained nuclei to the total nuclei in each field. At least 400 nuclei were calculated) revealed a significant increase in the number of MHC^{+} cells in

siG9a cells. Values obtained were represented as means with error bar indicating standard deviations. Statistical significance was determined by the two-tailed, un-paired Student's *t*-test and *p*-values of <0.05 were considered to be statistically significant. p-value obtained is shown as ******* [$p < 0.001$]

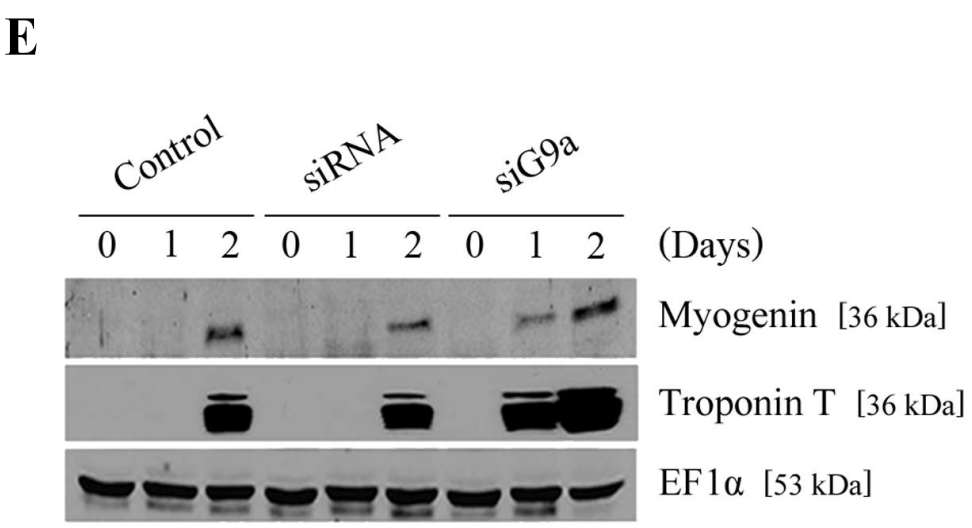


Fig 3.1.4E Knockdown of G9a enhances skeletal muscle differentiation: (E) Cell lysates were analyzed by western blot for myogenic markers myogenin and Troponin T expression at days 0, 1 and 2. EF1α (53Kda) was used as internal control.

3.1.5 G9a represses MRF transcriptional activity

Since G9a is expressed in proliferating myoblasts and represses muscle specific genes that are induced during muscle differentiation, its effect on the transcriptional activity of Myogenic Regulatory Factors (MRFs) was examined by luciferase assays in C2C12 cells. C2C12 myoblasts were transfected with the MRF reporter 4R-tk-Luc (which contains four tandem E boxes from the Muscle Creatine Kinase (MCK) enhancer upstream of the thymidine basal promoter) in the absence and presence of EGFP-G9a or EGFP-G9aΔSET. EGFP-G9a strongly repressed the reporter activity in a dose-dependent manner. The inhibition of MRF activity was methyltransferase

activity dependent, as EGFP-G9aΔSET had no effect on the reporter activity (**Fig 3.1.5**). Since C2C12 cells express Myf5 and MyoD, this suggested that G9a may inhibit endogenous MyoD and/or Myf5 activity.

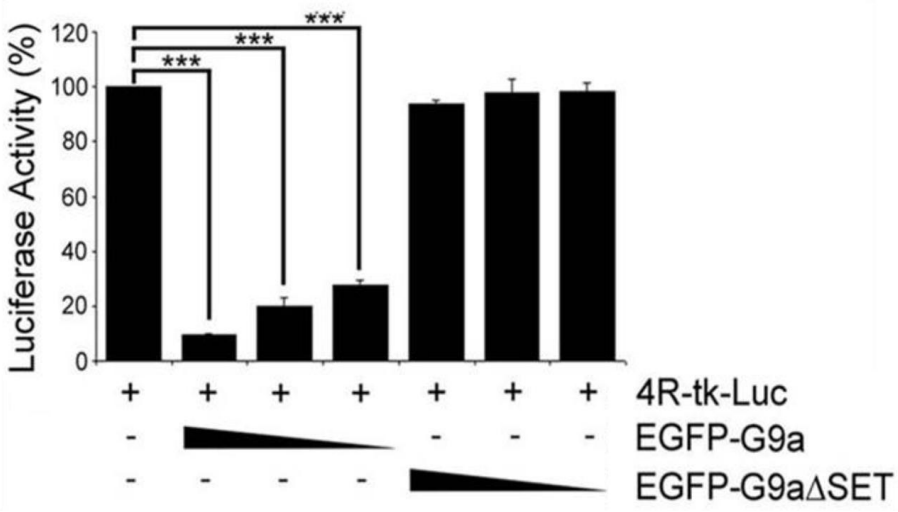


Figure 3.1.5 G9a represses MRF transcriptional activity: C2C12 cells were transfected with 4R-tk driven firefly luciferase reporter 4R-tk-Luc, 50 ng in the absence and presence of increasing amount of EGFP-G9a or EGFP-G9aΔset (50, 25 and 10 ng). Luciferase activity was measured 48 hours after transfection and was represented as percent luciferase activity in which the control was assigned a value of 100%. Data represent the mean ± SD. Statistical significance was determined by Student's *t* test and *p*-values of <0.05 were considered to be statistically significant. p-value obtained is shown as ******* [$p < 0.001$].

3.1.5.1 G9a represses MyoD transcriptional activity

To examine the effect of G9a specifically on MyoD activity, 10T1/2 cells (non-muscle cells) were transfected with 4R-tk-Luc in the absence and presence of MyoD, along with EGFP-G9a or EGFP-G9aΔSET. As expected, transfection of MyoD in 10T1/2 fibroblasts resulted in induction of reporter activity. Co-expression of EGFP-G9a blocked the MyoD-dependent activation of 4R-tk-Luc. However co-expression of EGFP-G9aΔSET, failed to inhibit MyoD dependent transcriptional activity (**Fig 3.1.5.1**).

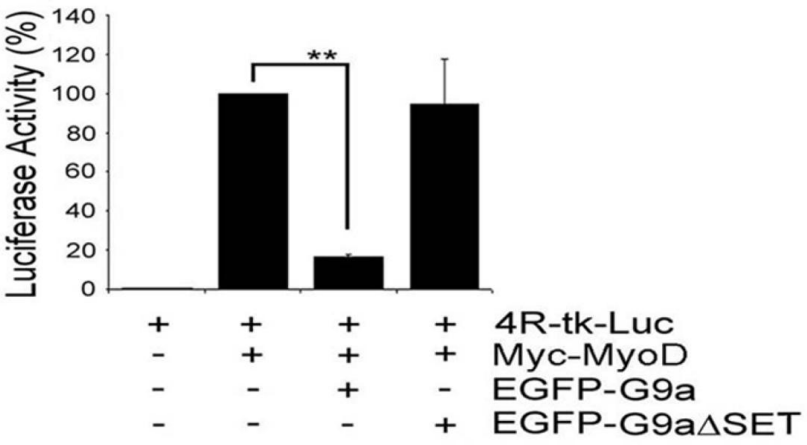


Figure 3.1.5.1 G9a represses MyoD transcriptional activity: 10T1/2 cells were transfected with 4R-tk-Luc (50ng) along with MyoD (50ng) in the absence and presence of EGFP-G9a or EGFP-G9aΔset (50ng). Luciferase activity was measured 48 hours after transfection and was represented as percent luciferase activity in which the control was assigned a value of 100%. Data represent the mean ± SD. Statistical significance was determined by Student's *t* test and p-value obtained is shown as ****** [$p < 0.01$].

From these results (**3.1.5 & 3.1.5.1**), it appeared that G9a inhibits MyoD transcriptional activity.

3.1.6 G9a interferes with the function of MyoD

Since G9a inhibits MRF activity and specifically MyoD activity, this raised the possibility that it may interfere with MyoD function. Thus to test whether MyoD loses its ability to activate the myogenic program in the presence of G9a, myogenic conversion assays were performed. 10T1/2 cells were transfected with MyoD alone, or together with equivalent amounts of EGFP-G9a or EGFP-G9aΔSET (**Fig 3.1.6.A**) and subjected to myogenic differentiation assays. Consistent with the effect of G9a on MyoD transcriptional activity, EGFP-G9a, but not EGFP-G9aΔSET mutant inhibited MyoD dependent myogenic conversion, again indicating that G9a blocked MyoD in a methyltransferase activity dependent manner. This was evidenced by a significant reduction in the presence of MHC^{+} myotubes (**Fig 3.1.6.B**) as well as myogenic index (**Fig 3.1.6.C**). To validate these findings, western blot analysis was performed for the expression of Troponin T. A significant reduction in the expression of Troponin T was apparent in cells co-transfected with MyoD and EGFP-G9a but not those expressing MyoD (**Fig 3.1.6.D**). Altogether, these results demonstrate that G9a interferes with MyoD function.

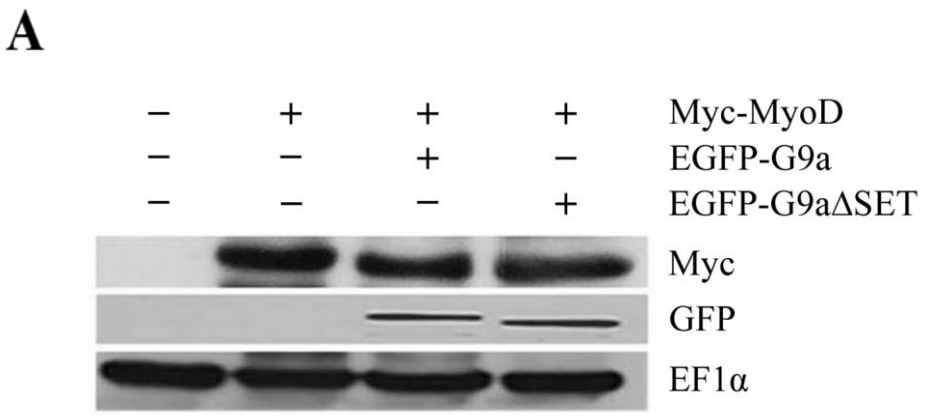


Figure 3.1.6A G9a interferes with the function of MyoD: (A) C3H10T1/2 fibroblasts were transiently transfected with MyoD alone or co-transfected with either EGFP-G9a or EGFP-G9aΔSET and analyzed for their expression by western blot. EGFP-G9a and EGFP-G9aΔSET were expressed at equivalent amount. EF1α was used as internal control.

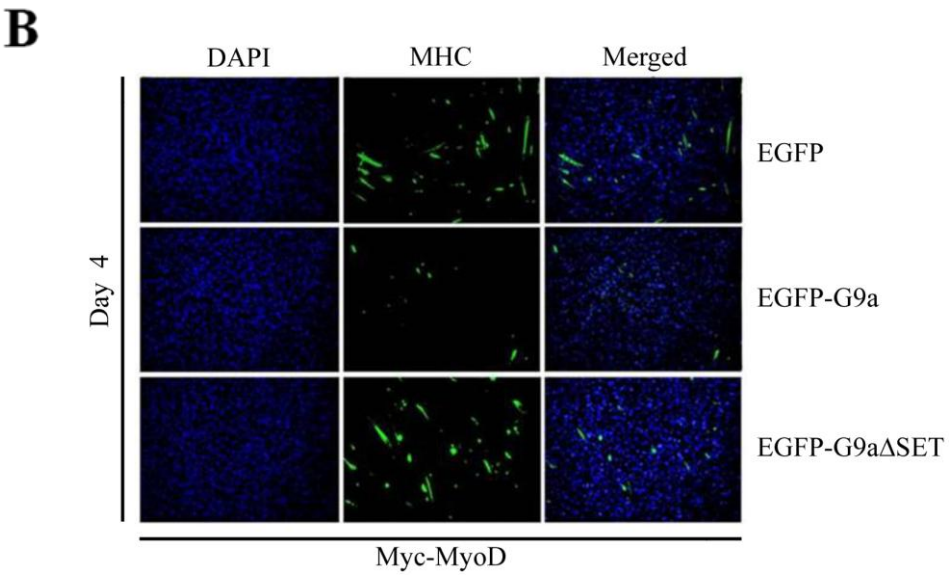


Figure 3.1.6B G9a interferes with the function of MyoD: (B) Cells were then subjected to MyoD dependent myogenic conversion assays by transferring to differentiation medium for 96 hours (Day 4). Differentiated cells (Day 4) were immuno-stained with anti-MHC (green) antibody. Nuclei were stained with DAPI (blue). Images were captured under fluorescent microscope using 10X magnification.

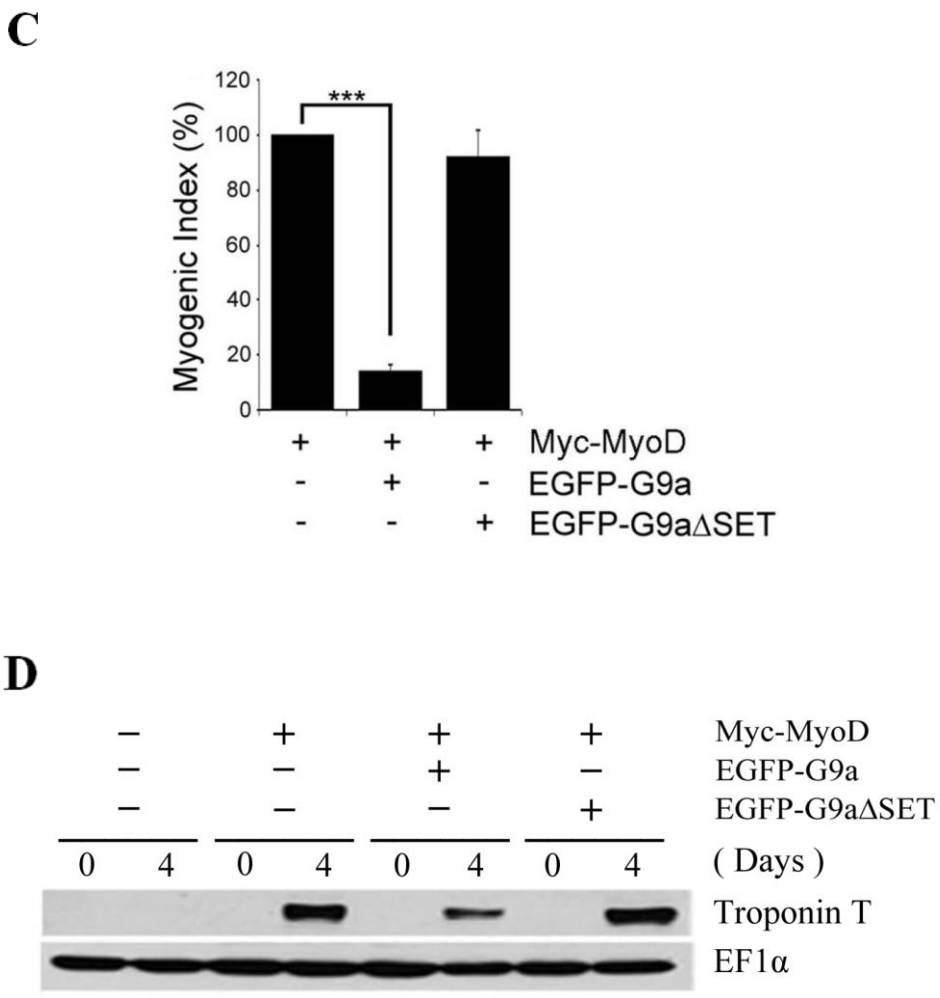


Figure 3.1.6 C&D G9a interferes with the function of MyoD: (C) The percentage of MHC^{+} cells was quantified (myogenic index) relative to MyoD, which was given a value of 100%. A significant decrease in myogenic index was apparent in cells expressing EGFP-G9a and Myc-MyoD compared to controls (Myc-MyoD and EGFP-G9aΔSET) over-expressing cells. Data represent the mean ± SD. Statistical significance was determined by Student's *t* test and p-value obtained is shown as ******* [p< 0.001]. (D) Cell lysates were analyzed by western blot for the expression of Troponin T at day 4, which was inhibited in cells over-expressing MyoD and G9a compared to controls.

3.1.7 G9a interacts physically with MyoD

Since G9a inhibits MyoD dependent gene transcription, this raised possibility that G9a may interact with MyoD. To test whether G9a associates with MyoD, co-immunoprecipitation assays were performed. 293T cells were transfected with full length Myc-tagged MyoD and full length Flag-tagged G9a, either individually or together. Immunoprecipitation of either Myc-tagged MyoD, or Flag-tagged G9a, revealed a strong interaction between G9a and MyoD (**Fig 3.1.7**).

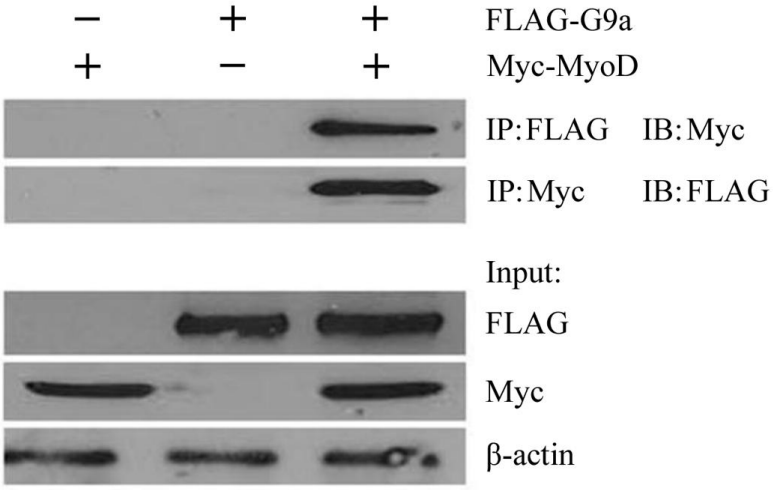


Figure 3.1.7 G9a and MyoD interact in cultured cells: HEK293T cells were transfected with Myc-tagged MyoD and/or FLAG-tagged G9a either individually or together. MyoD was immunoprecipitated using anti-Myc agarose beads, and anti-Flag antibodies were used to detect the presence of G9a in the immunoprecipitates by western blot analysis. Similarly, G9a was immunoprecipitated using anti-Flag agarose beads, and anti-Myc antibodies were used to detect the presence of MyoD in the immunoprecipitates by Western blot analysis. Immunoprecipitation of either G9a, or MyoD from 293T cells, revealed an interaction between the two proteins. Lysates (input) were analyzed for G9a and MyoD expression by western blot analysis. β-actin was used as internal control.

3.1.8 Endogenous MyoD associates with G9a in proliferating muscle cells

Although the previous experiments indicated that G9a interacts with MyoD, this interaction was assessed in non-muscle cells by exogenous co-expression of MyoD and G9a. As MyoD is exclusively expressed in skeletal muscle cells, it was important to test their association in skeletal muscle cells. To examine whether endogenous MyoD interacts with G9a in C2C12 cells, immunoprecipitation assays were carried out. Since G9a is expressed at high levels in proliferating myoblasts and its expression declines upon differentiation, C2C12 cell lysates were collected from cells cultured in GM (Day 0) and after induction of differentiation in DM (Day 2). Endogenous MyoD was immunoprecipitated from extracts of C2C12 cells with anti-MyoD antibody. The immunoprecipitated proteins were then immunoblotted with G9a antibody. The result showed that endogenous MyoD interacted with G9a in myoblasts; and this interaction diminished upon induction of differentiation (**Fig 3.1.8**).

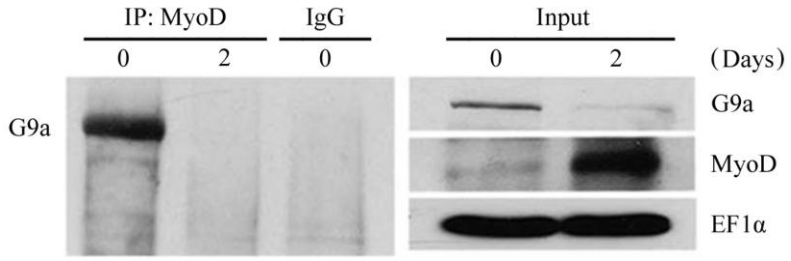


Figure 3.1.8 Endogenous MyoD associates with G9a: Endogenous MyoD was immunoprecipitated from lysates of C2C12 cells grown either in GM (Day 0) or DM for 48 hrs (Day 2) using anti-MyoD antibody (or IgG in control) and the presence of G9a in the immunoprecipitate detected by immunoblotting with anti-G9a antibody. Input shows expression of G9a and MyoD in the lysates. EF1α was used as internal control.

3.1.9 Endogenous G9a co-localizes with MyoD

As G9a interacts with MyoD endogenously, co-localization of these two endogenous proteins in C2C12 myoblasts was examined. C2C12 cells cultured in growth medium were fixed, and immunostained with anti-G9a and anti-MyoD antibody. Nuclei were stained with DAPI (Blue). Consistent with the endogenous interaction of G9a with MyoD, both the nuclear proteins were noticeably co-localized (**Fig.3.1.9**).

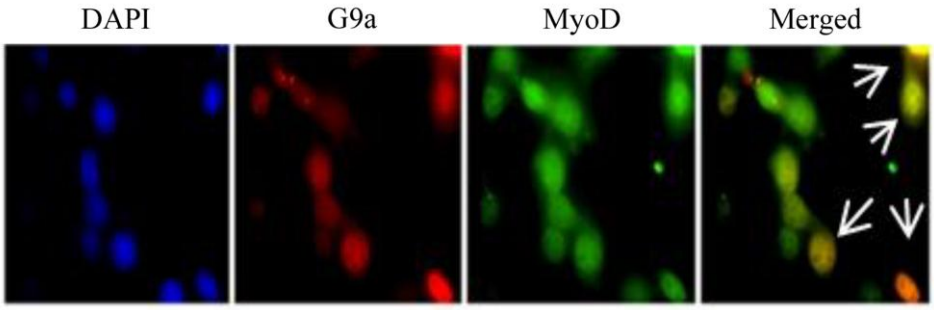


Figure 3.1.9 G9a co-localizes with MyoD: C2C12 cells were cultured in GM (Day 0). Cells were then immunostained with anti-G9a antibody and anti-MyoD antibody. Nuclei were stained with DAPI (blue). Images were captured under fluorescent microscope using 20X magnification. G9a (red) and MyoD (green) co-localization was apparent in myoblasts.

3.1.10 Over-expression of G9a increases H3K9me2 on myogenin promoter

It has been shown by previous studies that G9a preferentially di-methylates H3K9 (Tachibana *et al*., 2002, Rice *et al*., 2003) which is associated with transcriptional repression. To understand the mechanisms by which G9a brings about inhibition of MyoD activity and repression of muscle-specific

genes that are induced by MyoD, chromatin immunoprecipitation (ChIP) assays were performed to determine whether there is any enrichment of H3K9me2 on the MyoD target myogenin promoter. C2C12 cells over-expressing EGFP (vector control) and EGFP-G9a were cultured in GM (day 0) and differentiated for two days (day 2). Cell lysates were collected at the respective time points and subjected to sonication to shear chromatin and immunoprecipitated with H3K9me2 antibody. Consistent with previous reports, H3K9me2 levels were higher on the myogenin promoter in proliferating myoblasts which declined substantially upon differentiation (**Fig 3.1.10**) (Zhang *et al*., 2002). However, upon EGFP-G9a overexpression, a significant increase in the enrichment of H3K9me2 was seen in both proliferating myoblasts and differentiated myotubes on myogenin promoter (**Fig 3.1.10**).

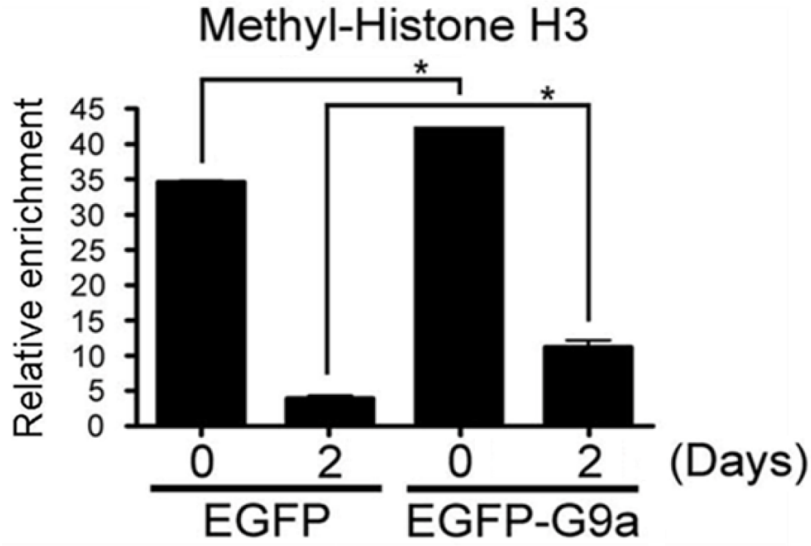


Figure 3.1.10 G9a alters H3K9me2 levels on myogenin promoter: C2C12 cells over-expressing EGFP- (vector control) or EGFP-G9a were cultured in GM (Day0) and induced to differentiate in DM for two days (Day 2). Cells were analyzed by ChIP assays to determine the H3K9me2 on the myogenin promoter. Cell lysates were subjected to immunoprecipitation with H3K9me2 antibody and the chromatin were analyzed by Q-PCR using primers for the myogenin promoter and β-actin. Data represent the mean ± SD. Statistical significance was determined by Student's *t* test and p-value obtained is shown as ***** [$p < 0.05$]. Data provided by Mr. Teng-Kai Chung.

3.1.11 Over-expression of G9a decreases H3K9K14ac mark on myogenin promoter

To assess for alteration in H3K9K14ac levels which are associated with transcriptional activation, similar ChIP assays were carried out with EGFP and EGFP-G9a over-expressing cells using acetylated H3K9K14ac antibody. There was a significant reduction in H3K9K14ac levels in G9a over-expressing cells compared to control cells (**Fig 3.1.11**).

The increase in H3K9me2 and reduction of H3K9K14ac levels (**3.1.10 & 3.1.11**) at the myogenin promoter upon G9a over expression correlates with repression of myogenin and MHC expression.

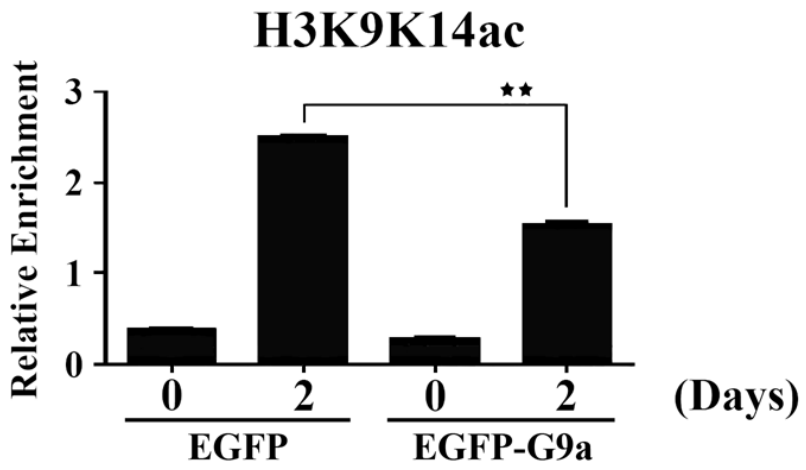


Figure 3.1.11 G9a alters H3K9K14ac levels on myogenin promoter
C2C12 cells over-expressing EGFP- (vector control) or EGFP-G9a were cultured in GM (Day0) and induced to differentiate in DM for two days (Day 2). Cells were analyzed by ChIP assays to determine the H3K9K14ac on the myogenin promoter. Cell lysates were subjected to immunoprecipitation with H3K9K14ac antibody and the chromatin were analyzed by Q-PCR using primers for the myogenin promoter and β-actin. Data represent the mean ± SD. Statistical significance was determined by Student's *t* test and p-value obtained is shown as****** [$p < 0.01$]. Data provided by Mr. Teng-Kai Chung.

3.1.12 MyoD has G9a methylation consensus at K104

Previous reports have shown that G9a methylates non-histone substrates including CDYL1, WIZ, ACINUS and C/EBPβ in addition to mediating H3K9me1 and H3K9me2 (Rathert *et al*., 2008). Since G9a interacts with MyoD and inhibits MyoD function in a methyltransferase activity dependent manner, this raised the possibility that MyoD could be a non-histone target of G9a mediated methylation. It has been shown that G9a mostly recognizes an

Arg-Lys (RK) sequence (Rathert *et al*., 2008). We analyzed MyoD cDNA across many species which revealed a single conserved G9a methylation consensus RK at K104 (**Fig 3.1.12**).

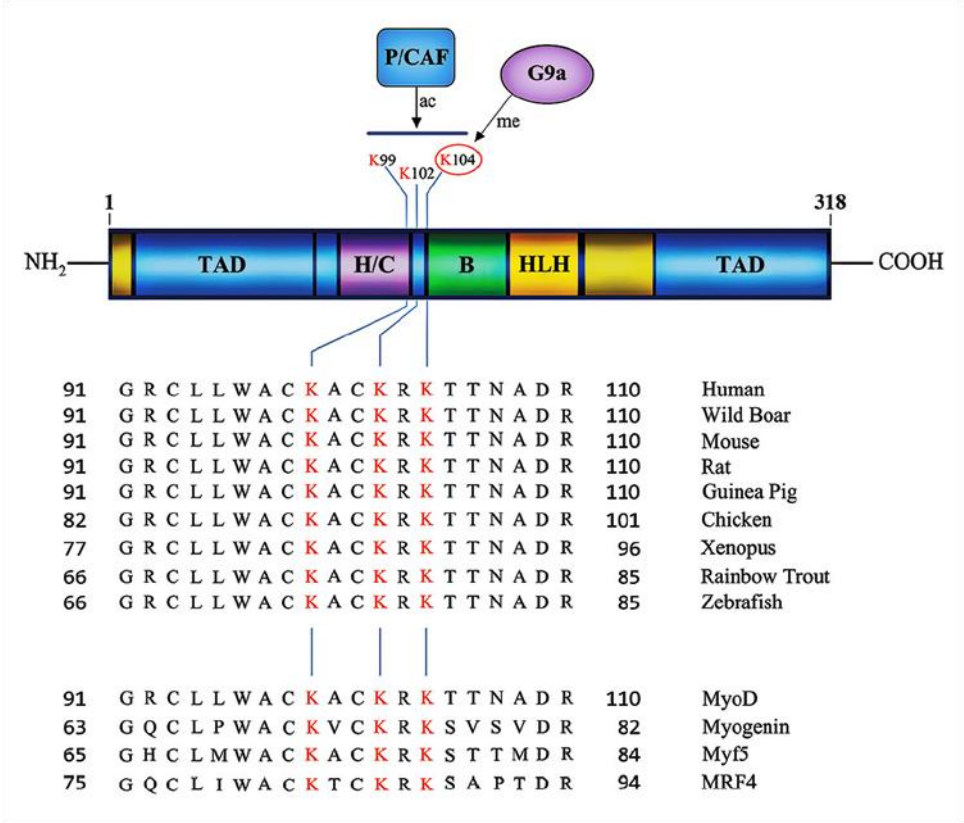


Figure 3.1.12 Schematic representation of MyoD domain structure with its G9a methylation consensus K104 and P/CAF acetylation consensus K99, K102 and K104 conserved across various species and through all MRFs (Transcription.2012; 3(5):1-6).

3.1.12.1 G9a methylates MyoD

To test the possibility whether G9a methylates at K104, immunoprecipitation assays were performed. 293T cells were transfected with full-length Myc-tagged MyoD and EGFP-tagged G9a, individually and together. In addition, EGFP-tagged G9aΔSET was co-transfected with Myc-MyoD.

Twenty-four hours post-transfection, cell lysates were harvested and checked for expression of the Myc-MyoD, EGFP-G9a and EGFP-G9aΔSET (**Fig 3.1.12.1**). Cell lysates were immunoprecipitated with Myc-agarose beads and immunoprecipitates were analyzed by western blot initially for the interaction of EGFP-G9a and EGFP-G9aΔSET with MyoD. Consistent with our previous result, G9a interacted with MyoD (**Fig 3.1.12.1**). In addition to this, G9aΔSET interacted with MyoD as well, clearly indicating that this association of G9a with MyoD is independent of the catalytic SET domain of G9a (**Fig 3.1.12.1**). To examine whether G9a methylates MyoD, the blot was then probed for MyoD methylation using anti-methyl-lysine (Me-Lys) antibody. The result revealed a strong methylation of MyoD by G9a (**Fig 3.1.12.1**), whereas there was no corresponding methylation band observed for the lane co-expressing MyoD and EGFP-G9aΔSET, indicating that MyoD is methylated by G9a through its SET domain (**Fig 3.1.12.1**).

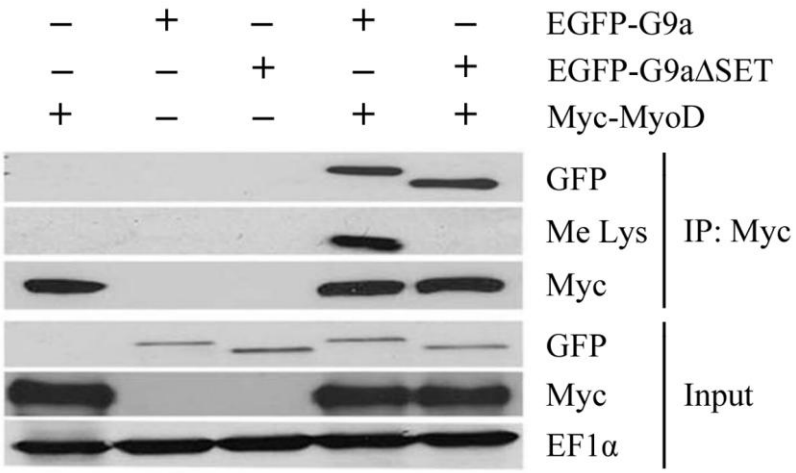


Figure 3.1.12.1 G9a methylates MyoD *in vivo*: HEK293T cells were transfected with full length Myc-tagged MyoD, EGFP-tagged G9a, SET domain deleted mutant EGFP-tagged G9aΔSET either individually or together. MyoD was immunoprecipitated using anti-Myc agarose beads followed by immuno-blotting with anti-GFP antibody to detect the association of MyoD with G9a and G9aΔSET in the immunoprecipitates as well as its methylation using anti-methyl lysine (Me-Lys) antibody. Lysates (input) were analyzed for G9a, G9aΔSET and MyoD expression by Western blot analysis. EF1α was used as internal control.

3.1.12.2 Endogenous MyoD methylated by G9a in proliferating myoblasts

From the above experiments it appeared that G9a associates with and may methylate MyoD. This work was further expanded in C2C12 cells to examine whether G9a can methylate MyoD endogenously in skeletal muscle cells. C2C12 cells were cultured in growth medium and differentiation medium and endogenous MyoD was immunoprecipitated from extracts of C2C12 cells at day 0 and day 2 with MyoD antibody. As a negative control IgG antibody was used to immunoprecipitate Day 0 lysates. The immunoprecipitated proteins were then immunoblotted with anti-Me-Lys antibody. Consistent with the

above result, endogenous MyoD was methylated by G9a at day 0 (**Fig 3.1.12.2**) and methylation declined upon induction of differentiation at day 2 (Fig 3.1.12.2). This may be due to decline in endogenous G9a expression.

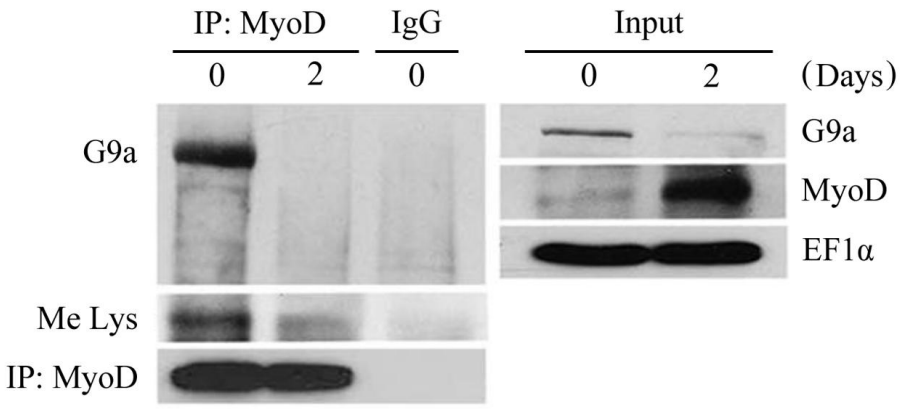


Figure 3.1.12.2 Endogenous MyoD methylated by G9a: Endogenous MyoD was immunoprecipitated from lysates of C2C12 cells grown either in GM (Day 0) or DM for 48 hrs (Day 2) using anti-MyoD antibody (or IgG in control lane) and the presence of G9a in the immunoprecipitate detected by immunoblotting with anti-G9a antibody. Then the blot was analyzed for MyoD methylation using anti-Me-Lys antibody. Input shows expression of G9a and MyoD in the lysates. EF1α was used as internal control.

3.1.12.3 Modulation of endogenous G9a levels impacts MyoD methylation

Although it is clear from the above experiment that MyoD is methylated in skeletal muscle cells, it was still not obvious whether methylation is mediated specifically by G9a.

To determine whether MyoD methylation requires G9a, endogenous G9a levels in myoblasts were modulated by two strategies and its corresponding impact on MyoD methylation was examined. First, G9a was over-expressed in proliferating myoblasts (Day 0) and endogenous MyoD was

immunoprecipitated 24 hours post transfection. As expected, over-expression of G9a resulted in significant increase in MyoD methylation compared to control C2C12 cells (**Fig 3.1.12.3A**).

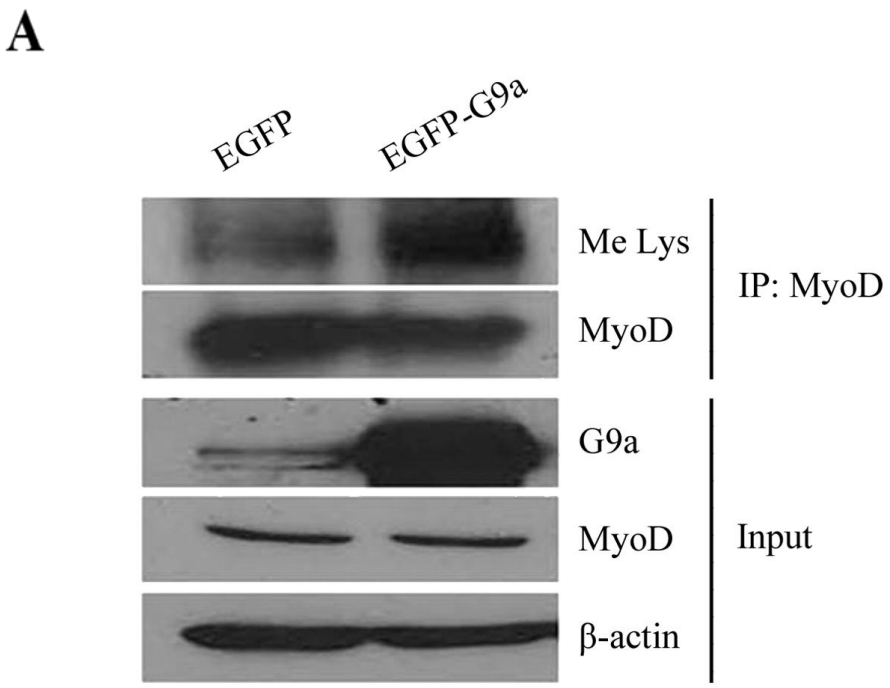


Figure 3.1.12.3A Overexpression of G9a increases MyoD methylation: (A) C2C12 myoblasts were transfected with EGFP-G9a and EGFP (control). Lysates were collected at day 0. Input shows the over-expression of G9a compared to endogenous G9a as well as MyoD expression at protein level. β-actin was used as internal control. Endogenous MyoD was immunoprecipitated from lysates of C2C12 myoblasts over-expressing EGFP-G9a and EGFP (control) using anti-MyoD antibody. It was followed by immuno-blotting with anti-Me-Lys antibody.

Second, endogenous G9a was targeted by siRNA knockdown. C2C12 cells were transfected with scrambled siRNA control or siRNA targeted to G9a (siG9a). Forty-eight hours post-transfection, cell lysates were collected and MyoD was immunoprecipitated using MyoD antibody and immuno-blotted with anti-Me-Lys antibody to assess whether the knockdown of G9a at protein

level modulates endogenous MyoD methylation. Compared to control (siRNA) cells, siG9a cells exhibited a marked reduction of MyoD methylation (**Fig 3.1.12.3B**).

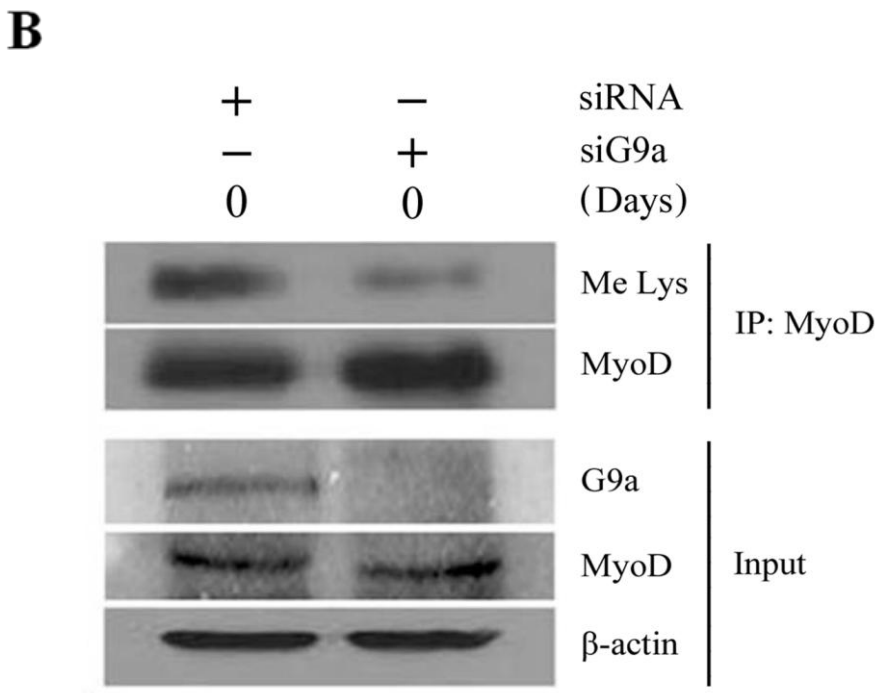


Figure 3.1.12.3B Knockdown of G9a decreases MyoD methylation: (B) C2C12 myoblasts were transfected with siG9a and control siRNA. Lysates were collected at day 0. Input shows the knockdown of endogenous G9a at protein level as well as the expression of MyoD. β-actin was used as internal control. Endogenous MyoD was immunoprecipitated from lysates of C2C12 myoblasts expressing siG9a and control siRNA using anti-MyoD antibody. Then the blot was analyzed for MyoD methylation using anti-Me-Lys antibody.

Thus it is clear from above strategies (**3.1.12.3A & B**) that targeting G9a expression modulates MyoD methylation.

3.1.13 G9a interacts with MyoD through its ankyrin repeats domain

It has been shown by previous studies that ankyrin repeats (ANK) domain of G9a is involved in protein-protein interaction. So to examine the possibility whether G9a associates with MyoD through its ankyrin repeats, 293T cells were transfected with full length Myc-tagged MyoD and co-transfected with either full-length Flag-tagged G9a or ANK domain deleted mutant Flag-tagged G9aΔANK.

Twenty-four hours post-transfection, cell lysates were harvested and then checked for the expression of Myc-MyoD, Flag-G9a and Flag-G9aΔANK. All proteins were expressed at equivalent amounts (**Fig 3.1.13**). Cell lysates were immunoprecipitated with Myc-agarose beads and immunoprecipitates were probed by western blot for interaction of G9a and G9aΔANK with MyoD using anti-Flag antibody. Immunoblot analysis revealed that wild-type G9a interacted with MyoD consistent with the previous results obtained. However, this interaction was disrupted completely as G9aΔANK failed to interact with MyoD (**Fig.3.1.13**) which indicates that ANK domain of G9a is necessary for its association with MyoD.

To further determine whether the association between these two proteins is essential for G9a mediated MyoD methylation, the same blot was then analyzed for its methylation using anti-Me-Lys antibody. There was no methylation signal detected with G9aΔANK compared to wild-type G9a suggesting the importance of interaction between these two proteins for methylation to occur (**Fig 3.1.13**).

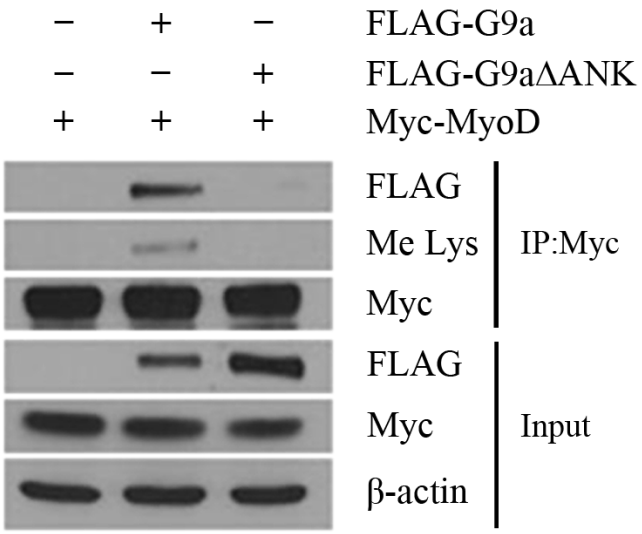


Figure 3.1.13 G9a interacts with MyoD through its ankyrin repeats domain: HEK293T cells were transfected with full length Myc-tagged MyoD, Flag-tagged G9a, Flag-tagged G9aΔANK either individually or together. MyoD was immunoprecipitated using anti-Myc agarose beads followed by immuno-blotting with anti-Flag antibody to detect the association of MyoD with G9a and G9aΔANK in the immunoprecipitates as well as its methylation using anti-Me-Lys antibody. Lysates (input) were analyzed for G9a, G9aΔANK and MyoD expression by Western blot analysis. β-actin was used as internal control.

3.1.14 G9a methylates MyoD directly

To examine whether G9a directly methylates MyoD, *in vitro* methylation assays were performed using 3µg of purified recombinant full length human G9a (hG9a), the SET mutant (hG9aΔSET) which was incubated with 2µg of purified full length human MyoD (hMyoD) fused to a GST affinity tag. GST-histone H3 tail (1-57) was used as a control. In addition to G9a, human Set7/9 (H3K4 methyltransferase) was also used. G9a methylated MyoD, although at a lower efficiency compared to H3 (**Fig 3.1.14**). However, there was no

methylation with G9aΔSET clearly indicating that G9a directly methylates MyoD in a SET domain dependent manner. Set7/9 also weakly methylated MyoD (**Fig 3.1.14**) suggesting the role of other methyltransferases in modulating MyoD by methylating it directly.

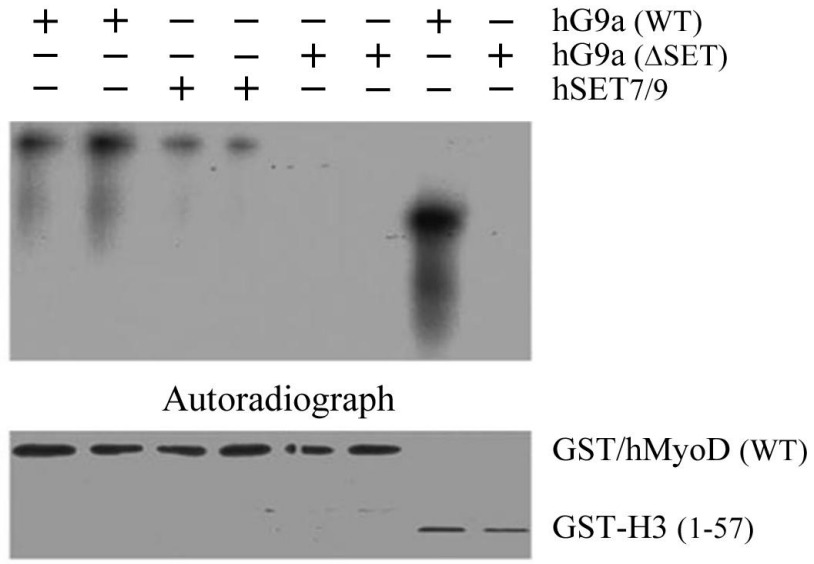


Figure 3.1.14 G9a methylates MyoD directly: *In vitro* methylation of 2µg of recombinant GST-tagged human MyoD by G9a using 3µg of purified poly-histidine-tagged-recombinant G9a, G9aΔSET and Set7/9. Proteins were separated on a 15% polyacrylamide gel and visualized by coomassie blue staining and fluorography. GST-H3 was used as a positive control. Data provided by SiDe Li from Dr. Martin Walsh's lab

3.1.15 G9a methylates MyoD at K104

To identify the site of MyoD methylation, tryptic peptides of recombinant MyoD were treated with full length G9a or with SET mutant (G9aΔSET). Liquid chromatography-mass spectrometry (LC-MS) identified K104 as the

site for methylation (**Fig 3.1.15**) which is consistent with a G9a methylation consensus at this residue.

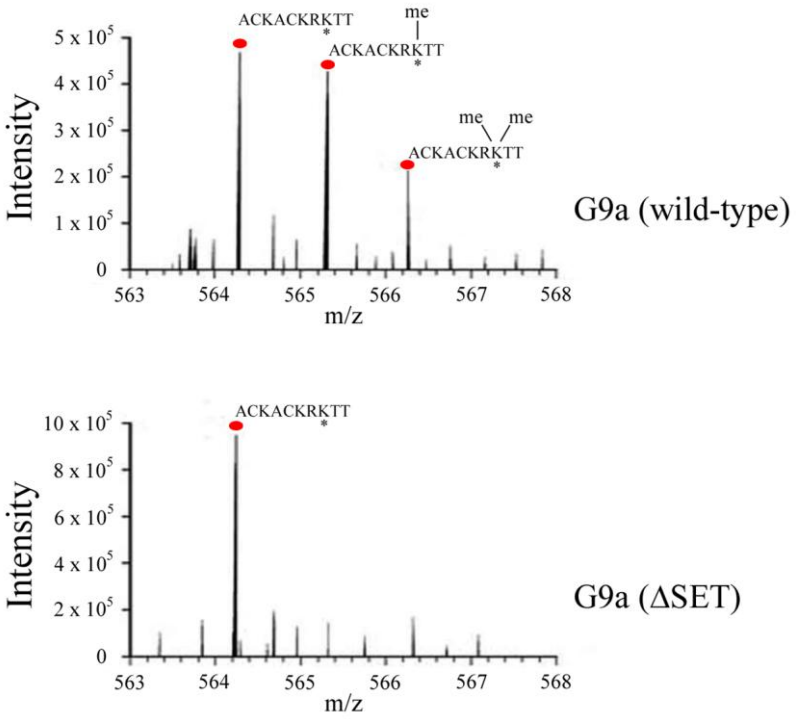


Figure 3.1.15 G9a methylates MyoD at K104: Liquid chromatography-mass spectrometry (LC-MS) analysis of tryptic peptides of the recombinant MyoD after treatment with wild-type G9a or mutant G9aΔSET. Data provided by SiDe Li from Dr. Martin Walsh's lab.

3.1.16 G9a methylates MyoD at K104 (RRR used) *in vivo*

To further validate that K104 is the methylation site on MyoD, 293T cells were transfected with full length Flag-tagged MyoD and Flag-tagged mutant MyoD (RRR) in which K99, K102 and K104 are mutated to arginine (Sartorelli *et al*., 1999) either alone or together with full length EGFP-tagged G9a.

Twenty-four hours post-transfection, cell lysates were harvested and checked for expression of Flag-MyoD, Flag-MyoD (RRR) and EGFP-G9a (**Fig 3.1.16**). Cell lysates were immunoprecipitated with Flag-agarose beads and immunoprecipitates were probed by western blot for methylation using anti-Me-Lys antibody. Consistent with the previous results, wild-type MyoD was methylated in the presence of G9a; however, G9a failed to methylate MyoD (RRR) (**Fig 3.1.16**).

Collectively (**3.1.15 & 3.1.16**), these data clearly show that G9a methylates MyoD at K104.

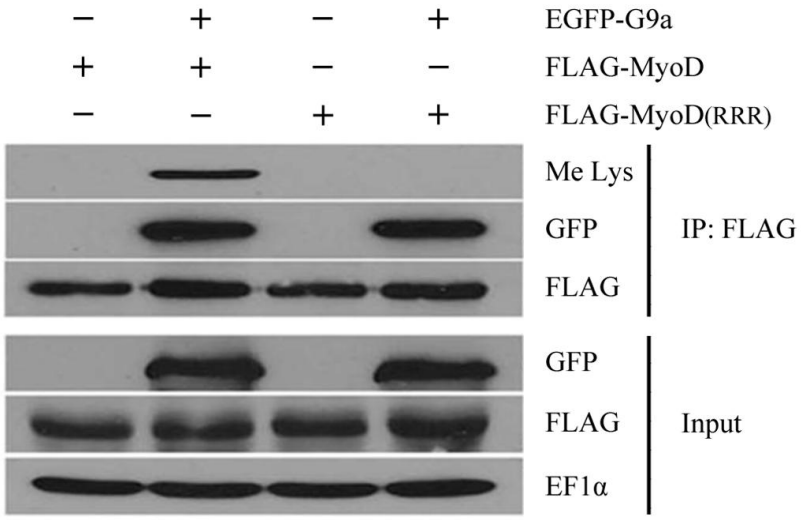


Figure 3.1.16 G9a methylates MyoD at K104 *in vivo*: HEK293T cells were transfected with full length Flag-tagged MyoD and point mutant Flag-tagged MyoD (RRR) either individually or together with EGFP-tagged G9a. MyoD was immunoprecipitated using anti-Flag agarose beads followed by immuno-blotting with anti-GFP antibody to detect the association of MyoD and MyoD (RRR) with G9a in the immunoprecipitates as well their methylation using anti-Me-Lys antibody. Lysates (input) were analyzed for G9a, MyoD and MyoD (RRR) expression by Western blot analysis. EF1α was used as internal control.

3.1.17 MyoD (K104R) has higher myogenic potential than wild-type MyoD

To address the relevance of G9a mediated MyoD methylation and H3K9me2 in G9a-dependent inhibition of differentiation, myogenic conversion assay was carried out. 10T1/2 cells were transfected with pCS2 (vector control) and equivalent amount of wild-type MyoD and MyoD (K104R) (**Fig 3.1.17A**). These cells were then differentiated for four days in the absence and presence of 2.5µM of BIX-01294. Cell lysates were collected at day 0 and day 4 and analyzed for the expression of Troponin T by western blot analysis. As expected, MyoD alone resulted in the myogenic conversion as well as the expression of Troponin T which was further augmented in the presence of 2.5µM of BIX-01294 (**Fig 3.1.17B**). In comparison to wild-type MyoD, MyoD K104R exhibited higher myogenic conversion potential reflected by the higher expression of Troponin T and myogenic index which, however, was unresponsive to BIX-01294 (**3.1.17B & C).**

These data point out that mutation of K104 to arginine (K104R) makes MyoD non-responsive to G9a mediated methylation, thereby enhancing MyoD dependent activation of myogenin and muscle differentiation. Thus it shows that MyoD methylation plays a dominant role in G9a dependent inhibition of skeletal muscle differentiation compared to its modulation of H3K9me2 mark on muscle promoters.

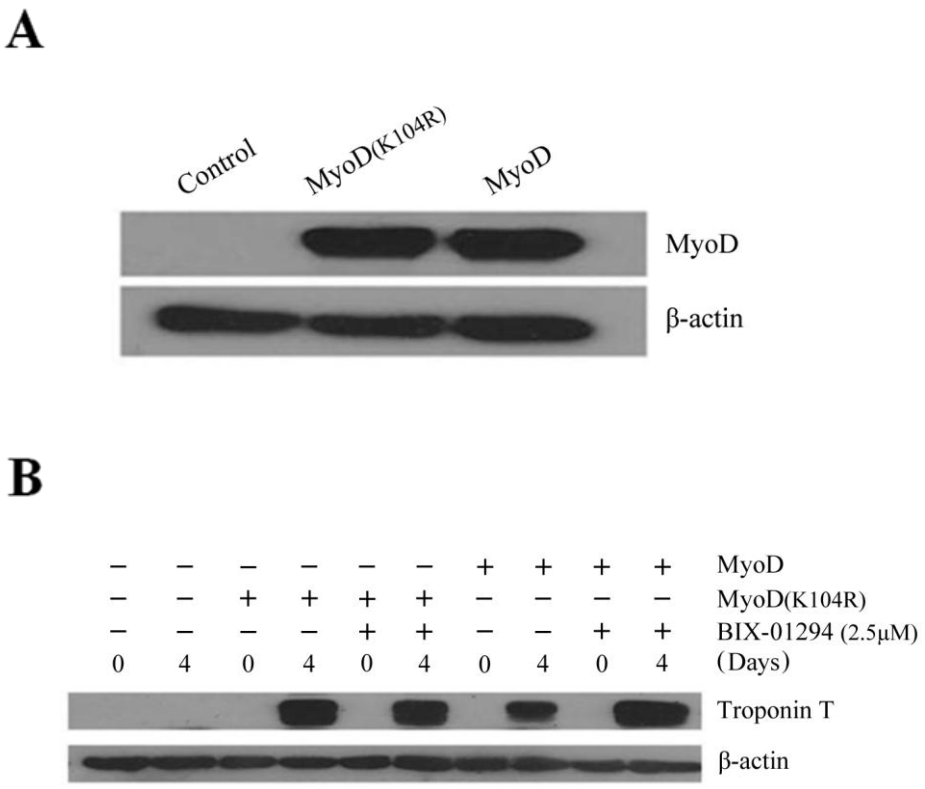


Figure 3.1.17A&B: MyoD (K104R) has higher myogenic potential than wild-type MyoD: (A) 10T1/2 fibroblasts were transiently transfected with either wild-type MyoD or mutant MyoD (K104R) and pCS2 (vector control) and analyzed for their expression by western blot. Cells were then subjected to MyoD dependent myogenic conversion assays by transferring to differentiation medium for 96 hours (Day 4) in the absence and presence of 2.5μM of BIX-01294. (B) Cell lysates were collected at day 0 and day 4 and analyzed for the expression of Troponin T in the MyoD and MyoD (K104R) over-expressing cells in the absence and presence of 2.5μM of BIX-01294.

C

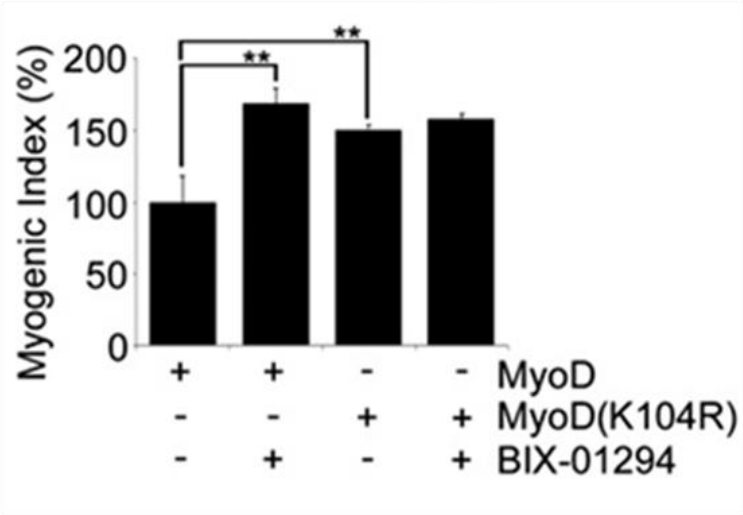


Figure 3.1.17C MyoD (K104R) has higher myogenic potential than wild-type MyoD: (C) Myogenic index was quantified and it reflected the higher myogenic potential of MyoD (K104R) compared to wild-type MyoD. Data represent the mean ± SD. Statistical significance was determined by Student's *t* test and p-value obtained is shown as ****** [$p < 0.01$].

3.2 Role of P/CAF in skeletal muscle differentiation

Previous studies have established a key and critical role for HATs p300 and P/CAF in skeletal myogenesis with their distinct functions. During differentiation, p300 first acetylates H3 and H4 within the promoter region and then recruits P/CAF which interacts with and directly acetylates MyoD at lysine residues 99, 102 and 104 (**Fig 3.1.12**) resulting in its transactivation (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010). This increases the affinity of MyoD for its DNA target, switching on genes for differentiation thereby controlling myogenic transcription and differentiation. Since my results showed that G9a methylates MyoD at lysine residue K104, which is one of the three sites for acetylation by P/CAF (**Fig 3.1.12**), this raised the

possibility that G9a might disrupt P/CAF-mediated MyoD acetylation which is critical for its transactivation function and for execution of the myogenic program. To investigate this possibility as well as to study the wider role of P/CAF in skeletal muscle differentiation we used a selective P/CAF inhibitor (Embelin) to determine its impact on MyoD and muscle differentiation.

3.2.1 G9a disrupts P/CAF mediated MyoD acetylation

G9a negatively impacts the transactivation ability of MyoD to execute the myogenic program by methylating it directly at K104 in undifferentiated myoblasts. On the other hand, P/CAF does the opposite by directly acetylating MyoD at K99, K102 and K104 during differentiation and thereby turning on MyoD transcriptional activity (Sartorelli *et al*., 1999; Duquet *et al*., 2006). It is interesting to note that the same lysine residue K104 which is conserved across species and through all MRFs (**Figure 3.1.12**) is subjected to both methylation and acetylation at different stages of differentiation. So to analyze whether G9a disrupts the ability of P/CAF to acetylate MyoD, immunoprecipitation assay was performed.

293T cells were transfected with full length Myc-tagged MyoD alone or together with EGFP-tagged G9a and Flag-tagged P/CAF. Twenty-four hours post-transfection, cell lysates were harvested and checked for expression of Myc-MyoD, EGFP-G9a and Flag-P/CAF (**Fig 3.2.1**). Cell lysates were immunoprecipitated with Myc-agarose beads and immunoprecipitates were probed by western blot for association of P/CAF with MyoD using anti-Flag antibody. Consistent with previous reports, MyoD immunoprecipitated with

P/CAF (Sartorelli *et al*., 1999). However, in the presence of G9a, MyoD-P/CAF interaction was reduced to an undetectable level (**Fig 3.2.1**). To determine whether this loss of association correlated with altered MyoD acetylation, we performed immunoblot analysis for P/CAF mediated MyoD acetylation in the presence of G9a using anti-acetyl lysine (Ac-Lys) antibody. Consistent with the negative impact on MyoD and P/CAF association, G9a was able to abrogate P/CAF mediated MyoD acetylation as well (**Fig 3.2.1**).

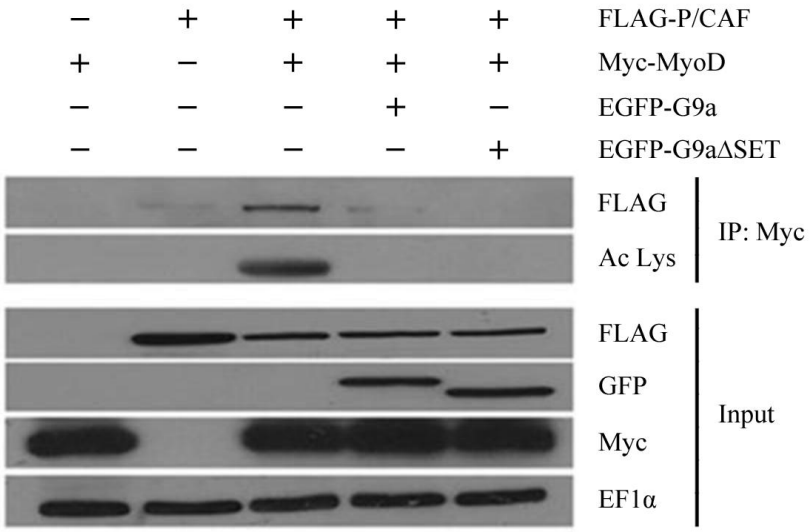


Figure 3.2.1 G9a abrogates P/CAF mediated MyoD acetylation: HEK293T cells were transfected with full length Myc-tagged MyoD either individually or together with Flag-tagged P/CAF and EGFP-tagged G9a. MyoD was immunoprecipitated using anti-Myc agarose beads followed by immuno-blotting with anti-Flag antibody to detect the association of MyoD and P/CAF in the presence of G9a compared to control. It was followed by immuno-blotting with anti-Ac-Lys) antibody. Lysates (input) were analyzed for G9a, MyoD and P/CAF expression by Western blot analysis. EF1α was used as internal control.

3.2.2 Modulation of endogenous G9a level and its impact on MyoD acetylation

To determine whether MyoD acetylation can be modulated by depletion of endogenous G9a levels in myoblasts, siRNA knockdown approach was carried out. C2C12 cells were transfected with scrambled siRNA control or siRNA targeted to G9a (siG9a). Forty-eight hours post-transfection, cells were switched to DM with 10mM each of nicotinamide (NAM), which is an inhibitor of class III NAD-dependent Sir-2 family deacetylases and sodium butyrate, an HDAC inhibitor, in order to preserve P/CAF mediated MyoD acetylation and induced to differentiate for two days. Cell lysates were collected at day 0 and day 2. MyoD was immunoprecipitated using anti-MyoD antibody and immuno-blotted with anti-acetyl-lysine antibody (Ac-Lys) to assess whether the knockdown of G9a modulates endogenous MyoD acetylation. Compared to control (siRNA) cells, siG9a cells showed an early induction of MyoD acetylation at Day 0 (**Fig 3.2.2**). Western blot analyses performed for Troponin T on cell extracts obtained from G9a depleted (siG9a) or control (siRNA) C2C12 cells showed that expression of TroponinT was increased in siG9a cells at Day 2.

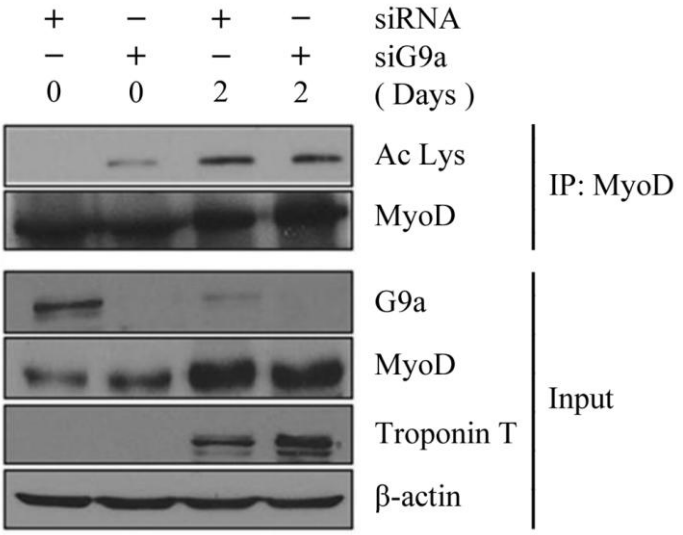


Figure 3.2.2 Modulation of endogenous G9a level and its impact on MyoD acetylation: C2C12 myoblasts were transfected with siG9a and control siRNA. Lysates were collected at day 0 and day 2. Endogenous MyoD was immunoprecipitated from lysates of C2C12 myoblasts expressing siG9a and control siRNA using anti-MyoD antibody. The blot was analyzed for MyoD acetylation using anti-Ac-Lys antibody. Input shows the knockdown of endogenous G9a as well as the expression of MyoD and Troponin T. β-actin was used as internal control.

3.2.3 Titration of embelin in C2C12 cells

Embelin, (a hydroxy benzoquinone class of natural compound) is a cell permeable small molecule which was originally identified by its pro-apoptotic, anti-tumourogenic, anti-cancer and anti-viral activity (Reuter *et al*., 2010; Hussein *et al*., 2000). However, the molecular mechanisms behind these biological activities are not well understood. Works done in collaboration with Prof Tapas Kundu's lab have established that embelin is a selective P/CAF inhibitor (Modak *et al*., submitted).

As embelin specifically targets P/CAF acetyltransferase activity, we wanted to analyze its impact on skeletal muscle differentiation. To first determine the concentration of embelin that could be used, C2C12 myoblasts were cultured with varying concentrations of embelin (0-20µM) and switched to DM and induced to differentiate for two days. As treatment with 10µM resulted in impairment of myotube formation (**Fig.3.2.3**) and a concentration higher than 10µM induced cytotoxicity (**Fig.3.2.3**), 10µM was used as an optimum concentration for subsequent experiments. An equivalent concentration of its analogue derivative, MJTK-4 (an inactive compound) was used as control.

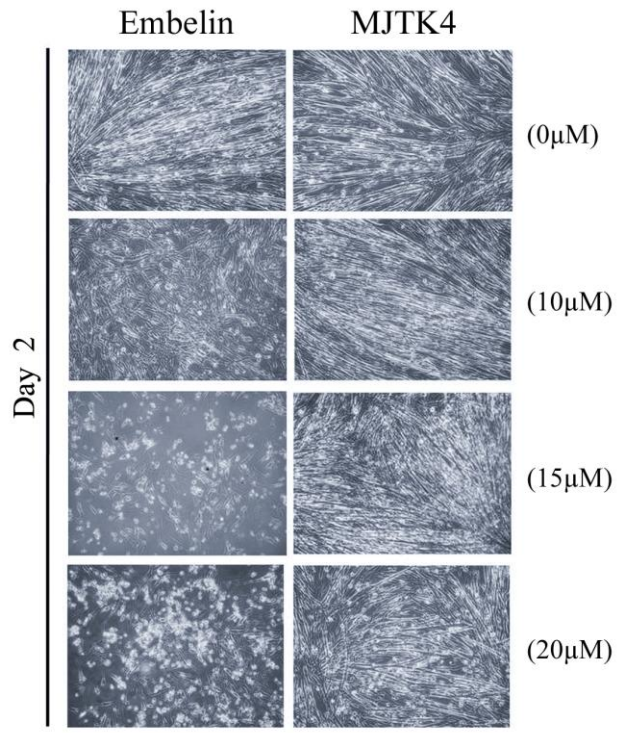


Figure 3.2.3 Titration of embelin in C2C12 cells: C2C12 cells were cultured in GM and switched to DM and treated with different concentrations of embelin or MJTK-4 (control) for 48 hours (Day2). Myoblast differentiation was observed by capturing the image at 10X magnification using phase-contrast microscopy.

3.2.4 Embelin inhibits muscle differentiation

To analyze the impact of targeting P/CAF acetyltransferase activity on muscle differentiation, C2C12 cells were cultured in GM and switched to DM with 10µM of embelin or its inactive analogue MJTK-4 (control) and induced to differentiate for two days. Untreated and 10 µM of DMSO treated cells were used as additional controls.

On day 2, differentiation was examined morphologically in embelin treated cells compared to MJTK-4, DMSO treated and untreated cells. We noticed an impairment of myotube formation in embelin treated cells compared to controls as evidenced by reduced myotube formation with overall defect in the formation of differentiated cells (**Fig 3.2.4A**).

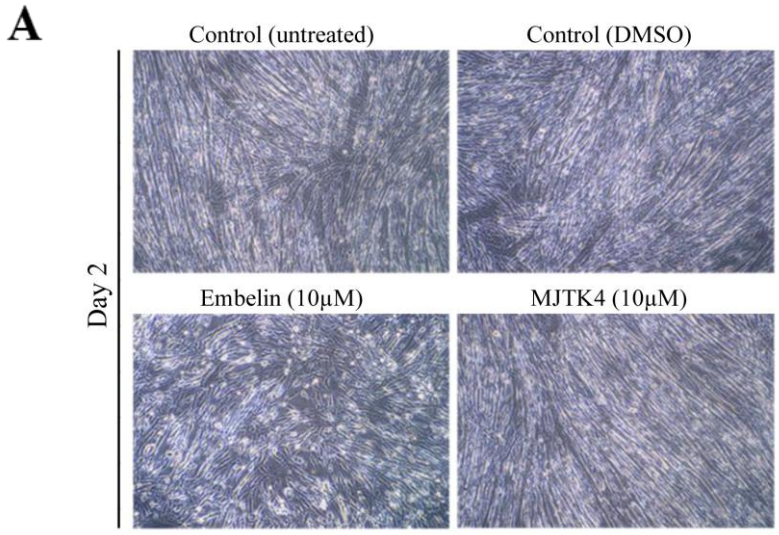


Figure 3.2.4A Embelin inhibits muscle differentiation: (A) C2C12 cells were cultured in GM (D0) and switched to DM and treated with 10µM of Embelin or MJTK-4 (control) for 48 hours (D2). Untreated and vehicle (DMSO) treated cells were used as additional controls. Myoblast differentiation was observed by phase-contrast microscopy. Impaired myotube formation was evident in embelin treated cells compared to controls.

This was also reflected by reduction in the number of (MHC^+) cells (**Fig 3.2.4.B**) as well as myogenic index at D3 (**Fig 3.2.4C**). To further investigate the mechanisms underlying the inhibition of myogenic differentiation, we analyzed expression of myogenin and Troponin T, which are MyoD target genes and are dependent on its transcriptional activity. Both genes were substantially down-regulated in embelin treated cells as shown in the western blot (**Fig 3.2.4D**).

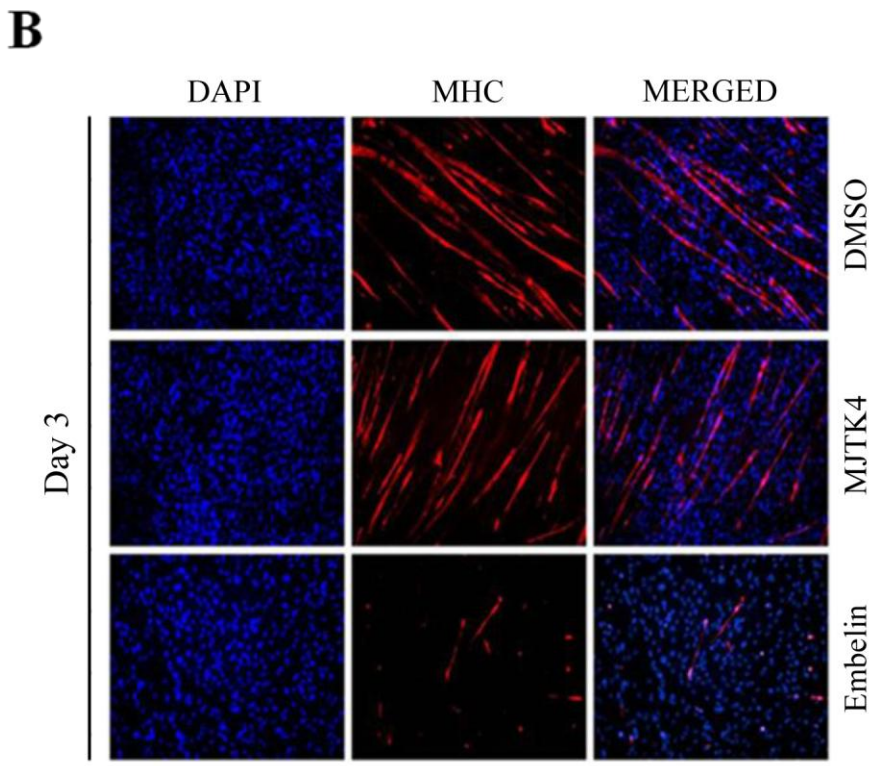


Figure 3.2.4B Embelin inhibits muscle differentiation: (B) For MHC staining, C2C12 cells were treated with embelin, MJTK-4 and vehicle (DMSO) for 72 hours (D3). The cells were then immuno-stained for MHC using anti-MHC (red) antibody and the nuclei were stained with DAPI (blue). Images were captured under fluorescent microscope using 10X magnification. There was a significant reduction in the number of MHC^+ cells.

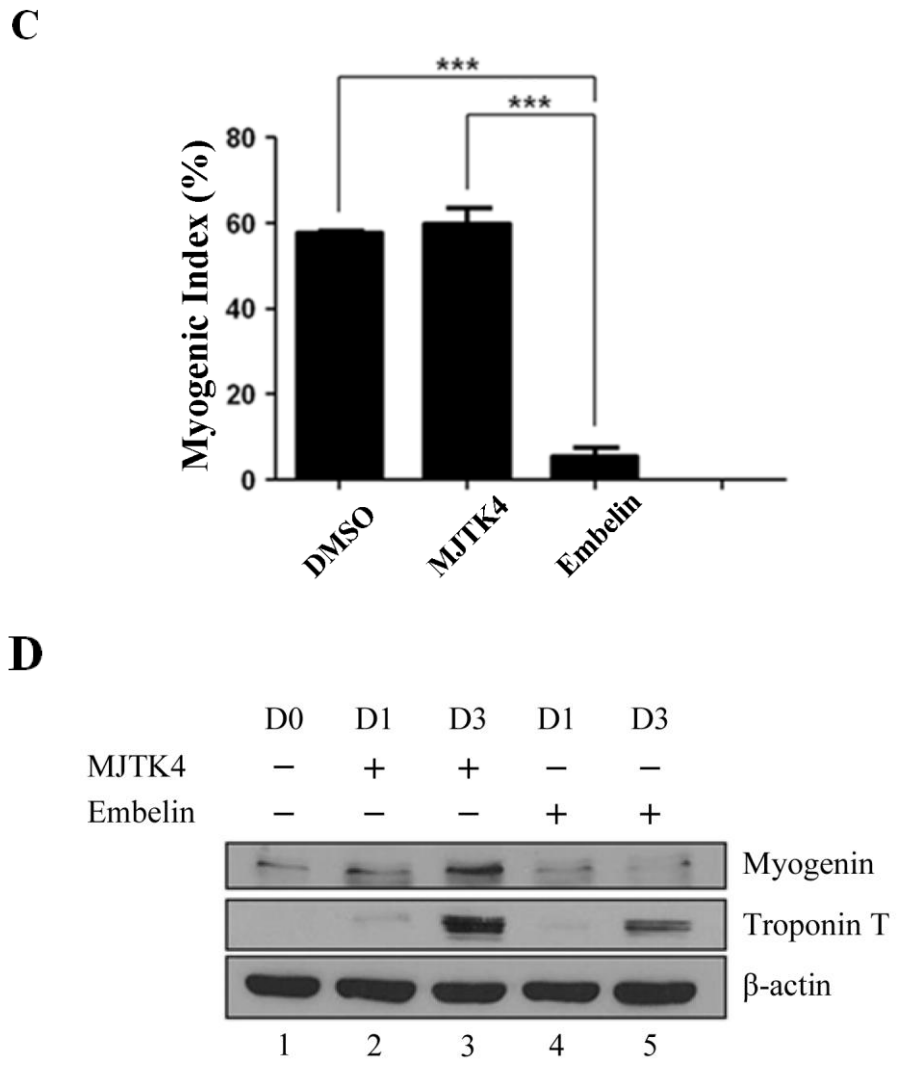


Figure 3.2.4 C&D Embelin inhibits muscle differentiation: (C) Quantification of myogenic index from 4 randomly chosen microscopic fields (at least 400 nuclei were calculated) revealed a significant decrease in the number of MHC^{+} cells in embelin treated cells compared to controls. Values obtained were represented as means with error bar indicating standard deviations. Statistical significance was determined by the two-tailed, un-paired Student's t test and *p*-values of <0.05 were considered to be statistically significant. p-value obtained is shown as ******* ($p < 0.001$). (D) C2C12 cells were left untreated (D0) or treated with10μM of Embelin or MJTK-4 for 24 hours (D1) or 72 hours (D3) in DM. Cell lysates were analyzed by western blot for myogenic markers such as myogenin and Troponin T expression at days 0, 1 and 3. β-actin was used as internal control.

3.2.5 Embelin affects P/CAF mediated MyoD acetylation

It has been shown by previous studies that p300 first acetylates H3 and H4 within the promoter region and then recruits P/CAF which interacts and directly acetylates MyoD to initiate skeletal myogenesis (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010). To examine whether treatment of cells with embelin results in altered MyoD acetylation, co-immunoprecipitation assays were performed. 293T cells were transfected with either Myc-tagged MyoD or Flag-tagged P/CAF alone or together in the presence of either 15μM embelin or MJTK-4. In addition, the cells were also treated with 10mM of NAM and sodium butyrate. Twenty-four hours post-transfection, cell lysates were harvested using RIPA lysis buffer, to which 10mM of NAM and sodium butyrate were added and checked for expression of Myc-MyoD and Flag-P/CAF. Cell lysates were immunoprecipitated with Myc-agarose beads and immunoprecipitates were probed by western blot for P/CAF mediated MyoD acetylation using anti-acetyl-lysine (Ac-Lys) antibody. As expected, MyoD was acetylated when it was co-expressed with P/CAF (**Fig 3.2.5**). However, treatment with embelin resulted in reduction in MyoD acetylation compared to untreated and MJTK-4 control. However, P/CAF interaction with MyoD remained unaltered (**Fig 3.2.5**).

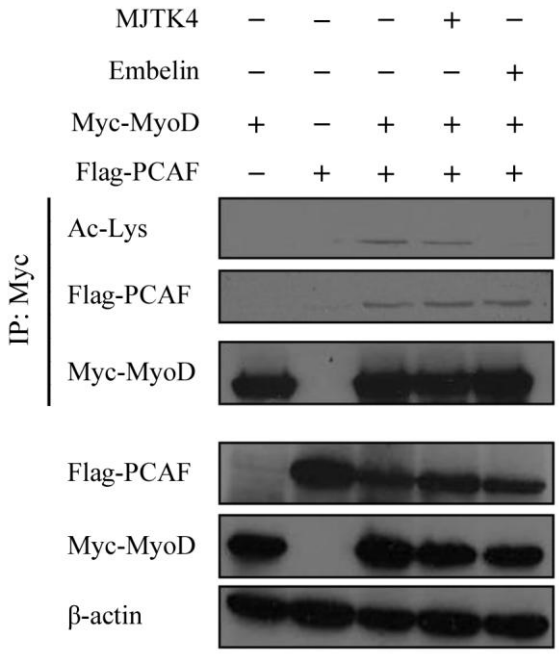


Figure 3.2.5 Embelin affects P/CAF mediated MyoD acetylation: HEK293T cells were transfected with full length Myc-tagged MyoD, Flag-tagged P/CAF either individually or together in the presence of 15μM of Embelin or MJTK-4 (control). Untreated cells were used as an additional control. MyoD was immunoprecipitated using anti-Myc agarose beads followed by immuno-blotting with anti-Flag, anti-Ac-Lys and anti-Myc antibody. Lysates (input) were analyzed for MyoD and P/CAF expression by western blot analysis. β-actin was used as internal control.

3.2.6 Embelin does not alter H3K9 acetylation on muscle promoters

To examine for any alteration in the level of histone H3K9 acetylation on muscle promoters upon embelin treatment, ChIP assays were carried out on myogenin (**Fig 3.2.6A**) and MCK promoter (**Fig 3.2.6B**) by treating C2C12 cells with10μM of embelin and MJTK-4 for two days in differentiation medium. D0 cells were left untreated. We observed no alteration in H3K9 acetylation on both the muscle promoters. Together these results (**Fig 3.2.5,**

3.2.6A & 3.2.6B) show the specificity of embelin as selective P/CAF inhibitor. In addition, these data are in clear agreement with and further validate the previous literature about distinct roles played by p300 and P/CAF, wherein p300 was shown to first acetylate H3 and H4 within the promoter region and then recruit P/CAF which then interacts and directly acetylates MyoD resulting in its transactivation (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010).

A

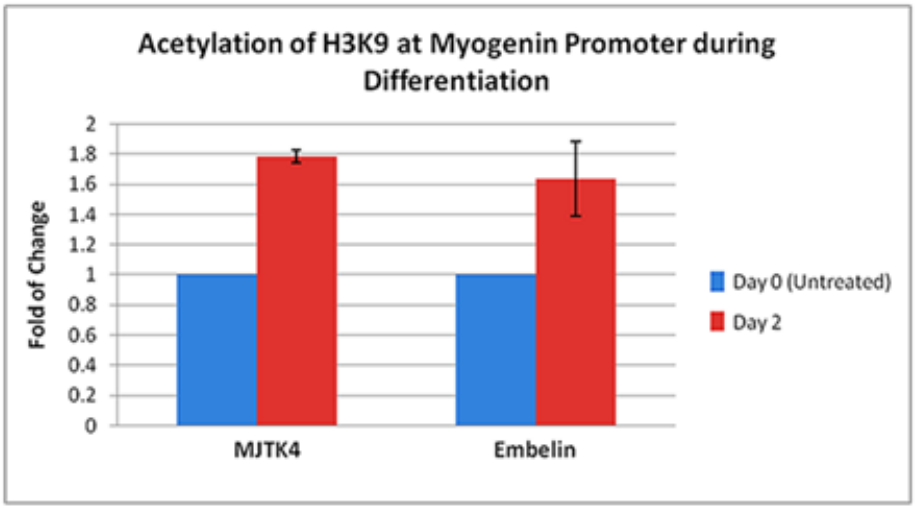


Figure 3.2.6A Embelin treatment does not alter H3K9 acetylation on myogenin promoter: (A) H3K9 acetylation of the myogenin promoter was analyzed by ChIP assays. C2C12 cells were cultured in GM (Day 0) and switched to DM and treated with 10µM of Embelin or MJTK-4 (control) for 48 hrs (Day 2). Cell lysates were subjected to ChIP assays using H3K9ac antibody. DNA was analyzed by Q-PCR using primer for the myogenin promoter and the values obtained were normalized to β-actin. Statistical significance was determined by Student's *t* test and p-value obtained was not statistically significant ($p < 0.22$). Experiments were done by Mr. Wai Kay Kok.

B

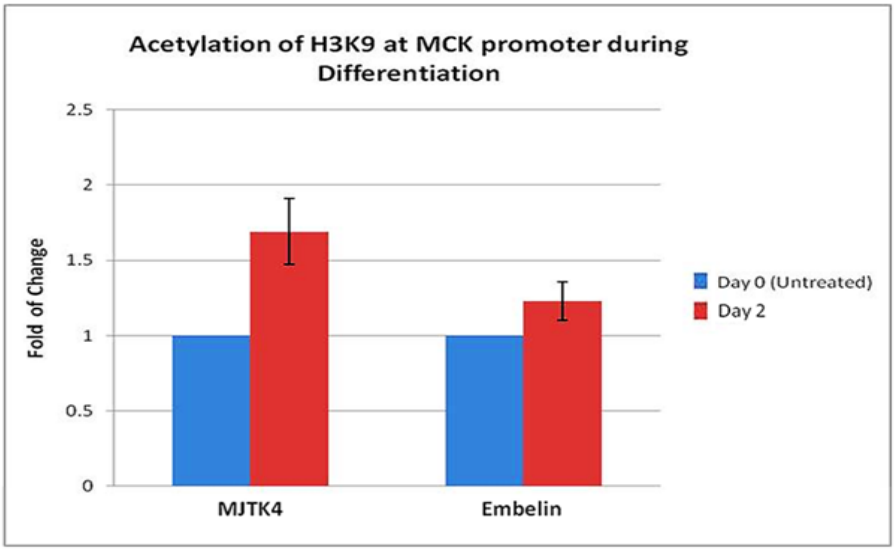


Figure 3.2.6B Embelin treatment does not alter H3K9 acetylation on MCK promoter: (B) H3K9 acetylation of the MCK promoter was analyzed by ChIP assays. C2C12 cells were cultured in GM (Day 0) and switched to DM and treated with 10μM of Embelin or MJTK-4 (control) for 48 hrs (Day 2). Cell lysates were subjected to ChIP assays using H3K9ac antibody. DNA was analyzed by Q-PCR using primer for the MCK promoter and the values obtained were normalized to β-actin. Statistical significance was determined by Student's *t* test and p-value obtained was not statistically significant ($p < 0.069$). Experiments were done by Mr. Wai Kay Kok.

3.2.7 Embelin treatment results in alteration of global H3K9 acetylation during differentiation

As the preceding result shows that embelin treatment results in no alteration of H3K9 acetylation on muscle promoters, we went on to check whether such treatment results in alteration of global H3K9ac. C2C12 cells were cultured in GM and then switched to DM with 10μM of embelin or MJTK-4 (control) and induced to differentiate for two days (D2). Lysates were collected and

analyzed by western blot for H3K9ac using anti-H3K9ac antibody. The level of H3K9ac increased upon differentiation (D2) in control cells treated with MJTK-4 compared to D0. Surprisingly, H3K9ac decreased in D2 cells upon embelin treatment to the level seen in D0 cells (**Fig 3.2.7**). This result is in contrast to what was seen by ChIP assays on the myogenin and MCK promoters upon embelin treatment, where the level of H3K9ac remains unaltered. This suggests that global H3K9ac is mediated by both by both p300 and P/CAF. This finding is interesting as it sheds light on the previously unreported role of P/CAF in mediating global H3K9ac in muscle cells.

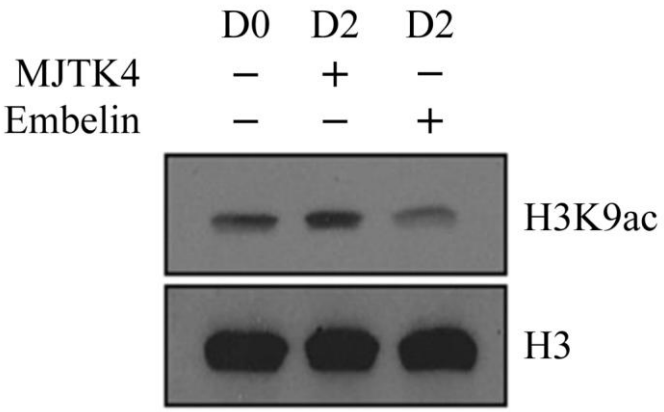


Figure 3.2.7 Embelin treatment results in alteration of global H3K9 acetylation during differentiation: C2C12 cells were cultured in GM (D0) and switched to DM with 10μM of embelin or MJTK-4 for 48 hrs (D2). Cell lysates were collected at day 0 and day 2. Global H3K9 acetylation was determined using anti H3K9ac antibody. Histone H3 levels were assayed as control.

3.2.8 Embelin affects a wide array of muscle specific gene expression during differentiation

While the role of P/CAF in acetylation and activation of MyoD has been reported, its genome wide targets in myogenesis have not been reported. To identify genes whose expression is altered upon embelin treatment by targeting P/CAF acetyltransferase activity, C2C12 cells were cultured in GM for 24 hours (Day 0) in the presence of 10μM of DMSO (control) or embelin and then switched to DM for another 24 hrs (Day 1) and 48 hrs (Day 2) respectively. From these cells, total RNA was isolated, reverse transcribed, labelled and subsequently hybridized to Illumina mouse WG-6 v2.0 array.

Our microarray data revealed that there was no significant difference in the global gene expression until day 1 in cells treated with embelin compared to control (DMSO) cells (**Fig 3.2.8**), although the absolute change in the expression pattern from day 0 to day 1 was slightly lower in embelin treated cells compared to control (**Fig 3.2.8**). However, a significant change was observed in gene expression pattern in cells that were treated with embelin for 2 days compared to control (DMSO) cells (**Fig 3.2.8**). This change in expression pattern of many muscle specific genes on day 2 upon embelin treatment is consistent with the impairment of myotube formation seen morphologically on day 2 in the earlier results (**Fig 3.2.4A**). As trans-activation of MyoD by P/CAF mediated acetylation is essential for MyoD to up-regulate its downstream target, we analyzed few MyoD responsive genes

myosin heavy chain (MYH1) and MRF4 and validated their significant reduction by Q-PCR (**Fig 3.2.8B & 3.2.8C**)

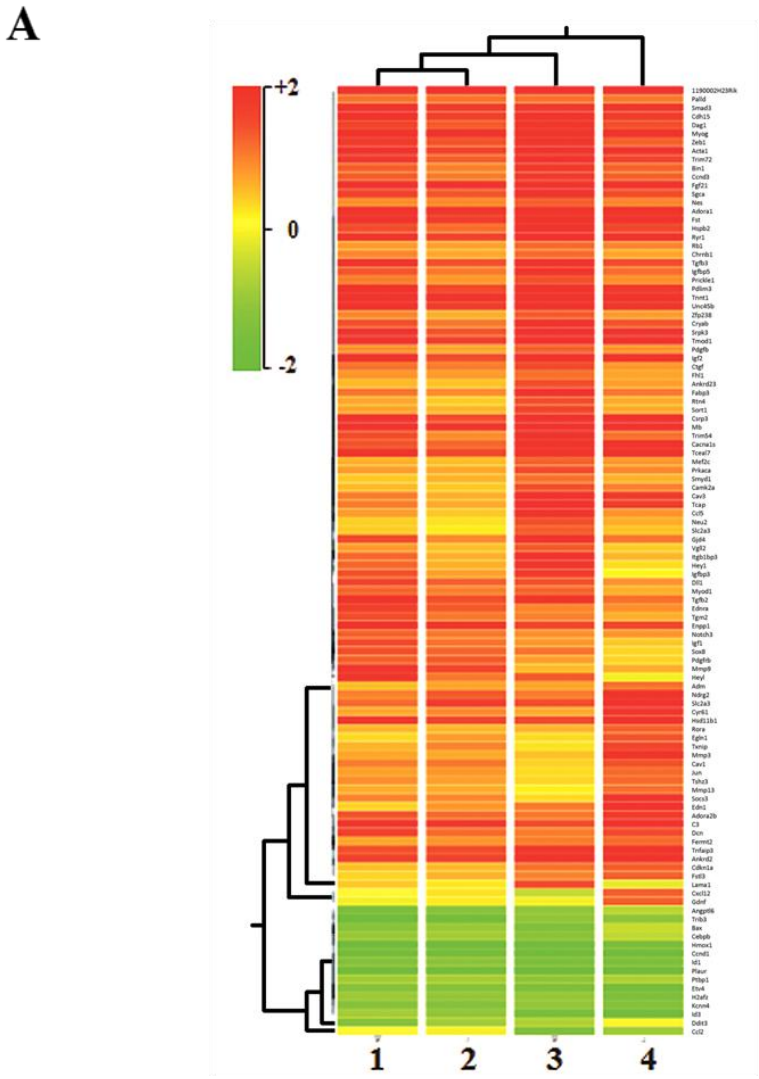


Figure 3.2.8A Embelin treatment results in alteration of a set of genes during differentiation: (A) C2C12 cells were cultured in GM for 24 hours (Day 0) in the presence of 10μM of DMSO or embelin and then switched to DM for another 24 hrs (Day 1) and 48 hrs (Day 2) respectively. From day 1 and day 2 DMSO and embelin treated cells, total RNA was isolated, reverse transcribed, labelled and subsequently hybridized to Illumina mouse WG-6 v2.0 array. The differential

expression of various genes after 24 hours and 48 hours treatment with DMSO (lane 1 and 3) and embelin (lane 2 and 4) upon induction of differentiation is presented.

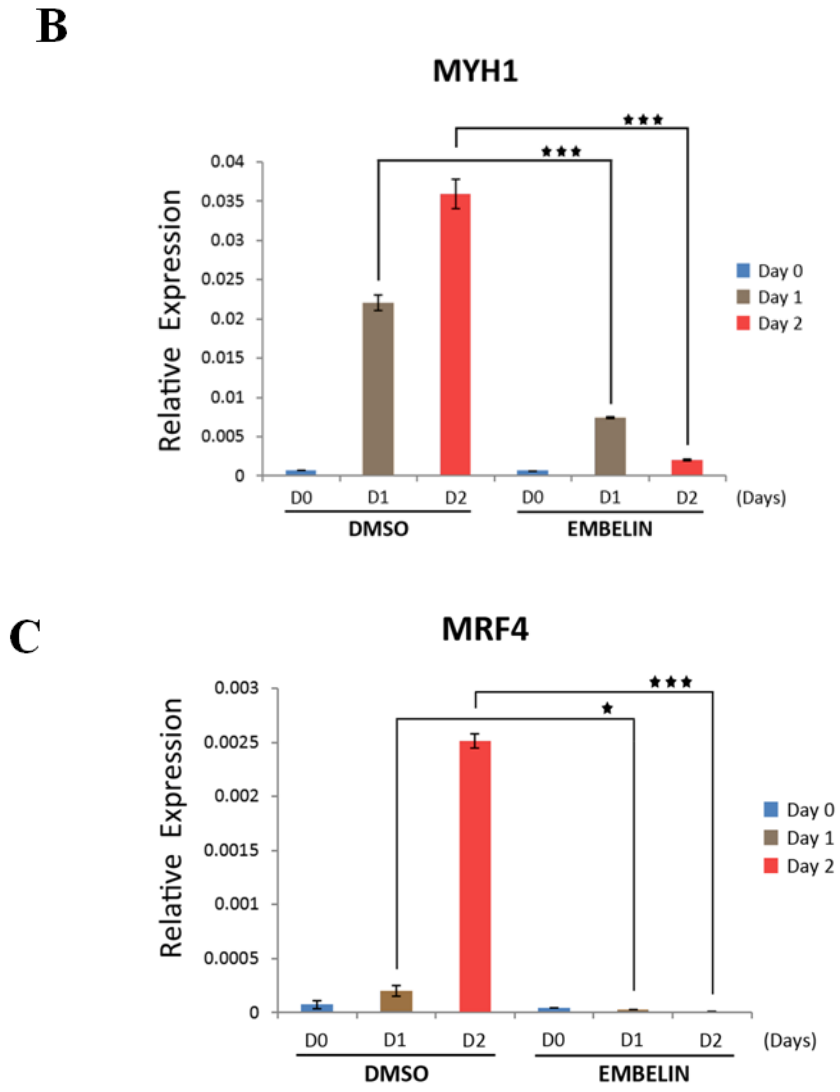


Figure 3.2.8B&C Embelin treatment results in alteration of a set of genes during differentiation: (B) Expression of few MyoD responsive genes like myosin heavy chain (MYH1) and MRF4 in the embelin treated C2C12 cells were validated by Q-PCR. The fold expression of muscle-specific genes in the embelin treated over DMSO (control) treated C2C12 cells was calculated and plotted as the average of three biological replicates. The relative mRNA

expression were quantified and standardized against the house-keeping gene GAPDH. Values obtained were represented as means with error bar indicating standard deviations. Statistical significance was determined by Student's *t* test and p-value obtained is shown as ***** [$p< 0.05$], ******* [$p< 0.001$]. Microarray data provided by Mr. Vinay Kumar.

3.2.9 Biological network analysis from microarray data obtained from embelin treated cells Vs DMSO treated cells

The *de novo* mouse muscle differentiation interactome map was generated by carrying out Biological network analysis from the microarray data using Genespring GX v.12.0 from a total of 171 genes that are annotated to be involved in myogenic differentiation. The analysis revealed that targeting P/CAF acetyltransferase activity by embelin leads to induction in the expression of several genes such as TNFα, NOS2, STAT3, PAX 7 [**Fig 3.2.9** and **Table 3.2.9** (**inner ring**)], NOS1, Sox9, BMP4, Socs3 [**Fig 3.2.9** and **Table 3.2.9** (**middle ring**)], JAK3, KLF4, NFκB [Fig 3.2.9 and **Table 3.2.9** (**outer ring**)], (Diao *et al*., 2009; Jang *et al*., 2012;Wang *et al*.,2008; Yang *et al*., 2009; Kataoka *et al*., 2003; Chandran *et al*., 2007; Kaliman *et al*., 1999; Soleimani *et al.*, 2012; Hernández-Hernández *et al*., 2009) which are known to be essential for myoblast survival, proliferation and inhibition of myogenic differentiation. On the other hand, it leads to repression of genes such as Adam12, Mef2c, SRF [**Fig 3.2.9** and **Table 3.2.9** (**middle ring**)], MyoD and laminin-α1[**Fig 3.2.9** and **Table 3.2.9** (**outer ring**)], which are essential for myogenesis (Lafuste *et al*., 2005; Braun and Gautel *et al*., 2011; Kuhl *et al*., 1982; Foster *et* al., 1987; Kroll *et al*., 1994). These results collectively suggest

that P/CAF regulates skeletal muscle differentiation through various MyoD dependent and independent pathways.

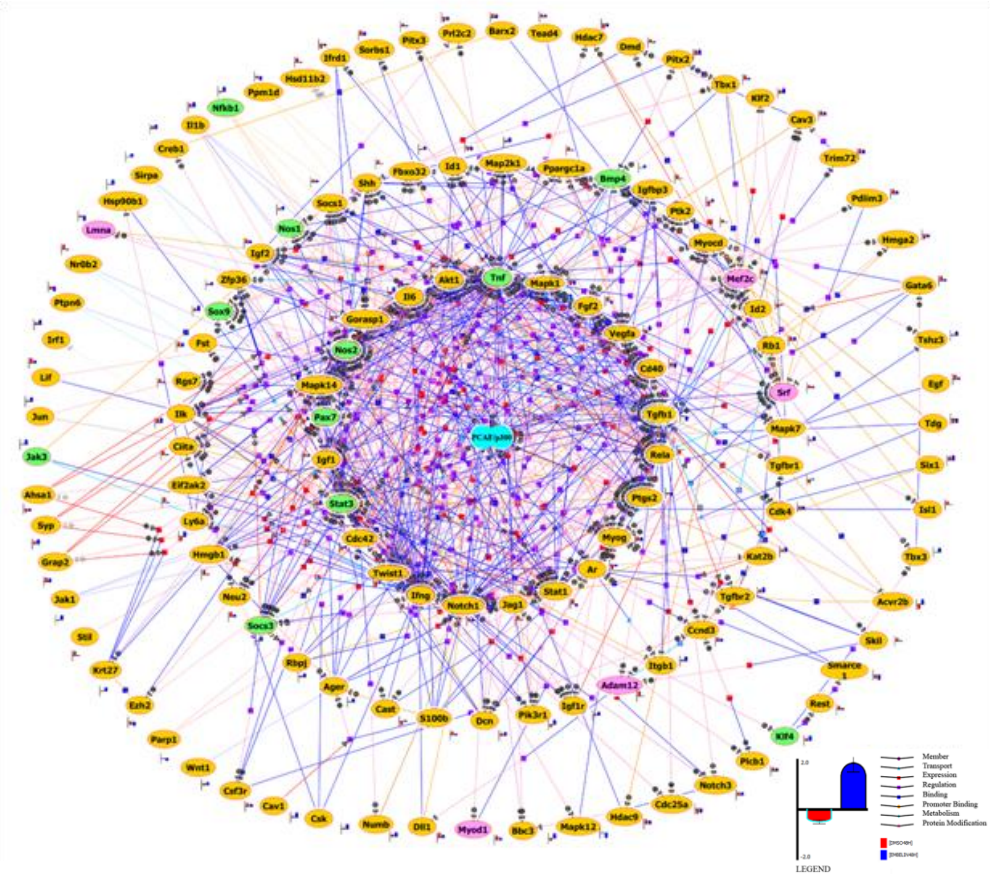


Figure 3.2.9 Biological network analysis from microarray data obtained from embelin treated cells vs. DMSO treated cells: Biological network analysis exhibiting the differential expression of several genes involved in myogenic differentiation in C2C12 cells cultured in DM for 48 hrs (Day 2) in the presence of 10µM of DMSO (red bar) or embelin (blue bar). Genes of interest analyzed under this result section is highlighted in green and pink for induction and repression respectively. Data provided by Rahul Modak from Dr. Tapas Kundu's lab.

Table 3.2.9 A total of 128 genes that are shown in Fig 3.2.9 is being tabulated here (genes are tabulated in the order of inner, middle and outer ring of the *de novo* mouse muscle differentiation interactome map shown in the Fig3.2.9 above).

3.3 Role of G9a in skeletal muscle pathology (Rhabdomyosarcoma)

Rhabdomyosarcoma is a highly malignant and most common soft tissue sarcoma of childhood and adolescence which accounts for approximately 5-8% of pediatric cancer. It originates as a consequence of disruption of growth and differentiation pathways of myogenic precursor cells and is characterized by the expression of MyoD and its downstream target genes such as myogenin (Scrable *et al*., 1989; Sebire *et al*., 2003; Ren *et al*., 2008; Tapscott *et al*., 1993). In spite of the expression of MyoD, it does not lead to cell cycle arrest or to terminal differentiation, implicating that MyoD pathway is functionally defective. As G9a inhibits myogenesis by affecting the transcriptional activity of MyoD, I wanted to know if these findings have any clinical significance; hence I analyzed for the relevance of G9a in rhabdomyosarcoma.

3.3.1 G9a is overexpressed in ARMS cells under differentiation-permissive conditions

To investigate the possible role of G9a in RMS, its expression was analyzed in Rh30 cells at protein level in proliferating and differentiation-permissive conditions by western blot analysis. Rh30 cells were cultured in GM for 24 hours (Day 0) and then switched to DM for another 24 hours (Day 1), 48 hours (Day 2) and 72 hours (Day 3) respectively. In addition, C2C12 cells were cultured in GM and then switched to DM for 48 hours (Day 2). Cell lysates were collected at day 0, 1, 2 and 3 for Rh30 cells and C2C12 cells respectively and analyzed for the expression of G9a by using anti-G9a antibody. Consistent with the result obtained earlier, G9a was expressed in C2C12 myoblasts at day

0 and declined upon differentiation (Day 2) (**Fig 3.3.1A**). In contrast, in Rh30 cells, G9a was expressed at day 0, 2 (**Fig 3.3.1B)** and its expression was sustained at high levels until day 3 of differentiation (**Fig 3.3.1C**) demonstrating deregulated expression of G9a in ARMS cells.

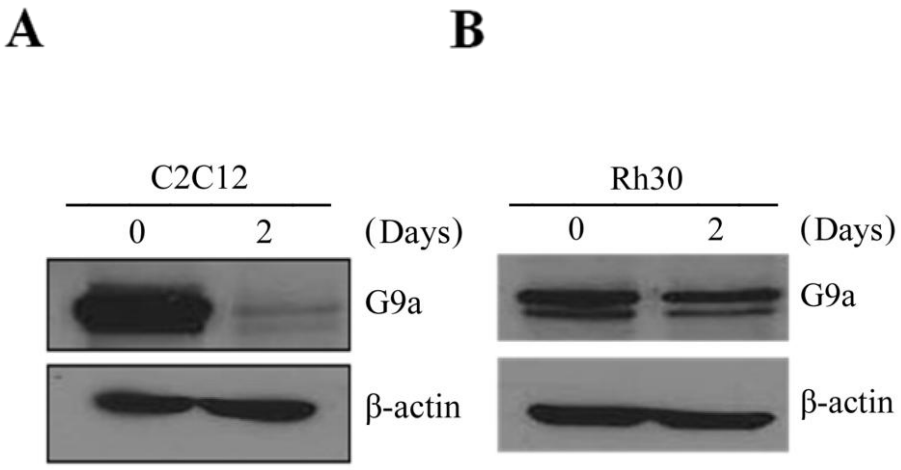


Figure 3.3.1A&B G9a is overexpressed in Rh30 cells: (A) C2C12 cells were grown either in GM (Day 0) or DM for 48 hrs (Day 2). Lysates were collected at day 0 and day 2 and analyzed for G9a expression by western blot analysis. β-actin was used as internal control. (B) Rh30 cells were grown either in GM (Day 0) or DM for 48 hrs (Day 2). Lysates were collected at day 0 and day 2. Lysates (input) were analyzed for G9a expression by western blot analysis. β-actin was used as internal control.

Since RMS is characterized by the expression of key early myogenic regulators of differentiation namely Myf5 and MyoD as well as its target myogenin (Scrable *et al*., 1989; Sebire *et al*., 2003; Ren *et al*., 2008; Tapscott *et al*., 1993), the immunoblot was probed for the expression of MyoD and myogenin in addition to G9a. Consistent with previous reports, these MRFs (**Fig 3.3.1D**) were found to be expressed in Rh30 cells although they have lost

the ability to terminally differentiate as evident by the lack of expression of terminal differentiation markers like Troponin T (**Fig 3.3.1C**).

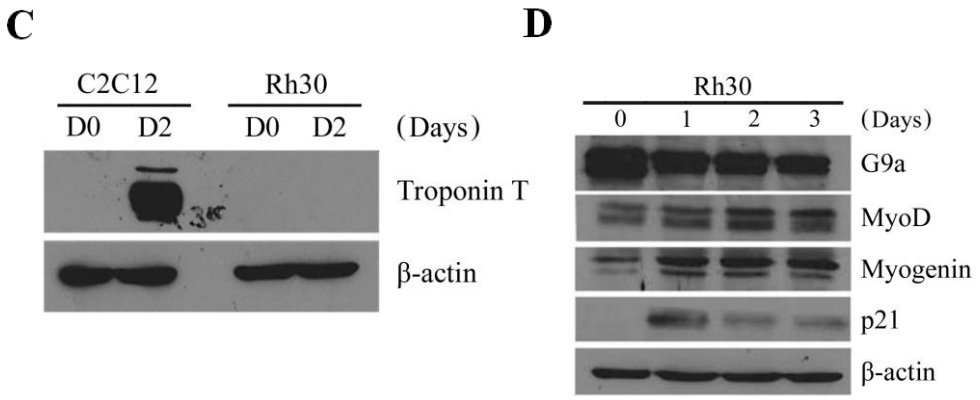


Figure 3.3.1C&D G9a is overexpressed in Rh30 cells: (C) Lystaes that were collected at day 0 and day 2 from C2C12 and Rh30 cells were then analyzed for Troponin-T expression. (D) Rh30 cells were grown either in GM (Day 0) or DM for 24, 48 and 72 hrs respectively (Day1, 2 and 3). Lysates were collected at day 0, 1, 2 and 3 and analyzed for G9a, MyoD, myogenin and p21 expression by western blot analysis. β-actin was used as internal control.

Studies have shown that the expression of p21 and MyoD has an inverse relationship in these tumors (Weintraub *et al*., 1997). For example, Rh30 cells which express high level of MyoD have lower p21 levels. Conversely, Rh28 cells which have lower expression of MyoD have higher level of p21. Hence, p21 expression was analyzed in Rh30 cells and found to be expressed at lower level consistent with previous reports (**Fig 3.3.1D**).

3.3.2 UNC0638 induces expression of myogenin and p21 in Rh30 cells

Since G9a expression is deregulated in ARMS cells (i.e., Rh 30 cells), its methyltransferase activity was targeted using UNC0638 to examine whether it rescues the differentiation defect. Rh30 cells were cultured in GM for 24 hours (Day 0) and then switched to DM for another 24 hours (Day 1) and48 hours (Day 2) respectively in the absence and presence of 1.5µM of UNC0638. Cell lysates were collected at day 0, 1 and 2 and analyzed for expression of myogenin and p21. Interestingly, Rh30 cells treated with 1.5µM of UNC0638 exhibited higher expression of myogenin and p21 (**Fig 3.3.2**) compared to untreated control cells at day 1 and day 2 suggesting the role of G9a in the differentiation defect of RMS.

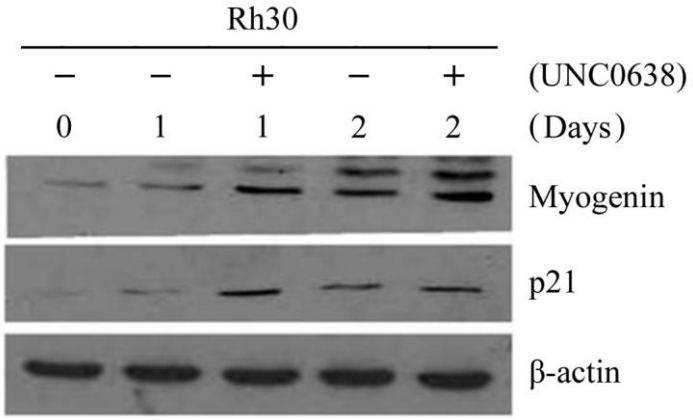


Figure 3.3.2 UNC0638 induces expression of myogenin and p21 in Rh30 cells
Rh30 cells were grown in GM (Day 0) and switched to DM for 24 hrs (Day 1) and 48 hrs (Day 2) respectively in the absence and presence of 1.5µM of UNC0638. Lysates were collected at day 0, 1 and 2 and analyzed for myogenin and p21 expression by western blot analysis. β-actin was used as the internal control.

3.3.3 UNC0638 induces early expression of myogenin in Rh30 cells

UNC0638 treatment of Rh30 cells in differentiation-permissive condition induced higher expression of myogenin at day 1 and day 2 (**Fig3.3.2**); hence, Rh30 cells were treated with UNC0638 at day 0 to examine whether it results in early induction of myogenin. Rh30 cells were cultured in GM for 24 hours (Day 0) in the absence and presence of 1.5µM of UNC0638 and then switched to DM for another 48 hours (Day 2) in the absence and presence of 1.5µM of UNC0638. Cell lysates were collected at day 0 and 2 and analyzed for the expression of myogenin. Interestingly, Rh30 cells treated with UNC0638 at day 0 resulted in early induction of myogenin (**Fig3.3.3**).

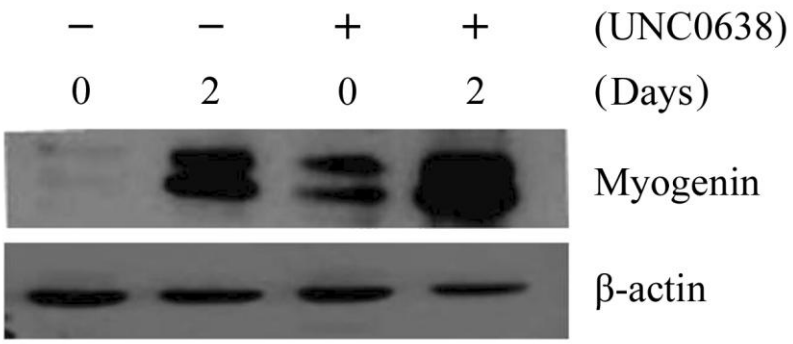


Figure 3.3.3 UNC0638 induces early expression of myogenin in Rh30 cells: Rh30 cells were grown for 24 hours in GM (Day 0) in the absence and presence of 1.5µM and switched to DM for another 48 hrs (Day 2) in the absence and presence of 1.5µM of UNC0638. Lysates were collected at day 0 and day 2 and analyzed for myogenin expression by Western blot analysis. β-actin was used as internal control.

3.3.4 Loss of G9a induces myogenin and p21 expression in Rh30 cells

Rh30 cells were transfected with 100nM of scrambled siRNA (control) or human G9a specific siG9a for 48 hrs in GM. As shown in **Fig 3.3.4**, siG9a reduced endogenous G9a levels in Rh30 cells. To assess the effect of G9a knockdown on control and siG9a cells, Rh30 cells were switched to DM for another 96 hrs (Day 4). Cell lysates were collected from G9a depleted (siG9a) or control (siRNA) Rh30 cells on day 0 and day 4 and western blot analyses performed for myogenin as well as p21showed an increase in the expression of myogenin and p21 on day 4 in siG9a cells compared to siRNA cells (**Fig 3.3.4**).

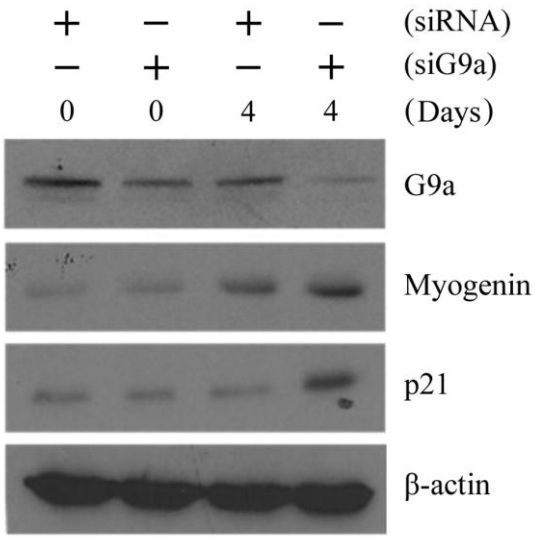


Figure 3.3.4 Loss of G9a induces higher expression of myogenin and p21 in Rh30 cells: Rh30 cells were transfected with human siRNA against G9a (siG9a), scrambled siRNA (siRNA) for 48 hours. Lysates were collected for day 0 and analyzed for the knockdown of endogenous G9a at protein level by western blot. Rh 30 cells were switched to DM for another 96 hours (Day 4) and lysates were collected. Cell lysates were then analyzed by western blot for myogenin and p21expression at days 0 and 4. β-actin was used as internal control.

CHAPTER 4

DISCUSSION

4. Discussion

Skeletal muscle development and differentiation is controlled by the combinatorial activity of two transcription factors families that include Myogenic Regulatory Factors (MRFs) and the Myocyte Enhancer Factor-2 (MEF2) which works in combination with various other transcription factors and epigenetic regulatory mechanisms. The goal of this project is to investigate the role of G9a in epigenetic regulation of skeletal muscle differentiation. I have identified G9a as a novel negative regulator of myogenesis which is expressed high in proliferating myoblasts and is down-regulated upon differentiation. Overexpression of G9a in skeletal muscle precursor cells blocks their ability to undergo differentiation. This repression of differentiation is dependent on its methyltransferase activity. Thus over-expression of mutant G9a lacking catalytically active SET domain fails to inhibit myogenesis unlike wild-type G9a. Conversely, knockdown of G9a in proliferating myoblasts by siRNA results in enhanced myogenic differentiation reflected by an increase in the number of myotubes as well as early induction of muscle specific genes such as myogenin and Troponin-T. At the molecular level, G9a mediated repression of muscle genes is achieved through enrichment of transcriptionally repressive H3K9me2 mark on the myogenin promoter which is a MyoD target gene. Over-expression of G9a results in significant increase in H3K9me2 both in undifferentiated as well as in differentiated myotubes. In addition to mediating H3K9me2 on muscle promoters, my findings show that G9a associates with the transcription factor

MyoD (**Fig.4**) through its ANK domain, and methylates at a single lysine (K) residue 104. The association of G9a with MyoD is essential for methylation, as protein interaction studies demonstrate that disruption of the association by the deletion of G9a ANK domain abrogates MyoD methylation. Methylation of MyoD inhibits its transactivational ability to upregulate myogenin expression. Thus mutation of K104 to arginine (K104R) makes MyoD non-responsive to methylation and this results in mutant MyoD (K104R) exhibiting higher myogenic potential compared to wild-type MyoD in myogenic conversion assays.

It is interesting to note that K104, which is subjected to methylation by G9a, also happens to be one of the three sites for acetylation by P/CAF. Previous studies have shown that P/CAF directly acetylates MyoD at K99, K102 and K104 during differentiation, resulting in its transactivation (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010). As K104 is an overlapping site for G9a mediated methylation and P/CAF mediated acetylation, this raised the possibility that G9a might disrupt P/CAF-mediated MyoD acetylation. Immunoprecipitation assays indeed confirmed this notion and revealed that G9a alters the ability of P/CAF to interact with MyoD and correspondingly MyoD acetylation. Moreover, depletion of G9a in proliferating myoblasts by siRNA mediated knockdown shifted the kinetics of acetylation to an early time point. These results demonstrate the potentiality of cross-talk between G9a and P/CAF in controlling MyoD transcriptional activity and as a consequence transition from myoblasts to myotubes.

To further understand the broader role played by P/CAF in regulating skeletal muscle differentiation, embelin, a natural compound which specifically targets the acetyltransferase activity of P/CAF was used. This enabled me to analyze its global impact on skeletal muscle differentiation. Treatment of C2C12 cells with embelin resulted in overall defect in skeletal muscle differentiation with lesser number of MHC positive cells as well as down-regulation of myogenin and Troponin-T. As P/CAF mediated MyoD acetylation is crucial for the differentiation of myoblasts to myotubes, immunoprecipitation assays were done to determine the impact of embelin on MyoD acetylation. In embelin treated cells, MyoD acetylation was reduced although MyoD-P/CAF interaction remained intact. This could attribute to the repression of MyoD target genes such as myogenin and Troponin-T and as a consequence impairment of myotube formation. Our result from ChIP assays showed that embelin treatment did not result in any alteration in the level of histone H3K9 acetylation at the myogenin and MCK promoters. These results validate previously documented studies that have demonstrated the distinct roles played by p300 and P/CAF, with p300 first acetylating H3 and H4 within the promoter region and then recruiting P/CAF which then interacts and directly acetylates MyoD (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010). Interestingly, the analysis for alteration in global H3K9ac in C2C12 cells upon embelin treatment yielded a contrasting result as it showed a decrease in the level of H3K9ac, suggesting the possibility that in skeletal muscle cells, global H3K9ac is mediated by both p300 and P/CAF. This result

is significant as it enables us to characterize the previously unexplored role of P/CAF in mediating global H3K9ac in muscle cells. Our micro-array data and subsequent biological network analysis enabled us to identify the wide array of genes that are known to be regulated by P/CAF during muscle differentiation. More importantly, several new and previously uncharacterized targets have been identified. In addition to reduction in the expression of MyoD responsive genes, such as myogenin, MRF4 and MHC, there was altered expression of muscle structural genes, histone modifiers, and many transcription factors. For example, targeting P/CAF acetyltransferase activity resulted in repression of genes like laminin-α1, which encodes for one of the three non-identical chains of laminin (laminin-1; alpha 1-beta 1-gamma 1), a basement membrane protein which plays a prominent role in facilitating myoblast adhesion, migration, proliferation and myotube formation (Kuhl et *al*., 1982; Foster *et al*., 1987; Kroll *et al*., 1994). In addition to laminin-α1, ADAM12 and SRF were found to be repressed. ADAM12 (a disintegrin and metalloproteinase domain containing protein 12) is a multi-domain protein which binds to $\alpha_9\beta_1$integrin, mediating cell-cell interaction resulting in fusion of myoblasts to form multinucleated myotubes (Lafuste *et al*., 2005). Likewise SRF (Serum responsive factor), a MADS box transcription factor related to Mef2 plays a central role in skeletal muscle growth and maturation (Li *et al*., 2005).

Embelin treated cells show higher expression of Pax7 which is essential for satellite cell survival and proliferation thereby maintenance of the

undifferentiated state (Soleimani *et al*., 2012; Wang and Rudnicki 2011). STAT3, a transcription factor which plays a central role in the regulation of growth, differentiation and survival in various cell types, is induced in the embelin treated cells. Studies have shown that STAT3 can interact with MyoD depriving it of transcriptional co-factors p300 and P/CAF thereby affecting its transcriptional activity resulting in inhibition of differentiation (Kataoka, *et al.*, 2003).

These results collectively suggest that P/CAF regulates skeletal muscle differentiation through various MyoD dependent and independent pathways.

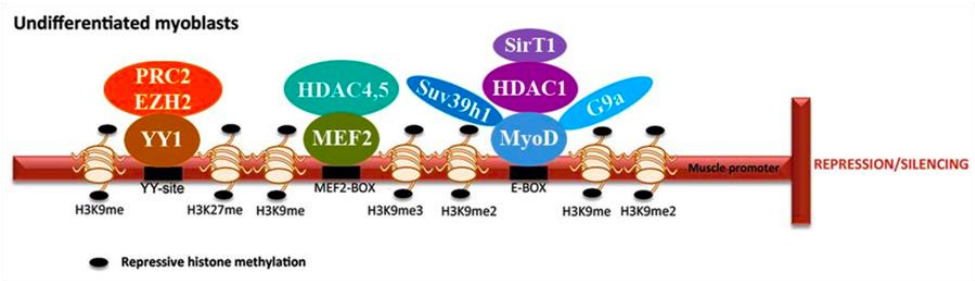


Figure 4 Model for epigenetic regulation of promoters in undifferentiated muscle cells (adapted from Transcription.2012; 3:1-6).

CONCLUSION AND FUTURE STUDIES

Conclusion and future studies

My work has revealed the previously not well-understood epigenetic mechanisms by which chromatin modifiers like G9a, an enzyme predominantly involved in H3K9me2, regulate the transcriptional activity of non-histone substrates such as MyoD by directly methylating them and controlling cellular differentiation programs. It is well known that P/CAF mediated acetylation of MyoD at three lysine residues is the key determinant of MyoD activity during differentiation (Sartorelli *et al*., 1999; Dilworth *et al*., 2004; Aziz *et al*., 2010). However, it is unclear why P/CAF mediated MyoD acetylation primarily occurs upon induction of differentiation although P/CAF is expressed even in undifferentiated cells (Sartorelli *et al*., 1999; Mal *et al*., 2001). My work provides a partial explanation for this, as it shows that K104 is an overlapping site for methylation by G9a and acetylation by P/CAF. G9a disrupts P/CAF mediated MyoD acetylation. This suggests that G9a, which is expressed in proliferating myoblasts methylates MyoD, and once methylated, K104 is unavailable for P/CAF mediated acetylation thereby restrains MyoD transcriptional activity in myoblasts. Thus there might be a regulatory axis involving G9a and P/CAF in controlling MyoD activity and thereby the transition from myoblasts to myotubes which needs to be further investigated. As other histone methyltransferases like Suv39h1 and Ezh2 are also expressed in proliferating myoblasts and block MyoD transcriptional activity (Mal *et al*., 2006; Caretti *et al*., 2004), it raises the possibility that G9a may be part of a large mutiprotein complex of chromatin modifiers that prevents premature

activation of muscle differentiation program. Since these HKMTs leave different repressive chromatin marks such as H3K9me2, H3K9me3 and H3K27me3, whether they act in unison or in sequence to mediate gene repression or silencing, to maintain an undifferentiated state, remains to be investigated. However, unlike G9a, which inhibits MyoD activity at multiple levels by directly methylating it as well as mediating H3K9me2 on muscle promoters, the repressive function of Suv39h1 and Ezh2 remains restricted to chromatin modification as they have not been reported to target MyoD for methylation. Interestingly, G9a methylation consensus extends beyond MyoD to include all other members of MRFs (**Fig 3.1.12**) and if all the MRFs are indeed the targets for its methylation, the consequences of such methylations need to be examined.

In addition, the analysis for a broader role of P/CAF in skeletal myogenesis by pharmacological inhibition of P/CAF activity enabled us to identify a wide array of genes that exhibited altered expression. Some of these genes may be direct P/CAF targets (those that are down regulated), whereas genes upregulated by embelin treatment likely reflect its indirect targets. Future studies aimed at dissecting the functional role of some previously unknown putative direct targets of P/CAF should shed insights into their role in myogenesis.

My preliminary result shows deregulated expression of G9a in rhabdomyosarcoma which are characterized by a differentiation defect despite expressing MyoD. Targeting G9a methyltransferase activity through the use of

chemical probe UNC0638 or depletion of G9a by siRNA mediated knockdown resulted in the induction of MyoD target genes such as myogenin and p21. These studies will be continued to determine whether targeting G9a expression/activity leads to induction of differentiation in cell culture and animal models. Through such studies we hope to have a better understanding about the role of deregulated expression of epigenome in skeletal muscle pathologies like rhabdomyosarcoma. Eventually, this might lead to identification of novel targets and the development of better therapeutic approaches.

APPENDIX

Table I. Primers for Q-PCR

Gene	Forward primer	Reverse primer	TM (°C)
mG9a (mouse G9a)	TCGGGCAATCAGTCAGACAG	TGAGGAACCCACACCATTCAC	60
MYH1	AACAGCAGCGGCTGATCAAT	GCTGCCTCTTCAGCTCCTCA	60
MRF4	CTACATTGAGCGTCTACAGGACC	CTGAAGACTGCTGGAGGCTG	60
Myogenin promoter	TGGCTATATTTATCTCTGGGTTCATG	GCTCCCGCAGCCCCT	60
MCK promoter	CGCCAGCTAGACTCAGCACT	CCCTGAGAGCAGATGAGCTT	60
GAPDH	ATCAACCGGGAAGCCCATCAC	CCTTTTGGCTCCACCCTTCA	60
β-actin	GCTTCTTTGCAGCTCCTTCGTTG	TTTGCACATGCCGGAGCCGTTGT	60

TABLE II. Primers for human siRNA

Smart pool siRNA sequences	sequence
Non-targeting siRNA -1	UGGUUUACAUGUCGACUAA
Non-targeting siRNA -2	UGGUUUACAUGUUGUGUGA
Non-targeting siRNA -3	UGGUUUACAUGUUUUCUGA
Non-targeting siRNA -4	UGGUUUACAUGUUUUCCUA
siG9a-1	GGACCUUCAUCUGCGAGUA
siG9a-2	GAACAUCGAUCGCAACAUC
siG9a-3	GGAGGUAGCCCGUUACAUG
siG9a-4	GGAGAGGUGUACUGCAUAG

TABLE III. Primers for mouse siRNA

Smart pool siRNA sequences	sequence
Non-targeting siRNA -1	UGGUUUACAUGUCGACUAA
Non-targeting siRNA -2	UGGUUUACAUGUUGUGUGA
Non-targeting siRNA -3	UGGUUUACAUGUUUUCUGA
Non-targeting siRNA -4	UGGUUUACAUGUUUUCCUA
siG9a-1	UAACAAGGAUGGCGAGGUU
siG9a-2	CCAUGAACAUCGACCGCAA
siG9a-3	CAGGACAGGUGGACGUCAA
siG9a-4	CCAAGAAGAAAUGGCGGAA

Table IV. Q-PCR analysis for G9a expression (Fig. 3.1.1A) (Raw Data)

		Average	Copy of GAPDH	Noramalize	Relative expression
			average	Delta CT	2^-Delta ct
A1 D0 GAP Unknown	23.420				
þ A2 Repl. of D0 GAPDH Unknown	23.510				
þ A3 Repl. of D0 GAPDH Unknown	23.520	23.483			
þ A4 D3 GAP Unknown	20.430				
þ A5 Repl. of D3 GAPDH Unknown	20.200				
þ A6 Repl. of D3 GAPDH Unknown	20.290	20.307			
þ B1 D0 G9A Unknown	28.220				
þ B2 Repl. of D0 G9A Unknown	28.230				
þ B3 Repl. of D0 G9A Unknown	28.170	28.207	23.483	4.723	0.038
þ B4 D3 G9A Unknown	27.600				
þ B5 Repl. of D3 G9A Unknown	27.550				
þ B6 Repl. of D3 G9A Unknown	27.680	27.610	20.307	7.303	0.006

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