CELL CYCLE REGULATORY MECHANISMS IN SKELETAL MUSCLE CELLS

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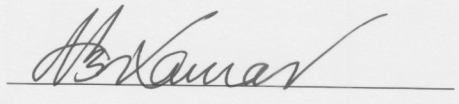
Declaration

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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Summary

During myogenic differentiation, proliferating myoblasts undergo a pre-requisite step of irreversible cell cycle exit prior to differentiation to form multinucleated myotubes. MyoD, a key regulator of muscle differentiation, mediates cell cycle exit as well as expression of differentiation specific genes. Studies from our lab have identified that G9a/EHMT2, a lysine methyltransferase, is expressed in skeletal myoblasts and inhibits myogenic differentiation. G9a, when overexpressed, mediates repressive histone-3 lysine-9-di-methylation (H3K9me2) on myogenin promoter. In addition, G9a also methylates MyoD and inhibits its transcriptional activity. While G9a overexpression inhibits myogenic differentiation, its role in regulating proliferation and cell cycle exit is not clear. Besides, the genome wide molecular targets of G9a in muscle cells are unknown.

In order to identify G9a targets globally, we performed gene expression analysis using microarrays. Interestingly, knockdown of G9a in myoblasts altered several genes involved in cell cycle control. Hence, we examined if G9a has a role in regulating proliferation of cells. Using cultured myoblast lines, as well as primary myoblasts from wild type and G9a conditional knockout mice, we found that G9a promotes proliferation of cells. G9a knockdown up regulated $p21^{Cip1/Waf1}$ (p21) and Rb1, which are required for cell cycle exit but also led to the down regulation of several E2F1 target genes. We therefore hypothesized that G9a may regulate myoblast proliferation in two distinct mechanisms: (1) Repression of p21 and Rb1 to prevent cell cycle exit; and (2) directly activating expression of E2F1 target genes.

Consistent with its function as a transcriptional repressor, G9a inhibited expression of p21 and Rb1 in methyltransferase-dependent manner during differentiation by mediating H3K9me2 marks on their promoters. Moreover, G9a mediated inhibition of myogenic differentiation was rescued by re-expression of p21 and Rb1.

To examine whether G9a actively promotes the expression of cyclins and other E2F1 target genes, we examined its occupancy on E2F1 target gene promoters. Remarkably, unlike p21 and Rb1 promoters, G9a occupancy was not correlated with repressive H3K9me2 on E2F1-target genes. Moreover, pharmacological inhibition of endogenous G9a methyltransferase activity did not significantly change expression of E2F1 target genes. Consistent with this, H3K9me2 on their promoters were unaltered. G9a occupancy was associated with H3K9ac instead. Furthermore, protein-protein interaction studies indicated that G9a complexes with the histone acetyltransferase P/CAF and E2F1 in myoblasts. Consistent with these findings, myoblasts isolated from G9a conditional knockout mice displayed reduced proliferation. Cell cycle exit genes were up-regulated and E2F1 target genes were down regulated validating our *in vitro* findings.

Overall our data support a model in which G9a both prevents cell cycle exit and promotes proliferation of muscle cells to block differentiation. These studies implicate that G9a may be de-regulated in myopathies associated with an imbalance of proliferation and differentiation, and suggest that targeting G9a may be a promising therapeutic approach

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Research articles:

Li L, Ng DS, Mah W, Almeida FF, Rahmat SA, **Rao VK**, Leow SC, Laudisi F, Peh MT, Goh AM, Lim JS, Wright GD, Mortellaro A, Taneja R, Ginhoux F, Lee CG, Moore PK, Lane DP. A unique role for p53 in the regulation of M2 macrophage polarization. Cell Death Differ. 2014 Dec 19 (IF- 8.3)

Modak R, Basha J, Bharathy N, Maity K, Mizar P, Bhat AV, Vasudevan M, **Rao VK**, Kok WK, Natesh N, Taneja R, Kundu TK. Probing p300/CBP associated factor (PCAF)-dependent pathways with a small molecule inhibitor. ACS Chem Biol. 2013;8(6):1311-23 (IF- 5.3)

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List of symbols and abbreviations

ANK	Ankyrin
bFGF	Basic fibroblast growth factor
b-HLH	Basic helix-loop-helix
BIX-01294	G9a/GLP methyltransferase inhibitor
BMP	Bone morphogenic protein
BrdU	5-bromo-2'-deoxyuridine
C2C12	Mouse myoblasts cell line
CARM1	Coactivator-associated arginine methyltransferase 1
CDKs	Cyclin dependent kinase
cDNA	Complementary DNA
CDP/cut	CCAAT displacement protein/cut homologue
ChIP	Chromatin immunoprecipitation
CIP	Cdk interacting protein
Co-IP	Co-immunoprecipitation
CSF2	Colony-stimulating factor-2
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbeco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
Dub1a	Deubiquitinating enzyme 1a
E days	Embryonic days
E2F1	E2F transcription factor 1
EHMT2	Euchromatic histone methyl transferase 2
Ep-CAM	Epithelial cell adhesion molecule
E^{y}	Embryonic β like globin

FBS	Fetal bovine serum
FGF	Fibroblasts growth factors
G1/S	Gap1/S phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gfi 1	Growth factor independent 1 protein
GLP	G9a-like protein
GRIP1	Glutamate receptor interacting protein 1
H3K9ac	Histone 3 lysine 9 acetylation
H3K9me2	Histone 3 lysine 9 di methylation
HATs	Histone acetyl transferase
HDACs	Histone deacetylases
HMTs	Histone methyltransferases
HIV1	Human immunodeficiency virus 1
HP1	Heterochromatin protein 1
Id	Inhibitor of DNA binding
LB broth	Luria-Bertani broth
Magea1	Melanoma Antigen Family A1
MEF2	Myocyte enhancer factor-2
MHC	Myosin heavy chain
MMP9	Matrix metalloproteinsase-9
MRF	Myogenic regulatory factor
Msx1	Msh homeobox 1
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein
NLS	Nuclear localization signal
PAX-3,-7	Paired homeobox transcription factor 3 and 7
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor

PPARγ	Peroxisome proliferator-activated receptor gamma
qRT-PCR	Quantitative real time Polymerase chain reaction
Rb1	Retinoblastoma protein 1
RUNX3	Runt related transcription factor 3
SAM	S-adenosyl-Lmethionine
SDF	Stromal differentiating factor-1
SET	SU (VAR) 3-9 Enhancer of Zeste [E (Z)] Trithorax (TRX)
SETDB-1,-2	SET Domain, Bifurcated- 1,-2
Sharp1	Enhancer-of-split and hairy-related protein 1
Shh	Sonic hedgehog
Sir2	Silent information regulator 2
Suv39h1	Suppressor of variegation 3-9 homolog 1
SWI/SNF	Switching and/or Sucrose fermentation
UHRF1	Ubiquitin-like with PHD and ring finger domains 1
UNC0638	G9a\GLP methyltransferase inhibitor
Waf1	Wild type p53 activated fragment 1
WIZ	Widely interspaced zinc finger motifs protein
Wnt	Wingless-Type MMTV Integration Site Family, Member 1
β^{maj}	β globin major

1. Introduction

The ability of living organisms to perform any type of body movement is attributed to the muscle tissue. Muscle tissue consists of cells arranged into muscle fibers which are capable of contracting and relaxing. Muscle tissue is categorized into three types – smooth muscle, cardiac muscle and skeletal muscle. Smooth muscles are non-striated muscle found intrinsically covering internal organs of the body. Cardiac muscles exhibit striations and are found in the heart. Skeletal muscle is also striated muscle, but unlike cardiac muscle, it can be voluntarily controlled. It is attached to the skeleton and is the most abundant tissue in the vertebrate body, accounting for approximately 40 percent of total body mass in adults.

During embryonic development, skeletal muscle is formed through a process called myogenesis (Bentzinger et al., 2012). Myogenesis is achieved through signaling molecules from adjacent tissues that specifies myogenic cell fate and requires spatial-temporal regulation of proliferation and differentiation of embryonic precursor cells (Buckingham, 2001; Zhang et al., 1999). In skeletal muscle cells proliferation and differentiation processes are coupled yet mutually exclusive. Cell cycle arrest is an essential step during muscle differentiation. Thus skeletal myogenesis serves as an exquisite paradigm to understand the cell cycle regulatory mechanisms during skeletal muscle differentiation.

1.1 Embryonic myogenesis

During gastrulation, the three germ layers ectoderm, endoderm and mesoderm are formed from pluripotent epiblast cells at the primitive streak (Beddington and Smith, 1993). Expression of key regulatory genes leads to the commitment of cells to different lineages. At the anterior part of primitive streak, paraxial mesoderm undergo segmentation, leading to the formation of an epithelial ball of cells called somites (Tajbakhsh and Cossu, 1997). In mice, somitogenesis begins from embryonic day eight (E8) and the newly formed somites mature in a rostro-caudal developmental gradient, differentiating to give rise to the dorsal epithelial dermomyotome and the ventral mesenchymal sclerotome (Fig 1.1A). Vertebral column, cartilage and ribs are formed from the sclerotome whereas skeletal muscles of the trunk and limbs are derived from skeletal muscle progenitor cells of the dermomyotome. A few cells migrate from the dorsomedial part of the somites under the dermomyotome to form the myotome. These cells undergo sequential steps of myoblast amplification, cell cycle arrest and differentiation to form the first skeletal muscle in the embryo (Buckingham and Rigby, 2014; Tajbakhsh and Cossu, 1997; Buckingham, 2001).

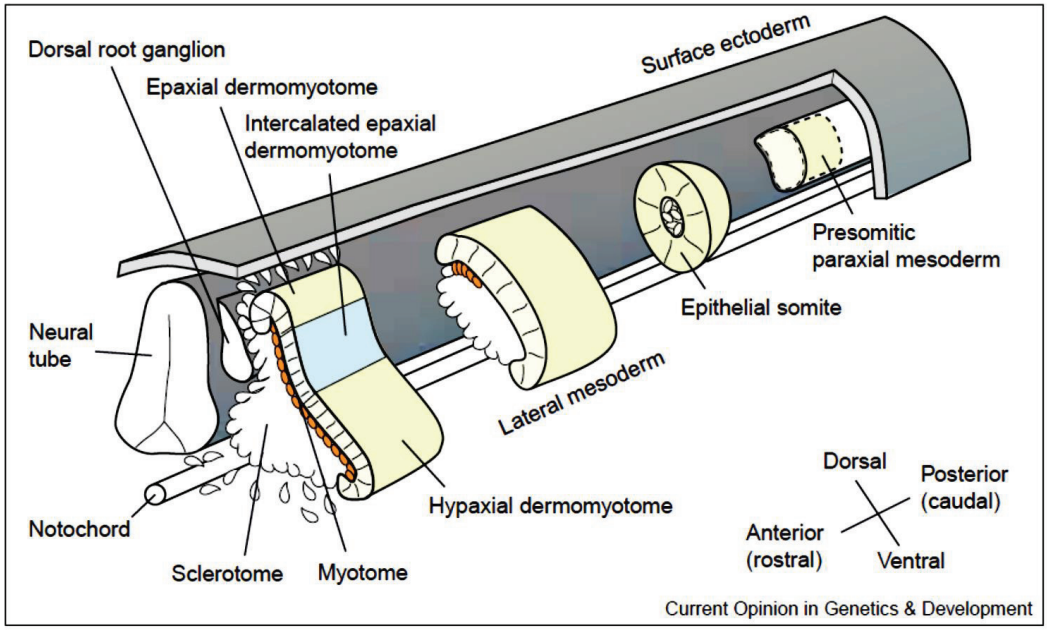


Figure 1.1A Schematic representation of vertebrate somitogenesis in mouse embryo (Buckingham, 2001). The paraxial mesoderm segments in a rostro-caudal gradient on either side of neural tube and notochord to form an epithelial ball of cells called somites. Skeletal muscle is formed from the embryonic progenitor cells arising from somites. Several signaling pathways arising from adjacent tissues lead to the specification of embryonic precursor cells to myogenic fate, forming dermomyotome and sclerotome. A few cells migrate under the dermomyotome to form the myotome which later differentiates to form skeletal muscle in the embryo.

The skeletal muscles at different stages - embryo, fetal and postnatal - appear to form through a sequence of steps involving embryonic myoblasts, fetal myoblasts and postnatal satellite cells (Tajbakhsh, 2003) (Fig 1.1B). Around embryonic day 11 (E11), the post-mitotic myocytes at the myotome fuse to form multinucleated primary myofibers, in the process known as primary or embryonic myogenesis. Later around embryonic day E14.5 and E17.5 a second wave of myogenesis called secondary myogenesis occurs, involving fusion of fetal myoblasts to form secondary myofibers. Each of these myofibers is surrounded by a basal lamina. At

this stage, muscle stem cells, or satellite cells, are formed beneath the basal lamina and myofiber plasma membrane (Tajbakhsh, 2003; Cossu et al., 1996) (Fig 1.1B).

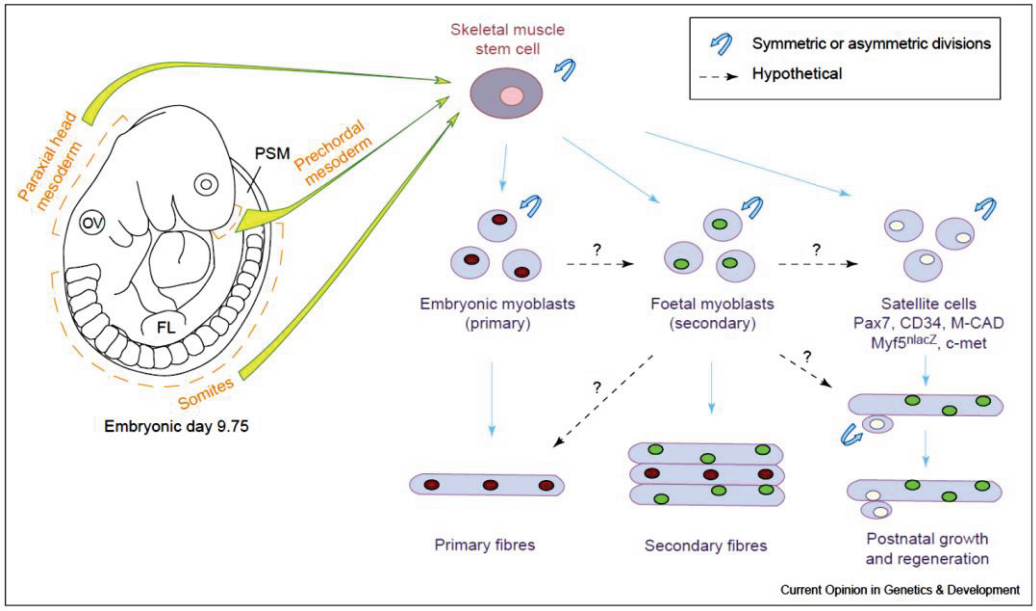


Figure 1.1B Schematic representation of muscle development from skeletal muscle stem cells (Tajbakhsh, 2003). Muscle progenitors at somites disperse as embryonic, fetal, and satellite cells leading to the formation of skeletal muscle in the body. The primary fibers formed from embryonic myoblasts act as scaffold for secondary fibers which arise from fetal myoblasts. These secondary fibers form the bulk of skeletal muscle in the postnatal period. Satellite cells are found in adults and are crucial for muscle regeneration.

1.2 Adult myogenesis

In 1961, Alexander Mauro first identified mononucleated cells attached to the basal lamina of the myofiber. Based on its anatomical location, sub laminar, these cells were named as satellite cells (Mauro, 1961). Satellite cells arise from muscle progenitor cells expressing Pax3 and Pax7 at the dermomyotome (Kassar-Duchossoy et al., 2005). Satellite cells are non-proliferative and quiescent in nature. However, emerging evidence indicates that these cells are metabolically and

transcriptionally active. The remarkable ability of skeletal muscles to regenerate is credited to the presence of satellite cells (Buckingham, 2001; Tajbakhsh, 2003).

In vivo, upon injury or trauma to the muscle tissue, satellite cells get activated to enter into the cell cycle, proliferate and give rise to myoblasts which later exit cell cycle irreversibly and fuse to repair the damaged myofiber (Schultz and McCormick, 1994) (Fig 1.2). Owing to its stem cell properties, satellite cells have the ability to self-renew (Potten and Loeffler, 1990). A few progenitor cells undergo reversible cell cycle exit to form new quiescent satellite cells, thus maintaining the satellite cell numbers (Dhawan and Rando, 2005). Whereas a few other cells undergo irreversible cell cycle exit leading to the activation of differentiation specific genes and form new myofibers

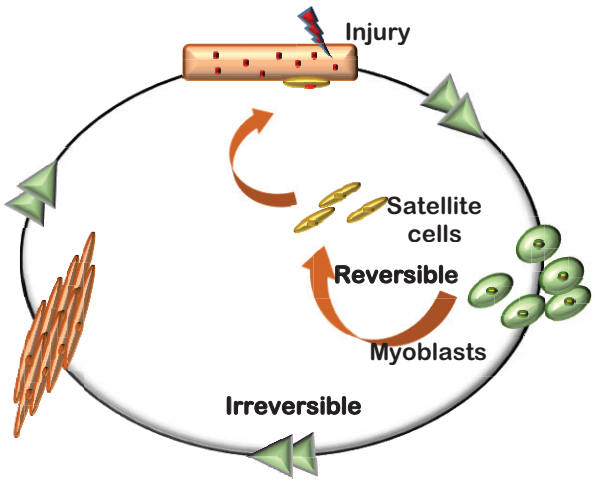


Figure 1.2 Schematic representation of muscle regeneration *in vivo*. During muscle regeneration quiescent satellite cells get activated to enter cell cycle and proliferate to form transit amplifying myoblasts. A few cells undergo reversible cell cycle exit and go back into quiescence, forming satellite cells. Majority of cells undergo irreversible exit forming new myofiber.

1.3 Signaling pathways regulating myogenesis

During somitogenesis, fine tuning of mesodermal progenitors to myogenic lineage has been attributed mainly to these signaling pathways: Wnt, Shh, Notch and BMP (Bentzinger et al., 2012). Wnt and Shh secreted by the dorsal neural tube and notochord are known to be involved in positive regulation of muscle differentiation and sclerotome formation. Wnt1 and Wnt3a are secreted from dorsal neural tube whereas Wnt4, Wnt6 and Wnt7a are secreted from surface ectoderm. Expression of Wnt receptors frizzled fzd7 is seen in the hypaxial region of somite whereas expression of fzd1 and fzd6 is seen in the epaxial region (Borello et al., 1999). At the myotome, Wnt1 and Wnt3a activate myogenesis through TCF/β-catenin pathway and Wnt7a is known to induce myogenesis through β-catenin independent signaling pathways (Brunelli et al., 2007; Borello et al., 2006). Wnt1 and Wnt3a deficient mice show defect in the formation of the dermomyotome (Ikeya and Takada, 1998), and consistent with this, Wnt1 or Wnt3a over expression in chick somites activates genes involved in myogenesis (Tajbakhsh et al., 1998). Collectively, these studies indicate the importance of Wnt signaling pathway in myogenesis.

Sonic hedgehog (Shh) released from the notochord of the neural tube is also involved in positive regulation of skeletal myogenesis. Shh knockout mice show defect in formation of distal limb structures and sclerotome formation (Zhang et al., 2001; Chiang et al., 1996). Wnt and Shh pathway positively regulates myogenesis whereas BMP and Notch pathway are known to be involved in negative regulation of myogenesis (Bentzinger et al., 2012). Bmp4, which is expressed in the

mesoderm, appears to inhibit the expression of myogenic determination genes, indicating that BMP signaling is involved in the expansion of progenitor population. Correspondingly, expression of BMP antagonist Noggin at the dermomyotome initiates the expression of myogenic transcription factors leading to formation of myotomal cells (Reshef et al., 1998). Notch signaling has been implicated in regulating somitogenesis. It is required for vertebrate segmentation and somite patterning (Lewis et al., 2009). It has been shown that Notch ligand Delta1 mutant embryos show more myoblasts differentiation and have excess myofibers (Schuster-Gossler et al., 2007), suggesting that Notch signaling is involved in inhibiting the differentiation program. Overall the above studies suggest that extrinsic signaling pathways play important roles in specifying embryonic precursor cells to myogenic lineage.

1.4 Transcription factors involved in skeletal myogenesis

1.4.1 Paired homeobox transcription factors (Pax3 and Pax7)

Along with extrinsic signaling pathways from adjacent tissues, several intrinsic regulatory factors are involved in specification and differentiation of skeletal muscle cells. The process of skeletal myogenesis is coordinated by several myogenic transcriptional factors. Pax3 and Pax7 mark progenitor cells in dermomyotome, and at embryonic day E9.75, almost all cells are positive for both these transcription factors. Higher expression of Pax3 is seen in dorsal and ventral dermomyotome lips whereas Pax7 expression is seen in central dermomyotome (Kassar-Duchossoy et al., 2005). Pax3 plays a role in progenitor cell formation as

well as in migration of cells to limb and other muscle structure in the body (Buckingham and Rigby, 2014; Cossu et al., 1996; Messina and Cossu, 2009). The mouse mutant Splotch (Pax3 null mice) fail to develop limb muscle indicating the importance of Pax3 in migration of progenitor cells (Daston et al., 1996; Strachan and Read, 1994). Moreover, ablation of $Pax3^{+}$ cells led to the loss of all embryonic myofibers indicating the importance of Pax3 in embryonic myogenesis (Hutcheson et al., 2009). However, Pax7 null mice display normal skeletal muscle development, but completely lack satellite cells (Seale et al., 2000). This indicates the redundancy in roles played between Pax3 and Pax7 during embryonic development. In Pax3 and Pax7 double knockout mice, muscle development is arrested and only the early myotome is formed (Relaix et al., 2005). $Pax3^{+}$ cells in the limb can give rise to both muscle as well as endothelial lineage cells whereas $Pax7^{+}$ cells can give rise to only myogenic cells (Hutcheson et al., 2009). $Pax7^{+}$ cells are derived from $Pax3^{+}$ cells and loss of Pax3 lineage is embryonically lethal whereas loss of Pax7 only leads to smaller muscle formation with fewer myofibers at birth (Seale et al., 2000). These studies suggest that Pax3 is critical for embryonic muscle formation and Pax7 for specification of myogenic satellite cells. Muscle precursor cells arising from dermomyotome express proto-oncogene c-Met which helps in cell migration and muscle formation (Cossu et al., 1996). Studies suggest that Pax3 regulate c-Met transcription, which is in turn required for cell migration of limb precursor cells (Epstein et al., 1996). Myogenic satellite cells are known to express c-Met receptors which is involved in hepatocyte growth factor signaling and activation of quiescent satellite cells (Allen et al., 1995).

1.4.2 Myogenic regulatory factors (MRFs)

Embryonic muscle progenitor cells expressing Pax3 and Pax7 cannot initiate the differentiation program, suggesting the involvement of other factors in driving the differentiation process. Isolation of myoblast specific cDNAs led to the discovery of MyoD1, Myoblasts determination gene number 1 (Davis et al., 1987). MyoD, when transfected into the fibroblasts, has the ability to convert them into fusion capable myoblasts (Davis et al., 1987). This discovery paved way to understand the molecular mechanisms behind myogenic differentiation. Similarly, identification of three other factors, namely Myf5, Myogenin and MRF4, helped in better understanding of the skeletal muscle differentiation program (Edmondson and Olson, 1989; Braun et al., 1989; Braun et al., 1990). All these factors MyoD, Myf5, Myogenin and MRF4 are highly conserved and are collectively called as Myogenic Regulatory Factors (MRFs). Each MRF, when expressed in fibroblasts, has the ability to convert them into myoblasts (Braun et al., 1990). MRFs are basic helix loop helix transcription factors which contain a DNA binding domain and a dimerization domain. MRFs, through their helix loop helix region, dimerize with ubiquitously expressed E proteins such as E47 and E12. Once dimerized, the basic region of MRF-E heterodimers binds to the E box elements CANNTG on the muscle promoters and drives the muscle differentiation program (Singh and Dilworth, 2013; Lassar et al., 1989).

In somites, Myf5 is expressed first in muscle progenitor cells followed by MyoD, leading to the formation of proliferating myoblasts (Buckingham and Tajbakhsh, 1993). Subsequently, Myogenin and MRF4 are expressed during terminal

differentiation (Cossu et al., 1996; Ott et al., 1991). However, expression of MRF4 has been observed in three waves during myotomal expansion (My1, My2 and My3). My1 begins at embryonic day 8 (E8), My2 at embryonic day 9 (E9) and the final one at embryonic day 16 (E16) (Patapoutian et al., 1995). The role of MRFs in muscle development has been well characterized. Mice lacking either Myf5 or MyoD have normal skeletal muscle development, suggesting the redundancy in roles played by these two factors (Braun et al., 1992). However, mice lacking both are devoid of skeletal muscle as well as myogenic precursor cells (Rudnicki et al., 1993). Mice lacking myogenin show normal muscle development at early stages, however, during later stages they show impairment in the myofiber formation and the mice die perinatally (Hasty et al., 1993; Nabeshima et al., 1993). This is highlighted by the fact that myogenin knockout myoblasts are able to undergo cell cycle exit, but cannot fuse to form myotubes. MRF4 null mice display multiple rib abnormalities with increased myogenin expression. This suggest that myogenin may compensate for the loss of MRF4 (Zhang et al., 1995).

Overall, Pax3 appears to be on top of hierarchy where Pax3 positive cells give rise to initial myofibers and Pax7 positive cells contribute to formation of secondary myofibers and are involved in adult muscle regeneration through maintenance of satellite cell population in adults (Figure 1.1B). Myf5 and MyoD act upstream of Myogenin and MRF4, where Myf5 and MyoD are involved in myogenic determination while Myogenin, MRF4 are involved in differentiation. However recent evidences indicate that sine oculis related homeobox 1 (Six1) and Six4 are involved in specifying dermomyotomal cells towards myogenic lineage and are

considered to be at the quintessence of this transcriptional regulatory cascade (Bentzinger et al., 2012; Kawakami et al., 2000) (Figure 1.4).

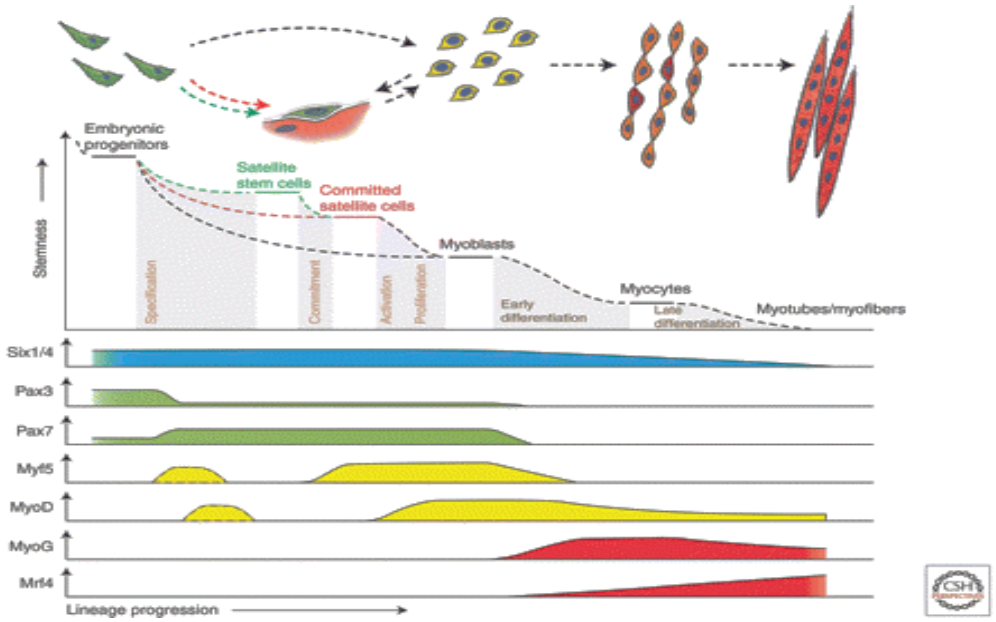


Figure 1.4 Schematic representation of transcription factors involved in myogenic lineage formation (Bentzinger et al., 2012). Myoblasts are formed from embryonic progenitor cells. A few progenitors remain as satellite cells in postnatal muscle forming adult muscle stem cells required for regeneration. During regeneration, some of the activated satellite cells can enter into quiescent state to maintain the satellite cell pool. Pax3, -7 and Six-1, -4 are involved in muscle lineage specification and stand at top of the hierarchy. Myf5 and MyoD commit cells to muscle differentiation program whereas Myogenin and MRF4 are expressed during terminal differentiation during muscle formation.

1.5 *In vitro* myogenic differentiation

In vitro, myogenic cell lines such as rat L6 and mouse C2C12 cells are commonly used to study muscle differentiation. C2C12 myoblasts were originally derived from satellite cells of thigh muscle of C3H mouse (Yaffe and Saxel, 1977). Later immortal sub-line C2C12 was selected (Blau et al., 1985). C2C12 cells are cultured

under high serum condition to maintain them in proliferation state. Upon serum withdrawal, myoblasts turn on differentiation specific genes and fuse to form multinucleated myotubes. The process of differentiation is orchestrated by the sequential expression of myogenic regulatory factors MRFs including MyoD, Myf5, Myogenin and MRF4 and MEF2 family of transcription factors which includes MEF2-A, -B, -C and -D (Black and Olson, 1998). In addition, it is now well established that chromatin regulators associate with MRFs to reprogram chromatin at the promoters of muscle specific genes driving the differentiation program (Bharathy et al., 2013).

Under *in vitro* conditions, proliferating myoblasts express MyoD and Myf5. Upon differentiation, myogenin is expressed at early stage, and then during myoblasts fusion, MRF4 and other structural proteins such as troponinT and myosin heavy chain (MHC) are expressed. MEF2 is expressed at lower levels compared to MRFs in proliferating myoblasts and their expression increases during differentiation. MEF2D is expressed at higher levels in undifferentiated cells compared to MEF2C. Myogenin and MEF2C are known to exist in a positive feedback loop promoting their expression and regulating the expression of structural genes required for terminal differentiation (Yee and Rigby, 1993).

For myogenic differentiation to occur, myoblasts need to exit the cell cycle irreversibly and like in most of the cells, proliferation promoting genes have to be suppressed. This type of exit is irreversible because the differentiated cells cannot re-enter the cell cycle or proliferate (Fig 1.5). Irreversible cell cycle arrest or permanent withdrawal from the cell cycle is an important pre-requisite step during skeletal muscle differentiation. Myoblasts also have the ability to undergo reversible exit leading to quiescence. Upon induction with quiescence signals, myoblasts exit the cell cycle reversibly, forming quiescent cells (Sachidanandan et al., 2002) (Fig 1.5). Upon addition of proliferation cues quiescent cells can reenter cell cycle and proliferate. Quiescent myoblasts mimic the characteristic of muscle progenitor cells.

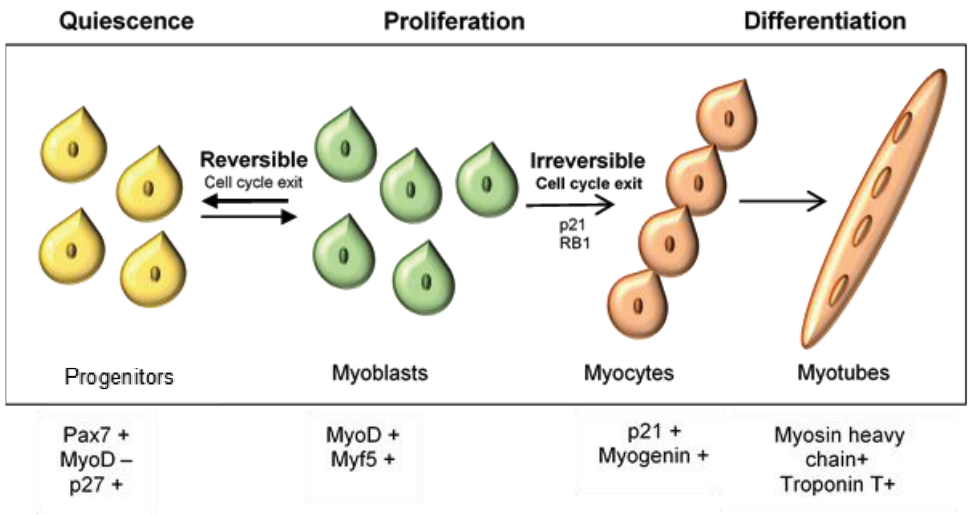


Figure 1.5 Schematic representation of in vitro myogenic differentiation. Proliferating myoblasts express MyoD and Myf5. Upon differentiation, cells undergo irreversible cell cycle exit mediated by p21 and Rb1. Subsequently, expression of Myogenin leads to differentiation of cells forming myocytes. Later myocytes fuse to form multinucleated myotubes expressing myosin heavy chain and troponin T. Proliferating myoblasts can also enter into quiescence through reversible cell cycle exit. These cells mimic the characteristics of muscle stem cells expressing Pax7and p27 that are negative for MyoD.

1.6 Mechanisms underlying irreversible cell cycle exit during differentiation

Apart from MRFs expression, cell cycle regulation seems to play a major role during muscle differentiation. Differentiation of myoblasts to multinucleated myotubes is coordinated by cell cycle genes as well as MRFs. Studies suggest that the process of cell cycle exit and differentiation are coupled in skeletal muscle cells (Nadal-Ginard, 1978). The role of cyclin kinase inhibitors such as p21 and Rb1 has been implicated in the process of cell cycle arrest during terminal differentiation (Mal et al., 2000). These proteins function by binding to Cyclin-CDK complexes and inhibiting their function leading to cell cycle arrest.

During differentiation RNA and protein levels of p21 is significantly upregulated (Parker et al., 1995). In general, p53 dependent induction of p21 is known to induce cell cycle arrest in various tissues. However, in skeletal muscle cells, MyoD upregulates p21 leading to the irreversible cell cycle exit. Indeed, over expression of MyoD in p53 null fibroblasts activates p21 promoter leading to terminal differentiation (Halevy et al., 1995). Although MyoD mediated activation of p21 in skeletal muscle cells is important for cell cycle exit and differentiation, p21 null mice develop normally and do not show any skeletal muscle defect, however mice lacking both p21 and p57 fail to form skeletal muscle (Deng et al., 1995; Zhang et al., 1999). This suggests that both p21 and p57 play redundant role in controlling muscle differentiation. High p21 expression in myotubes leads to reduced kinase activity and thereby reduces the phosphorylation of Rb1 and in part maintaining the

permanent cell cycle arrest (Guo et al., 1995). Hence, one of the other functions of p21 is to render Rb1 in hypo phosphorylated form.

In general, Rb/E2F1 pathway seems to be the major pathway controlling the G1/S transition of cells (Chan et al., 2001). E2F1 transcription factor helps in driving G1/S transition during proliferation by activating its target genes such as Cyclin D1, Cyclin E as well as genes involved in DNA synthesis such as DHFR (Watanabe et al., 1998; Ohtani et al., 1995; Nevins, 1998). During G1/S transition, hyper phosphorylation of Rb1 releases E2F1 from the Rb-E2F1 complex, which allows E2F1 to drive its target genes (Fig 1.6A).

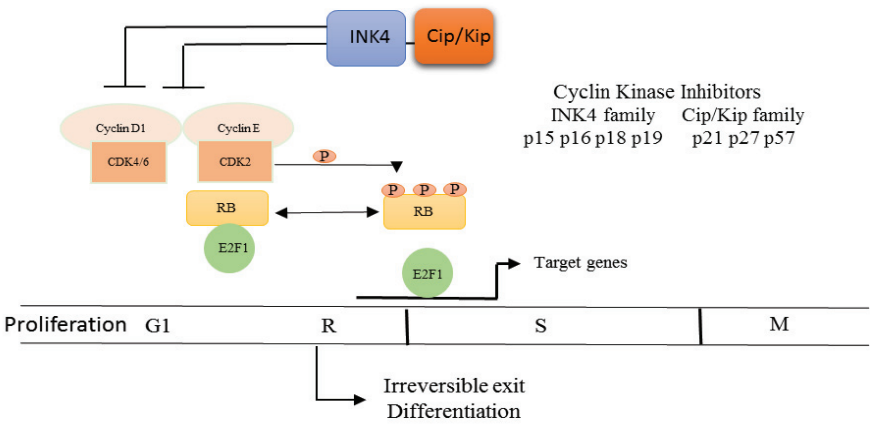


Figure 1.6A Schematic representation of role of Rb/E2F1 pathway in mammalian cell cycle. Cell cycle can be broadly classified into G1, S and G2/M phase. At late G1, just before restriction point (R) cells can enter into differentiation otherwise moves on to S phase. In G1 phase, cell cycle progression is controlled by CyclinD1/CDK4 and CyclinE/CDK2 complex which in turn are regulated by Cyclin dependent kinases belonging to INK4 and CIP/KIP family. Phosphorylation of Rb by CyclinD1/CDK4, CyclinD1/CDK6 and CyclinE/CDK2 complex releases E2F1 from Rb/E2F1 complex. Free E2F1 then induces the expression of its target genes required for cell cycle progression.

Since Rb phosphorylation is important for E2F1 to drive cell cycle, G1 Cyclins such as CyclinD1/CDK4, CyclinE/CDK2 complexes appear to play a significant role in Rb1 hyper phosphorylation (Mittnacht, 1998). G1 phase Cyclin/CDKs not only mediate Rb1 phosphorylation and help in G1/S transition, but they are also known to restrain MyoD activity in proliferating myoblasts and thereby avoid premature differentiation (Guo and Walsh, 1997). Indeed, forced expression of Cyclin D1 in cells has been shown to inhibit MyoD activity (Skapek et al., 1995). Therefore CyclinD1/CDK4 complex acts as one of the mechanisms restraining MyoD activity in myoblasts.

In addition to the role of Rb1 in negatively regulating E2F1 target genes, Rb1 has been shown to be involved in cell cycle exit as well as activating muscle specific genes, suggesting the importance of Rb1 as a key protein regulating cell cycle and differentiation (Gu et al., 1993; Novitch et al., 1996). Myogenic regulatory factors fail to mediate myogenic conversion and cell cycle exit in Rb null cells, indicating the importance of Rb during myogenic differentiation. Rb null mice show normal muscle development. However, early lethality in these mice before embryonic day E14.5 has restricted our understanding of the role of Rb in secondary myogenesis (Clarke et al., 1992). Rblox mice expressing low levels of Rb driven by Rb mini gene can be rescued to birth. The Rb mutant mice fetuses die at birth and show defect in skeletal muscle including shorter myotubes, fewer myofibrils, reduced myofibers and DNA synthesis in myotube nuclei (Zacksenhaus et al., 1996).

During differentiation, MyoD is involved in increase of Rb1 gene expression and studies also show that Rb interacts with MyoD to help in increasing MEF2

transcriptional activity (Gu et al., 1993; Novitch et al., 1999). The levels of hypo phosphorylated form of Rb1 increases during differentiation, which helps in blocking proliferation of cells. Myotubes derived from $Rb^{-/-}$ mice fail to maintain the permanent exit state and display DNA synthesis after re-addition of serum to the cultures (Mal et al., 2000). Furthermore, inactivation of p21 and Rb1 by binding of E1A adenovirus protein has been shown to induce DNA synthesis in myotubes (Mal et al., 2000). Hence both p21 and Rb1 are important for cell cycle exit and maintenance of permanent cell cycle arrest in differentiated myotubes.

To summarize, during the process of differentiation, proliferating myoblasts irreversibly exit the cell cycle, undergo permanent arrest and subsequently express differentiation specific genes. This indicates that for MyoD mediated differentiation, down regulation of E2F1 mediated proliferation genes is necessary. E2F1 is generally observed as master regulator for cell cycle control (La Thangue, 1994). Under growth conditions, E2F1 is active in myoblasts and drives the expression of its target genes required for proliferation, while MyoD activity is suppressed. However, upon differentiation cues, MyoD is activated and cell cycle arrest is achieved through induction of cell cycle inhibitors p21 and Rb1 while E2F1 activity is suppressed (Fig 1.6B). Therefore regulation of MyoD and E2F1 activity is required during proliferation and differentiation and epigenetic mechanisms appears to play a prominent role in regulating their activities.

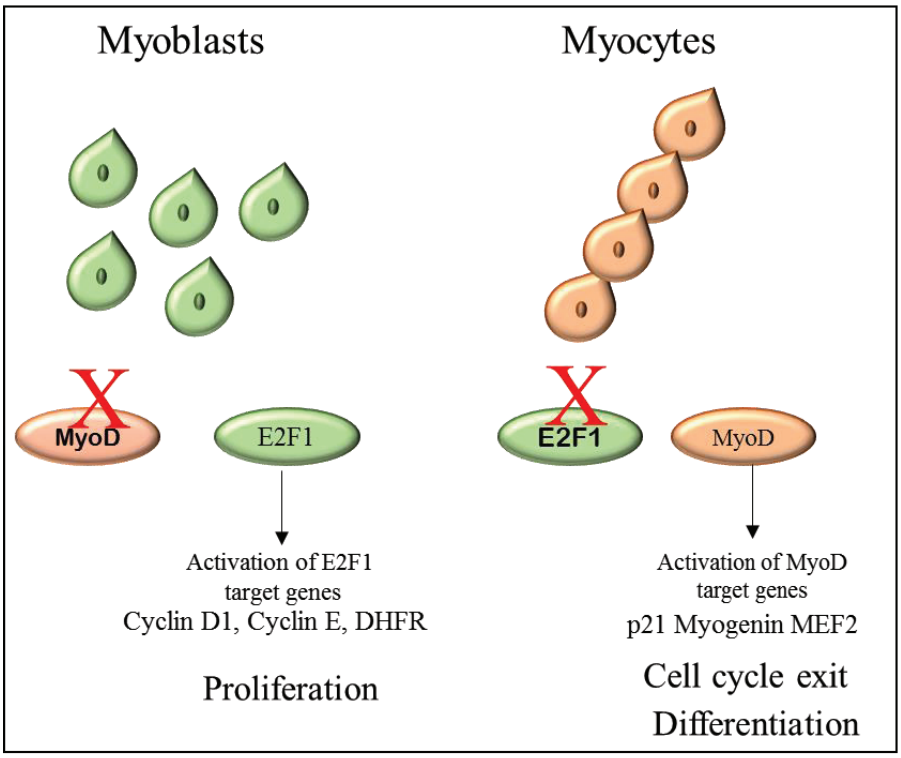


Figure 1.6B Schematic representation of E2F1 and MyoD function during proliferation and differentiation of myoblasts. In proliferating myoblasts, MyoD is inactive whereas E2F1 is active in transcribing genes required for proliferation. During differentiation, E2F1 is inactive and MyoD is activated to transcribe genes required for cell cycle exit and differentiation.

1.7 Transcriptional control and epigenetic regulation of proliferation and differentiation

Although MyoD, the master regulator of differentiation, is expressed in proliferating myoblasts, various mechanisms exist to regulate its activity to avoid premature differentiation. For instance, MyoD forms heterodimers with E proteins (E12 and E47) and bind to E box elements on muscle gene promoters and drives its target gene expression (Lassar et al., 1991). However, Id1 (inhibitor of DNA binding) proteins, prominently induced in high serum conditions, forms inactive heterodimers with E box proteins E12 and E47, thus sequestering the E proteins and preventing the formation of the functional MyoD-E heterodimers (Benezra et al., 1990). Other b-HLH transcription factors such as Twist are also known to inhibit MyoD activity through E protein sequestration as well as inhibiting MEF2 activity (Spicer et al., 1996). Similarly MyoR and several other signaling factors such as TGFs and FGFs seems to regulate myogenic differentiation (Florini and Magri, 1989). Sharp1, a basic helix loop helix transcription factor expressed in myoblasts, is also known to inhibit MyoD transcriptional activity through recruitment of chromatin modifiers (Azmi et al., 2004; Ling et al., 2012a). In addition, phosphorylation of MyoD by Cyclin kinases at different phases of cell cycle appears to negatively regulate the activity of MyoD as well as its turnover (Batonnet-Pichon et al., 2006). MyoD expression peaks at G1 as well as during early M phase of the cell cycle and myoblasts enter the differentiation program during G1 phase. During G1/S transition, MyoD becomes phosphorylated at serine residue 200, which signals for ubiquitination of MyoD and its degradation.

Similarly, MyoD phosphorylation during G2/M transition leads to its degradation (Batonnet-Pichon et al., 2006; Kitzmann et al., 1998).

At the chromatin level, muscle specific DNA sequences are packaged by histones and non-histone proteins to form chromatin. This condensed nucleosomal structure at muscle promoters prevents the access to the transcription factors such as MyoD resulting in transcriptional repression of muscle specific genes. Therefore dynamic changes at chromatin level are required for association or dissociation of transcription factors. These modifications are brought about through various post translational modifications of histone tails such as acetylation, phosphorylation and methylation (Lachner et al., 2003). In general, acetylation of histone tails leads to open chromatin conformation and hence is associated with transcriptional activation of genes whereas histone methylation is associated with transcriptional repression.

In undifferentiated proliferating myoblasts, various chromatin regulators create a repressive environment on muscle promoters, thereby restraining MyoD and MEF2 transcriptional activity. However, at the same time, E2F1 is active in driving the expression of proliferation genes (De Falco et al., 2006). Chromatin regulators and remodeling complexes such as HDACs, HATs, HMTs and SWI/SNF complexes cooperate with MyoD and E2F1 factors to bring changes at the promoters of differentiation as well as proliferation specific genes to regulate their expressions (Fig 1.7)

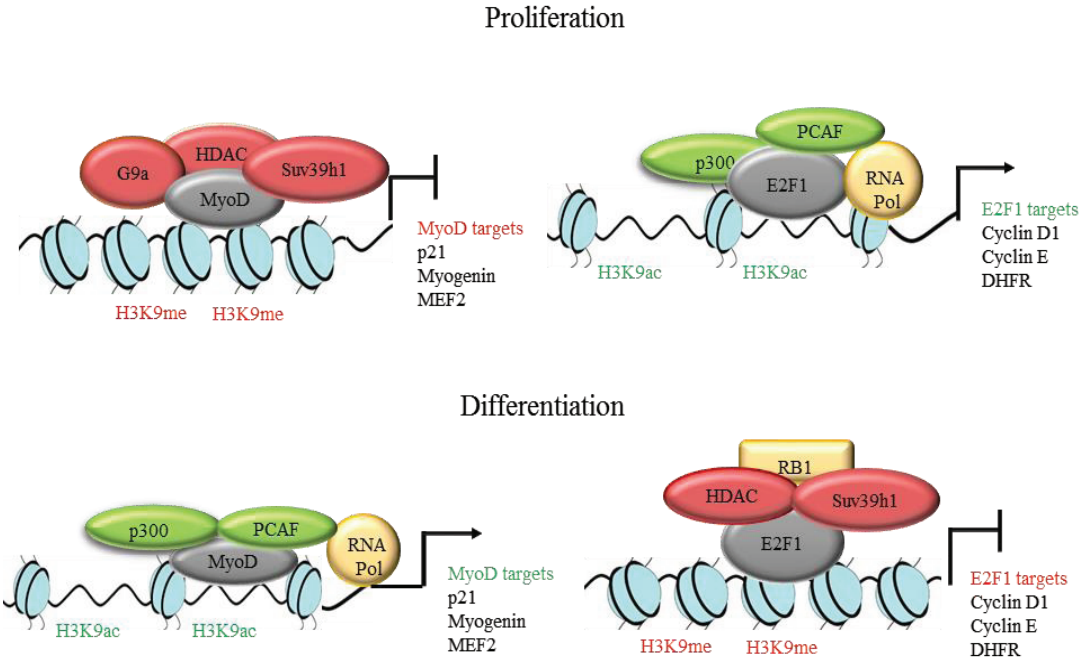


Figure 1.7 Schematic representation of epigenetic regulation of MyoD and E2F1 target genes during proliferation and differentiation. In proliferating myoblasts MyoD is associated with co-repressors rendering it transcriptionally inactive whereas E2F1 is associated with co-activators leading to the expression of its target gene. During differentiation MyoD associates with co-activators whereas E2F1 is inactive due to its association with co-repressors.

During proliferation, Class I and Class II HDACs which are expressed in proliferating myoblasts are known to negatively regulate MyoD activity. MyoD transcription activity is known to be repressed due to its association with class I HDACs in proliferating myoblasts, which leads to MyoD deacetylation as well as deacetylation of histone tails at target promoters (Puri et al., 2001; Mal and Harter, 2003; Mal et al., 2001). In addition, MEF2 associates with class II HDACs (HDAC 4 and HDAC 5) and thereby MEF2 transcriptional activity is repressed in undifferentiated cells (McKinsey et al., 2001). Similarly Class III HDACs (Sir2)

are also known to negatively regulate myogenesis by functioning as redox sensor. Sir2, when over expressed, associates with P/CAF and MyoD and inhibit muscle gene expression (Fulco et al., 2003).

Furthermore, in addition to HDACs, muscle promoters in proliferating myoblasts are repressed by SET domain containing histone methyltransferases (Zhang et al., 2002). Histone methyl transferases such as Suv39h1 and G9a mediate repressive H3K9 methylation on the muscle promoters and thereby represses muscle gene expression (Mal, 2006; Ling et al., 2012b). Suv39h1 is known to interact with MyoD and repress its activity as well as myogenic differentiation. H3K9 methylation of the muscle promoters signals the recruitment of HP1 leading to the formation of heterochromatic structure which represses muscle gene transcription (Mal, 2006). Recently our lab has found that G9a, a euchromatic HMTase, is expressed in undifferentiated myoblasts and its expression declines upon differentiation. G9a mediates H3K9me2 on myogenin promoter as well as methylates MyoD directly, thereby repressing its transcriptional activity (Ling et al., 2012b).

In contrast to the negative regulation of MyoD activity during proliferation, E2F1 actively transcribes genes involved in cell cycle progression. E2F1 is found in association with transcriptional co-activators such as p300/CBP and P/CAF (De Falco et al., 2006; Trouche et al., 1996). p300/CBP and P/CAF are histone acetyltransferases known to be involved in mediating acetylation (Ogryzko et al., 1996). Acetylation of E2F1 by P/CAF and p300 enhances DNA-binding ability of E2F1 and its transcriptional activity (Martínez-Balbás et al., 2000).

Differentiation requires replacement of all the repressive marks at muscle specific genes seen in undifferentiated myoblast with activation marks. Upon differentiation cues, the transition of cells from proliferation to differentiation requires permanent withdrawal from cell cycle as well as down regulation of inhibitory factors controlling MyoD activity. This transition is achieved through down regulation of Cyclins and inhibitory factors such as Id, Twist, and MyoR with up regulation of cell cycle inhibitors such as p21 and Rb1. MyoD becomes active whereas E2F1 activity is inhibited. This seems to be achieved by an interplay between the co-activator and co-repressor complexes on target promoters. MyoD complexes with p300/CBP and P/CAF and drives the muscle gene expression (Puri et al., 1997; Sartorelli et al., 1999) whereas E2F1 is found to be in repressive complex with Rb1 and HDAC leading to the repression of proliferation genes (Blais et al., 2007; De Falco et al., 2006). In addition to HDACs and Rb1, Suv39h1 is shown to be involved in silencing S phase genes in differentiated cells (Ait-Si-Ali et al., 2004).

Collectively, these studies indicate the importance of chromatin modifiers in regulating proliferation and differentiation of skeletal myoblasts. Our lab has been particularly interested in understanding the role of a chromatin modifier G9a in regulating differentiation of myoblasts (Ling et al., 2012a, 2012b) (Fig 1.7).

1.8 Mechanisms of repression by G9a, a lysine methyl transferase

G9a/EHMT2 belongs to the Su (var) 3-9 family of proteins which includes Suv39h1/h2, SETDB1and SETDB2 (Tachibana et al., 2001; Dillon et al., 2005). These proteins contain an evolutionarily conserved SET domain required for their methyltransferase activity and an ankyrin repeat domain required for protein-protein interaction (Brown et al., 2001; Milner and Campbell, 1993; Tachibana et al., 2001) (Fig 1.8). G9a is known to methylate histone 3 lysine 9 (H3K9me), generally associated with repression of gene expression. Endogenously, G9a is known to closely associate with GLP/EHMT1 and functions as an heteromeric complex (Dillon et al., 2005; Tachibana et al., 2005). G9a transfers a methyl group from S-adenosyl-l- methionine to ε- amino group of the substrate lysine residue causing mono and di methylation (H3K9me1 and H3K9me2).

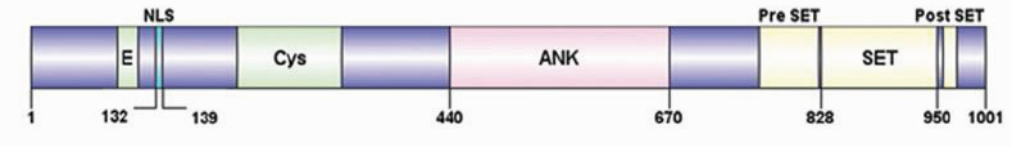


Figure 1.8 Schematic representation of domain structure of G9a. Adapted from (Shankar et al., 2013). G9a contains a nuclear localization signal (NLS) and cysteine and glutamic acid rich region at N terminus. It has Ankyrin repeat (ANK) region involved in protein interactions and catalytic SET domain required for methyltransferase activity

Apart from histones, G9a is also known to methylate non histone substrates including WIZ, ACINUS, HDAC and MYOD. Methylation of these factors by G9a generally associates with their transcriptional repression (Rathert et al., 2008; Shankar et al., 2013).

G9a is expressed in most of the tissues including fetal liver, bone marrow, spleen and skeletal muscles (Brown et al., 2001; Ling et al., 2012a; Shankar et al., 2013). Loss of G9a in mice results in early embryonic lethality between embryonic days at E9.5-E12. $G9a^{-/-}$ embryos show severe growth defects and increased apoptotic cells. $G9a^{-/-}$ embryonic stem cells display growth defects when induced to differentiate indicating the necessity of G9a during development and differentiation (Tachibana et al., 2002). More recently using G9a conditional knockout mice, G9a was found to inhibit adipogenic differentiation by mediating repressive H3K9me2 marks on promoters of PPARγ resulting in repression of its expression (Wang et al., 2013). G9a does not only mediates H3K9me, but is also capable of bringing about DNA methylation through its interaction with DNA methyltransferase DNMTs (Chin et al., 2007). For instance, LSH, a chromatin remodeling enzyme, recruits G9a to mediate DNA methylation and to silence genes involved in commitment and differentiation (Myant et al., 2011).

Our lab has been interested in understanding the role of G9a during muscle differentiation. Studies from our lab have shown that G9a is expressed in skeletal muscle and its expression declines during muscle differentiation (Ling et al., 2012a). G9a, when over expressed in myoblasts, inhibits myogenic differentiation through its ability to mediate repressive H3K9me2 on the promoter of myogenin.

Additionally G9a methylate MyoD and inhibits its ability to transcribe MyoD target genes required for differentiation (Ling et al., 2012b). G9a does not have the ability to directly bind to DNA. Sharp1 and MSX1 transcription factors are shown to be involved in recruiting G9a to the muscle promoters (Ling et al., 2012a; Wang and Abate-Shen, 2012).

1.9 Activator function of G9a

Although G9a is widely regarded as repressor of transcription, emerging evidence suggests a positive role for G9a as activator of gene expression which is independent of its methyltransferase activity (Bittencourt et al., 2012; Chaturvedi et al., 2009). It appears that repressor or activator function of G9a depends on its association with either repressor or activator complex (Shankar et al., 2013). In adult erythroid cells, G9a is shown to suppress the embryonic β globin gene while it also functions as an activator for adult β globin gene expression. Association of G9a with Jarid1 leads to repression of embryonic globin Ey gene expression whereas G9a association with Mediator leads to activation of adult β globin β-maj gene (Chaturvedi et al., 2009, 2012). G9a is recruited by ligand activated glucocorticoid receptor at the glucocorticoid receptor binding sites and functions as activator of gene expression. G9a in cooperation with GRIP1, CARM1 and p300 acts as a coactivator for nuclear receptors in a methyltransferase independent manner (Lee et al., 2006). Runx2 recruits G9a to the promoters of a subset of cancer related genes such as MMP9, CSF2 and SDF1 and activate their expression which does not require G9a methyltransferase activity (Purcell et al., 2012). From these studies it is clear that the role of G9a as co-activator does not require its

methyltransferase activity, and the function of G9a as repressor or activator might entirely depend upon its interacting partners (Shankar et al., 2013) (Fig 1.9).

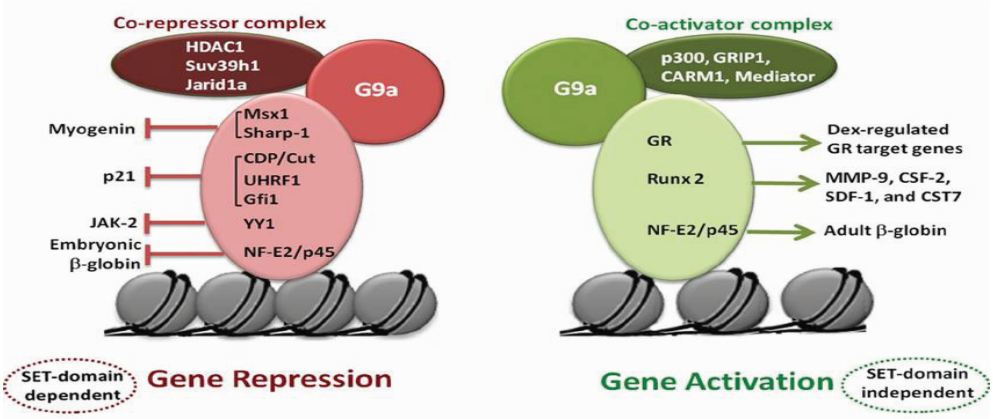


Figure 1.9 Schematic representation of G9a functioning as transcriptional repressor and activator (Shankar et al., 2013)**.** G9a associates with co-repressor and co-activator complex leading inhibition (p21, Myogenin Embryonic β-globin) or activation of gene expression (GR target genes adult β globin). Repressor function of G9a requires SET domain whereas activator function of G9a is independent of SET domain.

1.10 Role of G9a in cellular proliferation

Emerging evidence suggest a role for G9a in regulating proliferation of cells. p21, a tumor suppressor gene and a cyclin dependent kinase (Cdk) inhibitor, controls the Cyclin-CDK complex at G1 phase. It is a key cell cycle checkpoint regulator and several studies have demonstrated the role of G9a in regulating p21 expression. Several transcriptional factors recruit G9a to regulate p21 gene expression. For instance, CDP/cut a transcription factor involved in proliferation, differentiation and many cellular processes, is shown to interact with G9a *in vivo* and *in vitro*. p21 expression is repressed by CDP/cut and this transcriptional repression function of CDP/cut is due to its association with G9a and the methyltransferase activity of

G9a (Nishio and Walsh, 2004). Similarly UHRF1, (ubiquitin-like containing PHD and RING finger domains 1), a protein associated with cell proliferation and epigenetic regulation, interacts with G9a and is found to be co-localized in nucleus in a cell cycle dependent manner. UHRF1 recruits G9a to the promoter of p21 to repress its expression (Kim et al., 2009). Gif1, growth factor dependent 1, a transcriptional regulator oncoprotein, is also shown to recruit G9a to modify its target genes. Gif1 associates with HDAC1 and G9a on p21 promoter resulting in its repression (Duan et al., 2005). Studies have shown that BIX-01294, a potent inhibitor of G9a activity, reduces the proliferation in ovine fetal pulmonary arterial smooth muscle cells, suggesting the importance of G9a in proliferation. BIX-01294 treatment did not only induced G1 cell cycle arrest characterized by higher p21 expression, but also inhibited migration, contractility and altered global methylation levels (Yang et al., 2012).

Several studies have highlighted the role for G9a during replication. Gene expression analysis on G9a conditional knockout mouse ESCs found that significant numbers of late replicating genes including Magea1 and Dub1a were repressed by G9a, and its loss led to reduced H3K9me2 and de-repression of these genes that were found at the nuclear periphery. This suggest that G9a might be important to create a facultative heterochromatin at the nuclear periphery (Yokochi et al., 2009). During replication DNMT1, DNA methyltransferase 1, interacts with G9a and is co-localized in nucleus. DNMT1 forms a complex with G9a at replication foci, directing DNA and H3K9 methylation during cell division,

suggesting that both molecules work together to restore heterochromatin at replication fork during S phase (Estève et al., 2006).

Despite its vital requirement at the early embryonic developmental stages, and for proliferation of cells, a few studies have looked into role of G9a in regulating quiescence (G0 phase). Quiescence requires active repressive settings at the chromatin level to keep a check on proliferation and differentiation specific genes to maintain the G0 state. G9a was shown to be in complex with E2F6 (E2F6.com1) and other Polycomb group proteins to form a repressor complex on E2F responsive promoters in G0 phase (Ogawa et al., 2002). G9a mediated chromatin silencing has also been implicated in establishment of latent HIV 1 provirus. Viral latency requires modifications of the integrated viral gene to maintain quiescence. G9a has been shown to repress HIV 1 gene expression and is responsible for transcriptional quiescence of latent HIV 1 provirus by mediating H3K9me2 on HIV 1 long terminal repeat promoter, which is dependent on the methyltransferase activity of G9a (Imai et al., 2010).

1.11 Role of G9a in cancer

High G9a expression has been correlated with several cancers including prostate, lung and hepatocellular carcinoma (Shankar et al., 2013). G9a has been shown to repress Ep-CAM in lung cancer cells leading to higher migration and invasion (Chen et al., 2010) and suppresses Runx3 expression in gastric cancer cells in a methyltansferase activity dependent manner (Chen et al., 2006). G9a suppresses p53 activity by methylating its lysine residue 373, suggesting another level of control over p21 expression by G9a and indicating that G9a could be a potential inhibitory target in the treatment for cancer (Huang et al., 2010).

Overall, the above mentioned studies indicate that G9a has an important role to play in oncogenesis. High G9a expression is correlated with cancer progression and poor prognosis in cancer patients**.** G9a has been functionally linked to proliferation, cancer invasiveness and cancer progression. However, the mechanisms behind G9a regulation of cell cycle and proliferation are not clear. Although G9a association with few cell cycle regulators have been studied, finding the targets of G9a at global level is crucial for better understanding of its function. This might provide critical information on cell cycle regulation by G9a and also help us to gain more insights into various diseases which involve cell cycle deregulation such as cancer**.**

1.12 Rationale and Objectives of the study

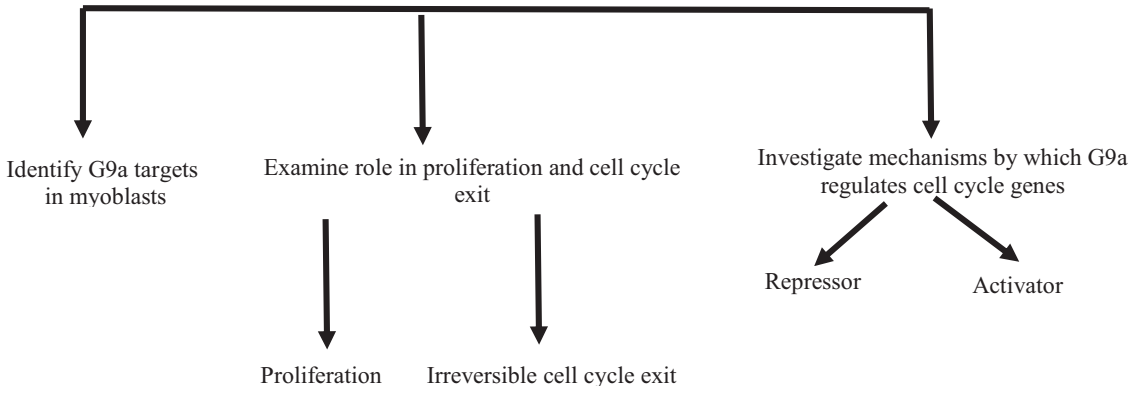
1.12.1 Rationale

Myogenic differentiation requires irreversible cell cycle exit. Previous studies from our lab have shown that G9a is expressed in proliferating myoblast and its expression declines during differentiation. Moreover, over expression of G9a was found to inhibit myogenic differentiation (Ling et al., 2012a, 2012b). Mechanistically, G9a was found to be complex with MyoD in myoblasts and repress its transcriptional activity and consequently expression of its downstream targets. However, whether G9a has an impact on proliferation of myoblasts, and their ability to irreversibly exit the cell cycle has not been addressed. Moreover, genome wide targets of G9a in skeletal muscle cells have not been identified

1.12.2 Objectives

1. To identify genome wide targets of G9a in skeletal muscle cells
2. To examine whether G9a has a role in regulating proliferation and cell cycle exit during myoblast differentiation
3. To investigate the mechanisms by which G9a regulates proliferation and cell cycle exit

1.12.3 Schematic representation of approach towards understanding aims



2. Materials and methods

2.1 Mice

$G9a^{fl/fl}$ mice were kindly provided by Alexander Tarakhovsky. $G9a^{fl/fl}$ mice were crossed with tamoxifen inducible $Pax7^{Cre\text{-}ERT2}$ Cre mice (Jackson Laboratory). All mice used for experiments carried the genotype $G9a^{fl/fl}$; $Pax7Cre^{ERT2/+}$. To induce Cre recombinase activity in $Pax7^{+}$ muscle satellite cells, tamoxifen (1mg/10g body weight) was injected intraperitoneally into 1 month old $G9a^{fl/fl}$;$Pax7^{Cre\ ERT2/+}$ mice for 5 consecutive days. Control mice with same genotype were injected with vehicle (corn oil). 10 days after the last injection, mice were euthanized using CO_2 asphyxiation and skeletal muscle from hind limbs were harvested for myoblast isolation. Genotyping was done using DNA from tail biopsies. Tail was digested in DNA digestion buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS) with proteinase K (0.3mg/ml) at 55^0C overnight. DNA was isolated using phenol-chloroform extraction method. Genotype was confirmed by PCR with following conditions (94^0C- 3min, 94^0C-20sec, 60^0C-20sec, 72^0C-30sec, 35 cycles, 72^0C-10min). Primers sequences are provided in table III. All mice were housed in sterile well ventilated cages under 12 hour light dark cycle in an animal facility. All mice experiments were performed in accordance to protocols approved by Institutional Animal Care and Use Committee (IACUC), National University of Singapore.

2.2 Primary myoblast isolation and culture

Primary myoblasts were isolated from hind limb muscles both from control and tamoxifen injected mice. In addition, for knockdown studies, primary myoblasts were isolated from hind limb muscle from wild type C57BL/6 mice. Mice were euthanized using carbon dioxide asphyxiation. Limbs were disinfected with 70% alcohol and using sterile scissors and forceps, outer skin was removed. Quadriceps and tibialis anterior (TA) muscle were dissected away from bone and was collected in sterile PBS with 1X penicillin-streptomycin. All procedures were carried out in sterile conditions in a tissue culture hood. Muscle tissue was minced to slurry with blade in a petri dish. 500ul of collagenase solution containing 1.5U/ml collagenase (Sigma), 2.5U/ml dispase (Roche) and 2.5mM $CaCl_2$ was added on to the slurry and minced for few minutes. Tissue slurry was pipetted out into falcon tube and incubated at 37^0C for 15 - 20 minutes. Every 5 min the tissue slurry was triturated with pipette to break the clumps. Slurry was diluted with plain F10 media (Gibco) and filtered through the 100um cell strainer. Next, the slurry in media was centrifuged at 1,200rpm for 5 min to pellet the cells. The pellet was suspended in F10 media supplemented with 20% FBS and 5ng/ml basic fibroblast growth factor (bFGF), and plated on to collagen coated petri dishes (tissue culture petri dishes were coated with 0.01% collagen from calf skin (Sigma) overnight and dried). Cells were incubated at 37^0C with 5% CO_2 and media was changed after 48hr. For myoblast enrichment, heterogeneous population of cells were trypsinized and pre-plated onto culture dish for 15-30 minutes to remove strongly adherent fibroblasts. Unattached floating cells were collected and plated onto new collagen coated dish.

Pure myoblast population was confirmed by staining cells with Pax7. More than 95% of cells stained positive for Pax7 and were used for further experiments.

2.3 Cell lines and culture conditions

2.3.1 C2C12 (mouse myoblast cell line)

C2C12 mouse myoblast cells were cultured in growth medium comprising of Dulbecco's Modified Eagle Medium (DMEM) high glucose (Sigma) supplemented with 20% fetal bovine serum (FBS) (Hyclone) and 1X penicillin-streptomycin (Gibco). For proliferation assays, C2C12 cells were cultured at 70% confluence and for differentiation assay, 80-90% confluent cells were provided with DMEM high glucose supplemented with 2% horse serum (Gibco) for different time points of 12, 24 and 36 hrs. Differentiation medium was changed every day.

2.3.2 Phoenix cells

Phoenix cells were cultured in DMEM high glucose supplemented with 10% FBS with penicillin-streptomycin (1X). Growth medium was changed every alternate day. All the cells were incubated at 37^0C with 5% CO_2 in a humidified incubator.

2.3.3 Cryopreservation of cells

Cells were trypsinized and pelleted using centrifugation. Pelleted cells were suspended in freezing medium (90% FBS with 10% dimethylsulfoxide (DMSO). Freezing mixture was aliquoted into cryovials and stored at -80^0C in an insulated box. After 24hrs, vials were transferred to liquid nitrogen for long term storage.

2.4 G_0 synchronization (quiescence)

Quiescence was induced in C2C12 cells using suspension culture method (Milasincic et al., 1996). Adherent proliferating C2C12 cells were washed with PBS, trypsinized and cell count was performed. Cell suspension was prepared with $1X10^5$ cells per ml along with 1.4% (final) methylcellulose (Sigma) medium containing 20% FBS, 10mM HEPES pH 7.4 (Sigma), 1X penicillin-streptomycin. Cell suspension was cultured in 50ml polypropylene falcon tubes with loosened cap and was incubated at 37^0C for 48hrs. Cells were harvested by diluting the cell suspension with PBS, washing it 3 times. Pelleted cells were then suspended in C2C12 growth medium and plated on to petri dish or were taken for further analysis.

2.5 Plasmids

pBabe and pBabe-G9a retroviral vectors, Flag-G9a (1,001aa), EGFP-G9a were kindly provided by Dr. Martin J Walsh (Mt Sinai School of Medicine New York NY 10029). Flag-P/CAF was provided by Dr. Yoshihiro Nakatani (NIH, USA). For luciferase reporter assay, a firefly luciferase reporter construct pD1luc containing E2F1 binding site in CyclinD1 promoter was provided by Dr. Michael Strauss.

Site directed mutagenesis: The E2F1 binding site in pD1luc was mutated (TTTG*GC*GC to TTTGG*AT*GC) using the QuickChangeTM site-directed mutagenesis kit (Agilent). The mutants were generated from pD1luc using the primers listed in the table III. The cDNA was sequenced to confirm the presence of directed mutations. Flag-p21 and Flag-Rb1 was constructed as below.

2.5.1 Cloning and transformation

Mouse p21 and Rb1 cDNA was separately cloned into pCMV 3X Flag (10) vector flanking restriction sites HindIII and BamHI. Ligation was carried out using T4 DNA ligase (Thermo scientific) and transformation was carried out using competent cells DH5α. Ligated DNA and competent cells were mixed and incubated on ice for 30min. Cells were subjected to heat shock at 42^0C for 90 seconds and cooled on ice for 5 minutes. Cells were recovered in 1ml broth at 37^0 C for 1hr. Cells were pelleted by centrifugation at 6,000rpm for 5 min and then inoculated on to the bacterial culture plates with ampicillin overnight at 37^0C. Single clones were isolated and inoculated into 10ml of broth and incubated overnight. Plasmid extraction was carried out using Promega Miniprep kit as per manufacturer's protocol.

Plasmid midi preparation was carried out using Qiagen kit. In brief, 10ul of bacterial glycerol stock was inoculated into 2ml broth as starter culture for overnight with suitable antibiotics. Next day morning, culture were stored at 4^0C, later in the evening again 1ml of the culture was inoculated into 200ml broth containing antibiotics. Flasks were incubated at 37^0C for overnight in an orbital shaker. Bacterial cells were pelleted at 6,000rpm for 15 min, resuspended in P1 buffer, lysed in P2 buffer and mixed with P3 buffer. Bacterial lysate clearing step was carried out by centrifuging cells at 20,000g for 30 min at 4^0C. Plasmid was washed, eluted using Qiagen tip columns as per the manufacturer's instructions. DNA was quantified using Nanodrop.

2.6 DNA Transfections

2.6.1 Retroviral transduction

Phoenix packaging cells were used to produce retroviral supernatant. Briefly, 1.5 million cells were seeded in a 10cm dish. Next day, transfection was carried out with 30ug of pBabe and pBabe-G9a plasmid separately using $CaPO_4$ transfection kit (Invitrogen) according to manufacturer's instructions. Briefly, 40ul of 2M $CaCl_2$ was mixed with 30ug of DNA in an Eppendorf tube. The volume was adjusted to 300ul with sterile water. While vortexing, 300ul of 2X HBS was added slowly, bubbling air through to the DNA mixture. The resulting DNA complex was incubated for 15min at room temperature. The precipitate was added onto the Phoenix cells with 10ml of media and incubated for 24hrs. Later, culture medium was changed to C2C12 growth medium for virus collection from the transfected Phoenix cells. Every 24hrs, media containing virus was collected and filtered using 0.45um syringe filter and stored at -80^0C.

G9a over expressing C2C12 stable cell line was generated by transducing C2C12 cells with virus generated from pBabe vector alone or pBabe-G9a. Infection was carried out using C2C12 growth medium containing the virus, for which 8ug/ml polybrene (Sigma) was added and incubated for 8hrs at 37^0C. Media was changed with normal C2C12 growth media after 8hrs of infection. Cells were allowed to grow for another 24hrs before selecting with 2ug/ml puromycin (Sigma) for 48hrs.

2.6.2 Transient transfections

Transient transfections were carried out using Lipofectamine and Plus reagent (Invitrogen). A day before transfection, cells were trypsinized and plated at desired density so that on the day of the transfection, the cells would reach 40% confluence. Briefly, DNA (for instance 2ug for a 10cm dish) was mixed with 250ul of DMEM without FBS and antibiotics in a 1.5ml Eppendorf tube. 2ul Plus reagent/µg of DNA was added to the DNA mix and incubated for 5 min. In a separate tube, Lipofectamine (3ul of Lipofectamine for 1µg DNA) was mixed with 250ul plain DMEM and incubated for 5 minutes. DMEM-Lipofectamine mixture was added to the mixture containing the DNA-Plus complex to a final volume of 500ul and incubated for 20 minutes at room temperature. DNA-Lipofectamine transfection mixture was added on to the cells for which medium had been changed to DMEM, and incubated for 3-4hrs at 37^0C in CO_2 incubator. After 4hrs, transfection media was removed and cells were fed with growth media.

2.6.3 siRNA transfection

Knockdown experiments were performed using 100nm scrambled siRNA (on-target plus control pool) or siRNA specific for G9a (siG9a; on-target plus smart pool, Mouse BAT8; accession number: NM_147151; NM_145830) from Dharmacon using Lipofectamine RNAiMax (Invitrogen) according to manufacturer's instruction. Sequences for siRNA are provided in the table (Table I). 5µl (100nm) of each siRNA was mixed separately with 100µl plain DMEM medium. 3.5µl of RNAiMax Lipofectamine was added to DMEM in a separate tube

and incubated for 5 min. Both siRNA mix and RNAiMax were mixed and incubated for 20 minutes and later, the complex was added to the cells in DMEM. Transfection was carried out for 3-4hrs and later cells were fed with growth medium. For C2C12, cells were taken for experiments after 48hrs knockdown. For primary myoblasts, cells were taken for analysis after 72hr of knockdown.

Table I: siRNA sequences

Smart pool siRNA	Sequences
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

2.7 Luciferase reporter assay

Luciferase reporter assay was performed using the Dual Luciferase Reporter System (Promega). pBabe and pBabe-G9a cells were transfected with 200ng of cyclinD1 reporter construct in a 24-well plate. Transfection was carried out in triplicates with Lipofectamine Plus. 2ng of Renilla reporter construct was also transfected as a normalization control. 24hrs after transfection, cells were lysed for 30 min using 1X passive lysis buffer provided in the kit. 100 µl of LAR II enzyme was added to 20µl of protein in a 96 well plate to measure the firefly luciferase signal and later, 100µl of stop and glow buffer was added to measure the Renilla activity. Luminescence reading was carried out using Varioskan plate reader using SkanIt software.

2.8 RNA isolation and Microarray

Total RNA was extracted from proliferating C2C12 cells (Day0) and differentiated (Day1) cells transfected with scrambled siRNA or siG9a. Briefly, cells were lysed in 500ul Trizol (Invitrogen) per million cells in 1.5ml Eppendorf tubes and vortexed. Chloroform was added to separate the phases and RNA was precipitated from the aqueous phase using isopropanol. RNA pellet was washed using 70% alcohol, and pellet was air dried and dissolved in nuclease free water. RNA was cleaned up using RNeasy MiniElute Cleanup Kit (Qiagen), was then quantified using Nanodrop. For microarray analysis, RNA quality was checked using Bioanalyzer (Agilent technologies).

Microarray was performed with RNA from two biological replicates. RNA was reverse transcribed and cRNA was synthesized using Total prep RNA amplification kit (Amibion). cRNA was labelled and subsequently hybridized to Illumina mouse WG-6 v2.0 array (Illumina). Partek Genomics Suite version 6.5 0 (Partek Inc., MO, USA) was used to perform gene expression analysis. Analysis of Variance (ANOVA) was applied on the data set from two samples and differentially expressed gene list was generated using p value < 0.05 with 1.3 fold change cut off.

2.9 Quantitative real time PCR (q-RTPCR)

To validate the results from the microarray analysis, quantitative real time PCR was performed on a few selected genes. Briefly, 1 µg of RNA was used to convert into cDNA using first strand cDNA synthesis kit (Invitrogen). cDNA was prepared according the manufacturer's protocol. Quantitative real time PCR was carried out using Light cycler 480 SYBR green I master (Roche). Reactions were done in triplicates, loaded onto the 384 well plate and PCR was performed in the Light cycler 480 II machine (Roche). ΔCT and 2^-ΔCT method was used to analyze the relative expression from the CT values. Primers used in the study are mentioned in the table (Table III)

2.10 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was carried out using Millipore ChIP kit. Chromatin was cross-linked to protein by adding formaldehyde (Sigma) (1% final concentration in media) for 10 min at 37^0C. Media was removed and cells were washed twice with cold 1X PBS buffer. Cells were removed/scraped out into a conical tube and centrifuged for 5 minutes at 2,000rpm, 4^0C.

Cells were lysed with 200µl SDS lysis buffer (with Protease Inhibitors) per $1X10^6$ cells. Lysate was incubated on ice for 10–30 min (depending on cell type) - for myoblasts, 10 min on ice, for day 1 myocytes, 20 min on ice. Cell lysate was sonicated using Bioruptor (Diagenode) with the below settings. $1X10^6$ cells per 200ul lysis buffer was maintained and samples were kept on ice throughout.

For day 0 myoblasts the following setting was used: Bioruptor power setting HIGH; 30s ON / 30s OFF; 10 cycles. For day 1 myocytes: power setting HIGH; 30s ON/ 30 s OFF; 12 cycles. For day 2 myotubes: power setting HIGH; 30s ON/ 30 s OFF; 14 cycles. Sonicated samples were centrifuged at 4^0C for 10 minutes and supernatant was collected.

ChIP was carried out according to kit protocol (Millipore) using antibodies mentioned in the table (Table II). Beads washing was carried out using wash buffers as per kit protocol. Reverse cross linking was performed at 65^0C overnight with 5M NaCl. Phenol-chloroform isolation method was used to extract DNA.

qRT-PCR was performed as explained previously. DNA isolated from 10% input was used as control. Relative enrichment was calculated using 2^-ΔCT method.

Table II: Antibodies used for ChIP

ANTIBODY	COMPANY	DILUTION
Anti H3K9me2	Millipore	2ug
Anti-G9a	Abcam	8ul
Anti-E2F1	Abcam	2ug
Anti H3K9ac	Millipore	2ug
Anti P/CAF	Abcam	2ug

Table III: Primers used in this study

Primers for qRT- PCR	Sequence 5' to 3'	Annealing temperature
Mouse Gapdh	F- ATCAACCGGGAAGCCCATCAC R -CCTTTTGGCTCCACCCTTCA	60
Mouse Cyclin E	F-TGTCCTCGCTGCTTCTGCTTTGTATCAT R -GGCTTTCTTTGCTTGGGCTTTGTCC	60
Mouse Cyclin D1	F-AAGTGCGTGCAGAAGGAGATTGTG R -TCGGGCCGGATAGAGTTGTCAGT	60
Mouse p21	F-GCAGCCGAGAGGTGTGAGC R-ACGGGACCGAAGAGACAACG	60
Mouse G9a	F-TCGGGCAATCAGTCAGACAG R-TGAGGAACCCACACCATTCAC	60
Mouse Dhfr	F-GGTAGGAAAACCTGGTTCTC R-CAGAACTGCCTCCGACTATC	60
Mouse Rb1	F-ACGCTGCCCAGGAGACCTTT R-AGGGCTTCGAGGAATGTGAGGT	60
Primers for ChIP qRT-PCR		
Mouse Cyclin D1	F-GAGAGCTTAGGGCTCGTCTG R-TGGGTGCGTTTCCGAGTAC	60
Mouse p21	F-CCCCGCATGCCCAGTTTATGG R-GGTCTGTCCCTGACCAACTGTG	60
Mouse Rb1	F-AGCCCAGGCTTGCAACCTACCC R-CCGCGTCACATAGCAGGTCCC	60
Mouse Dhfr	F-GCCTAAGCTGCGCAAGTGGT R-GTCTCCGTTCTTGCCAATCC	60
Mouse Cyclin E2	F-GAGCCGAACCGTAGCCTGA R-CTCCTGGACCGTGCTCCTC	60

Primers for cloning		
p21	F-CCCAAGCTTATGTCCAATCCTGGTG R-CGGGATCCTCAGGGTTTTCTCTTGC	
Rb1	F-CCAAGCTTATGCCGCCCAAAGCC R-CGCGGATCCTCACTTTTCCTCCTT	
pD1luc Pd1luc E2F1 mutant	F-CTCCCGGCGTTTGATGCCCGCGCC R-GGCGCGGGCATCAAACGCCGGGAG	
Primers for mice genotyping		
G9a-F1 G9a-F2 G9a-R $Pax7^{cre}$ F $Pax7^{cre}$ WT R $Pax7^{cre}$ Mut R	F1- CACGCTGCCTAGATGGAGCATGCC F2-AGGCTATGAGAATGTACCCATCCCCTG R-GTGTGAGCCTGTGTTCTGGGGATTA F-TACCAGAGGCAACAAACAGG R-TTGATGAAGACCCCACCAAG R-CAAAGGTGGCTAAGGTGGAG	60

2.11 G9a methyltransferase activity inhibition

G9a methyltransferase activity was blocked using UNC0638 compound (Sigma) known to selectively inhibit G9a methyltransferase activity (Vedadi et al., 2011). UNC0638 effect on myoblasts differentiation was checked using different concentrations and a concentration of 0.25μM was found to be optimal. As a control, cells were treated with DMSO. Treatment was carried out for at least 36hr before harvesting the cells for further analysis. For differentiation assay, cells were pretreated for 24hrs in growth medium and then treatment was continued in the differentiation medium containing UNC0638 as well.

2.12 BrdU incorporation assay

Proliferation of cells was measured by pulsing cells with 10mM BrdU for 30 minutes. Cells were fixed with 70% alcohol with 50mM glycine at -20^{0}C for 20 minutes. BrdU staining was carried out using BrdU staining kit according to manufacturer's protocol (Roche). Cells were incubated for 1hr at room temperature with anti-BrdU antibody (1:100) diluted in incubation buffer provided in the kit. After 3 washes with PBS, secondary antibody (1:100) labelled with FITC was added for 1hr at room temperature. Cells were again rinsed with PBS 3 times, nuclei was counter stained with DAPI and cells were mounted with mounting agent (Vectashield) and visualized using Olympus microscope (DP72). BrdU positive cells were quantified by ratio of BrdU positive cells to the total number of DAPI positive cells. At least 500 BrdU positive cells were counted from different fields.

2.13 Flow cytometry analysis

Cells were trypsinized and collected by centrifugation. Cell pellet was washed once using PBS and centrifuged again at 1,200rpm for 3 minutes. Cells were fixed with 70% alcohol while vortexing to avoid clumping of cells. Cells with alcohol was stored at -20^{0}C for at least 24hr. Later, cells were washed with PBS and centrifuged at 1,200rpm for 5 min. Cells were stained with 300µl propidium iodide (Sigma) mix (10µg/ml propidium iodide solution with RNAase A) for at least 30 minutes at room temperature. Cells were strained using 40um filters to avoid clumps and run through BD FACS machine. At least 10,000 cells were acquired during the run.

2.14 Immunofluorescence imaging

Cells were cultured on sterile cover slips placed in 6-well dishes. Cells were fixed with 4% paraformaldehyde (Sigma) for 20 minutes at room temperature. Cells were gently rinsed with PBS for 3 times. Fixed cells were blocked and permeabilized using PBS containing 10% horse serum (Gibco) and 0.1% tritonX 100 (Biorad) for 1hr at room temperature. Cells were then incubated with antibody in the blocking solution (PBS with 10% horse serum) at desired concentration at 4^{0}C for overnight. Next day, cells were gently rinsed 3 times with PBS, and incubated with secondary antibody tagged with fluorophore (Alexa fluor 488 or Alexa fluor 565) at 1:250 dilution. Cells were incubated for 1hr at room temperature. Later, cells were again rinsed with PBS and DNA was counter stained with DAPI, and mounted using mounting agent (Vectashield). Images were obtained using Olympus microscope (DP72).

Table IV: Antibodies used for Immunofluorescence staining

ANTIBODY	COMPANY	DILUTION
Anti- Myosin heavy chain (My32)	Sigma	1:300
PAX7	DSHB	1:10
Alexa fluor 488 goat anti rabbit	Invitrogen	1:250
Alexa fluor 568 goat anti mouse	Invitrogen	1:250

For staining with anti-myosin heavy chain antibody (MY32; Sigma), incubation were done for 1hr at room temperature, after which nucleus was counterstained with mounting agent containing DAPI (Vectashield). Myogenic index was calculated by quantifying the ratio of total nuclei within myotubes to total nuclei. At least 500 nuclei was counted.

2.15 SDS PAGE and Western blotting

Cells were lysed in NP40 buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA, 15mM MgCl2, 1% NP-40, 0.75% Sodium deoxycholate, 1mM DTT) with 1X protease inhibitor (Roche). Protein quantification was carried out using Bradford reagent (Biorad). 1 part of 5X Bradford reagent was diluted with 4 parts of MilliQ water to get 1X reagent. 1µl of protein was added to Bradford reagent in a cuvette and mixed, incubated for 3-5 min before taking the absorbance at 595nm using spectrophotometer. Quantified protein was denatured using SDS loading dye at 98^0C for 5 minutes. Protein was run on SDS polyacrylamide gels at 90 Volts.

Protein was transferred (wet transfer at 100V for 2hr) onto nitrocellulose membrane (Amershan hybond ECL). Transfer was carried out in cold conditions using ice packets.

Membrane was blocked with 5% skimmed milk (blotto, Santa Cruz) in 0.1% Tween in PBS (PBST) for 1hr. Primary antibodies were diluted in 5% milk and incubated either 1hr room temperature or overnight at 4^0C depending on the antibody. Membrane was washed 3 times with PBST 5 minutes each to remove the unbound antibody. Blots were incubated with horse radish peroxidase conjugated secondary antibody for 1hr at room temperature and again washed 3 times with PBST. Bands were visualized either using detection reagents from Amershan ECL detection system or West Dura from thermos scientific.

Table V: Antibodies used for western blotting

ANTIBODY	COMPANY	DILUTION
Rabbit polyclonal anti-G9a	Cell signaling	1:300
Rabbit polyclonal anti-p21	Santa Cruz	1:500
Mouse monoclonal anti-Rb1	BD bioscience	1:500
Mouse monoclonal anti-Rb1	Santa Cruz	1:500
Rabbit polyclonal anti-MyoD (M-318)	Santa Cruz	1:500
Rabbit polyclonal anti-myogenin	Santa Cruz	1:500
Rabbit polyclonal anti-Cyclin A	Santa Cruz	1:100
Rabbit polyclonal anti-CyclinD1	Santa Cruz	1:500
Anti-troponin-T	Sigma	1:1000
Anti-βactin	Sigma	1:10000
Anti-Flag	Sigma	1:1000

2.16 Co-immunoprecipitation (Co-IP)

For endogenous Co-IP, nuclear extracts were prepared from proliferating C2C12 myoblasts by following modified Dignam's protocol. Briefly, 100 million cells were trypsinized and washed twice with ice cold PBS. Cell pellet was incubated not more than 2 minutes by resuspending gently in 10ml of ice cold homogenization buffer with DTT and protease inhibitors (For the buffers recipe, see below). Cell suspension was slowly layered onto cold sucrose pad (8-10ml). Cells were centrifuged at 900rpm for 10 minutes at 4^0 C with no brakes. Supernatant was aspirated and nuclei at the bottom were washed with wash buffer (10ml) before

centrifugation at 14,000 rpm at 4^0C for 45 minutes to get rid of all cytoplasmic residues. Intact nuclei were observed under microscope with trypan blue. Nuclei pellet was resuspended in 1 packed volume of cold buffer C 0mM. Next, 2 packed volume of cold buffer C 840mM was added drop by drop while vortexing slowly. Every 5 drops, nuclei was incubated on ice. Nuclei were further lysed by 15 strokes with dounce homogenizer (Pestle B). Lysed nuclei were observed with trypan blue under microscope. The homogenate was gently stirred with a magnetic bar for half an hour at 4^0C and then centrifuged at 14,000rpm for 30 minutes at 4^0C. Supernatant was carefully recovered and diluted in 2 volumes of buffer D (for e.g. for 1ml supernatant 2ml buffer D). Pellet containing DNA and histone were left behind.

For endogenous co-immunoprecipitation, at least 1mg of protein was used to pull down with desired antibody. Protein lysate was pre-cleared using 30ul of protein A/G agarose beads (Santa Cruz) for 45minutes. Lysate was incubated with desired antibodies for overnight at 4^0C under rotation. Protein A/G agarose beads was then added (30ul) the following morning to the lysate for 2hr and kept for rotation at 4^0C. Beads were pelleted by centrifugation at low speed (2,000rpm) at 4^0C and washed with NP40 buffer with protease inhibitors for 3 washes lasting 5 minutes each. After final wash, sample loading dye (10ul) was added to the beads, heated at 98^0C for 8 minutes, and loaded on to the gel for SDS-PAGE.

Table VI: Antibodies used for Co-IP

ANTIBODY	COMPANY	CONCENTRATION
Anti-MyoD	Santa Cruz	2ug
Anti-G9a	Abcam	8ul
Anti-E2F1	Abcam	2ug
Anti-Flag beads	Sigma	20ul
Normal rabbit IgG	Santa Cruz	2ug

2.16.1 Solutions for nuclear extract

Salt stock (100ml): 10ml 1M Tris-HCl, pH 7.5, 3ml 5M NaCl, 60ml 1M KCl, top up with water to 100ml and store at RT.

Homogenization buffer (50ml): 5ml salt stock, 7.35ml 1M (34%) sucrose, 250ul 20% NP40, 100ul 0.5M EDTA, 10ul 0.5M EGTA, 32.3ml water. (Prior to use, add to per ml of buffer: 0.5ul 1M DTT, 1X PI)

Sucrose pad (10ml): 1ml salt stock, 2.94ml 1ml sucrose, 5.06ml water. (Prior to use, add to per ml of buffer: 0.5ul 1M DTT, 1X PI)

Wash buffer (50ml): 5ml salt stock, 100ul 0.5M EDTA, 10ul 0.5M EGTA, 40ml water. (Prior to use, add to per ml of buffer: 0.5ul 1M DTT, 1X PI)

Buffer C 0mM (9ml): 5ml 20mM HEPES pH 7.9, 25% glycerol, 0.2mM EDTA , 1.5mM MgCl2. (Prior to use, add to per ml of buffer: 0.5ul 1M DTT, 1X PI)

Buffer C 840mM : 20mM HEPES, pH 7.9, 25% glycerol, 0.2mM EDTA, 1.5mM MgCl2, 840mM KCL. (Prior to use, add to per ml of buffer: 0.5ul 1M DTT, 1X PI)

Buffer D: 20mM HEPES, pH 7.9, 20% glycerol, 0.2mM EDTA, 0.3% triton X 100. (Prior to use, add to per ml of buffer: 0.5ul 1M DTT, 1X PI)

2.17 Statistical analysis

Error bars represent mean standard deviation (SD). Significance was calculated using student t test (two tailed paired unless specified others) with p value <0.05 was considered to be statistically significant. (*p<0.05 **p<0.01 ***p<0.001).

3. Results

3.1 G9a expression correlates with proliferation of myoblasts

In order to find out if G9a has a role in proliferation and cell cycle exit of myoblasts, we examined its expression in different cellular states – proliferation, irreversible cell cycle exit (differentiation); and reversible cell cycle exit (quiescence). RNA and protein was extracted from proliferating C2C12 myoblasts, differentiated myocytes (Day 1) and quiescent myoblasts. G9a mRNA and protein expression was analyzed by quantitative real time PCR and western blot respectively. G9a mRNA level was higher in proliferating cells compared to cell cycle exit states (Fig 3.1A&B). Similarly, G9a protein expression was also high in myoblasts and declined upon differentiation and quiescence (Fig 3.1C&D). Thus G9a is expressed at higher levels in proliferating cells compared to cell cycle exit states, and suggest that a decline in G9a expression may be required for the cells to exit the cell cycle.

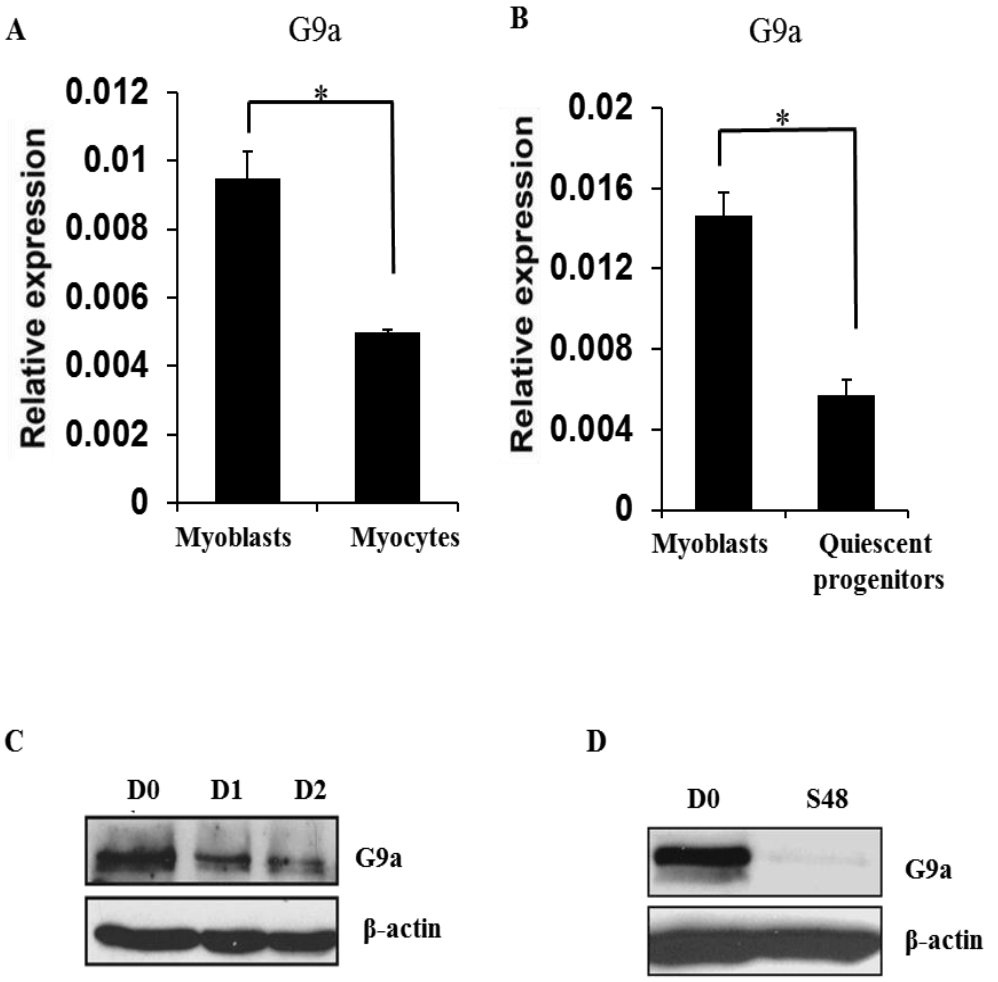


Figure 3.1. G9a expression decreases during myoblast cell cycle exit. (A & B) Relative quantification of G9a expression in proliferating myoblasts (D0), differentiated (Day 1) myocytes and quiescent C2C12 myoblasts (Quiescent progenitors) by qRT PCR. Gapdh was used to normalize the expression. (C & D) G9a protein level was analyzed in myoblasts (D0), differentiating myocytes (D1 & D2) and quiescent cells S48 (myoblasts cultured in suspension medium for 48hr) using western blot. β-actin was used as loading control. Error bars indicate mean ± SD. * indicates *p*-value <0.05.

3.2 Identification of genome wide targets of G9a

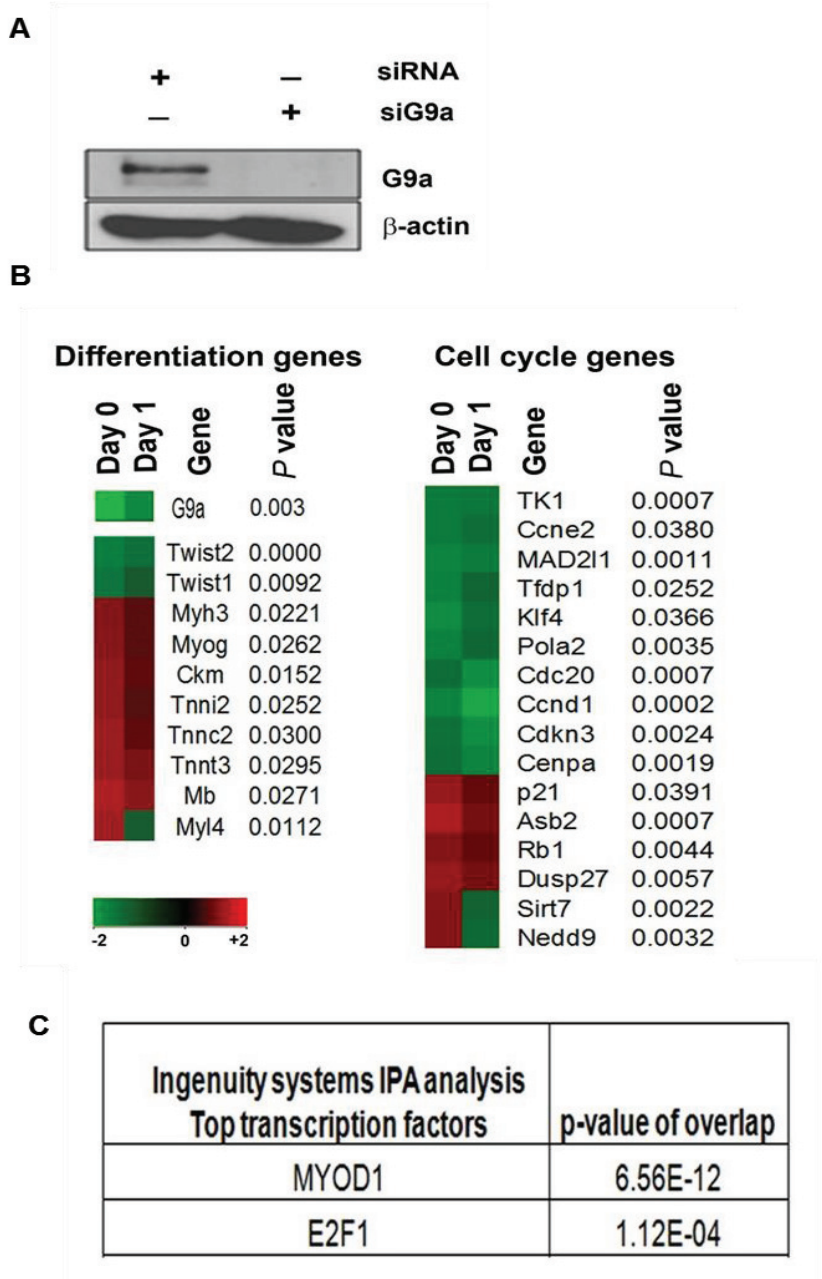
3.2.1 Gene expression studies using microarray

Previous studies from our lab have shown that G9a methylates MyoD in myoblasts to inhibit MyoD function. This is correlated with H3K9me2 repression marks at the myogenin promoter indicating that G9a functions as a negative regulator of myogenic differentiation (Ling et al., 2012b). While these studies established a role for G9a in myogenic differentiation, genome-wide targets of G9a in myoblasts have not been identified.

To identify G9a target genes, we performed gene expression analysis using microarray with G9a knock-down myoblasts. Endogenous G9a knockdown was performed using siRNA specific for G9a (siG9a cells). Control cells were transfected with scrambled siRNA (siRNA cells). G9a knockdown was confirmed 48hr post-transfection at protein level by western blot (Fig 3.2.1A). RNA was isolated from proliferating day 0 (Day 0) and differentiated day 1 (Day 1) from siG9a cells and siRNA cells. Two independent knockdown experiments were performed on two different days to achieve better reproducibility. RNA from both sets was reverse transcribed, labelled and hybridized to mouse WG6v2.0 array from Illumina.

From the microarray analysis, we identified 311 unique genes which were differentially regulated by 1.3 fold with *p*-value <0.05. 173 genes were up regulated whereas 138 genes were down regulated. G9a knockdown significantly altered

genes involved in different cellular pathways among which cell cycle control and muscle differentiation pathway genes were highly enriched (Fig 3.2.1B).



Ingenuity systems IPA analysis Top transcription factors	p-value of overlap
MYOD1	6.56E-12
E2F1	1.12E-04

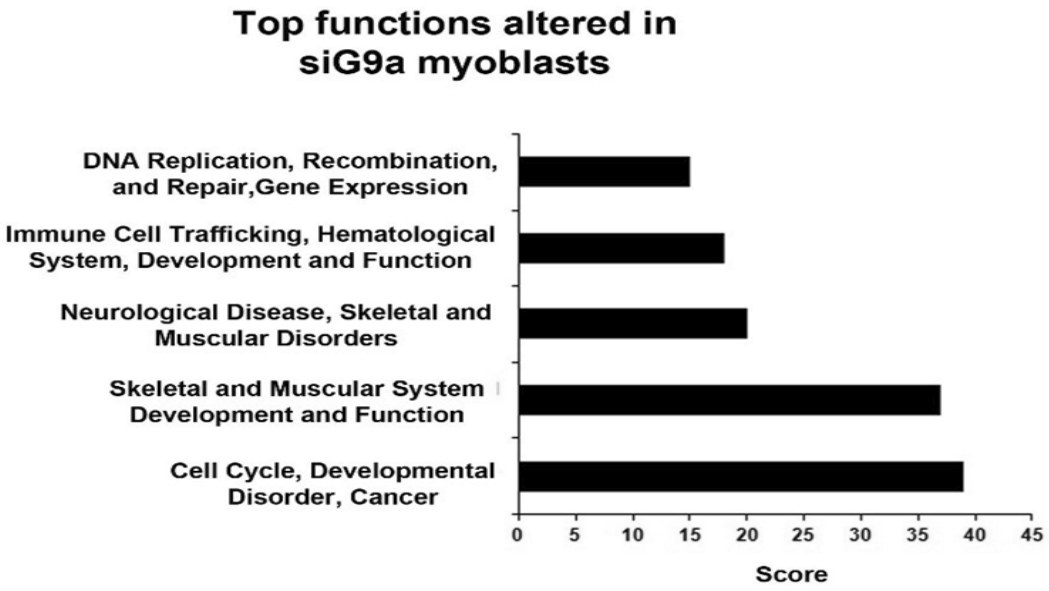
Figure 3.2.1A,B&C. Gene expression studies using microarray. (A) G9a protein expression was analyzed in siRNA and siG9a cells by western blot. β-actin was used as loading control. (B) List of G9a target genes involved in differentiation and cell cycle control in proliferating (Day 0) and differentiated (Day 1) cells with corresponding *p*-value. Green indicates down regulation and red indicates up regulation of gene expression. (C) Activity of transcription factors altered in siG9a cells. (Bioinformatics for microarrays was done by Jayapal Manikandan).

Consistent with our previous findings which showed that G9a inhibits myogenic differentiation (Ling et al., 2012b), MyoD dependent genes involved in differentiation such myogenin were up regulated in siG9a cells. Interestingly, in addition to differentiation related genes, the expression of many genes involved in cell cycle regulation was differentially altered. For instance, the expression of p21 and Rb1 genes which are involved in irreversible cell cycle exit during differentiation was up regulated. On the other hand, E2F1 target genes involved in cell cycle control such as CyclinD1, CyclinE and Thymidine Kinase were down regulated.

Ingenuity pathway analysis showed that the activities of MyoD and E2F1 transcription factors were altered in G9a knockdown cells (Fig 3.2.1C). This indicated that G9a in addition to its impact on myogenic differentiation through regulation of MyoD activity, G9a may control proliferation through its association E2F1.

Gene Ontology (GO) analysis for top canonical pathways and top biological functions showed that cell cycle regulation was altered upon G9a knockdown (Fig 3.2.1D&E). A set of genes from gene ontology was used to create a biological network. The biological network indicated that cell cycle progression and muscle differentiation was affected in siG9a cells (Fig 3.2.1F). This suggested that G9a has a major impact on the cell cycle control in addition to differentiation.

D



E

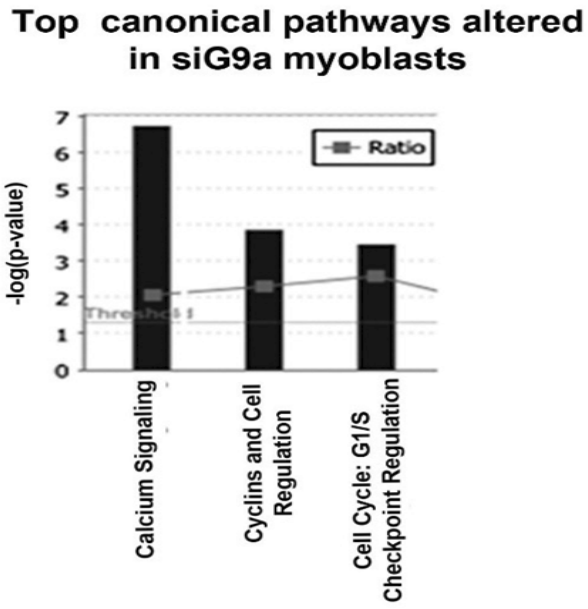


Figure 3.2.1D&E. Gene expression studies using microarray. Ingenuity pathway analysis. (D) Bar chart indicating top functions altered in siG9a myoblasts. Cell cycle control was found to be one among the top functions altered in siG9a cells. (E) Bar chart indicating top canonical pathways altered in siG9a myoblasts. Apart from calcium signaling, Cyclins and cell cycle regulation was found to be top canonical pathway altered upon G9a knockdown.

F

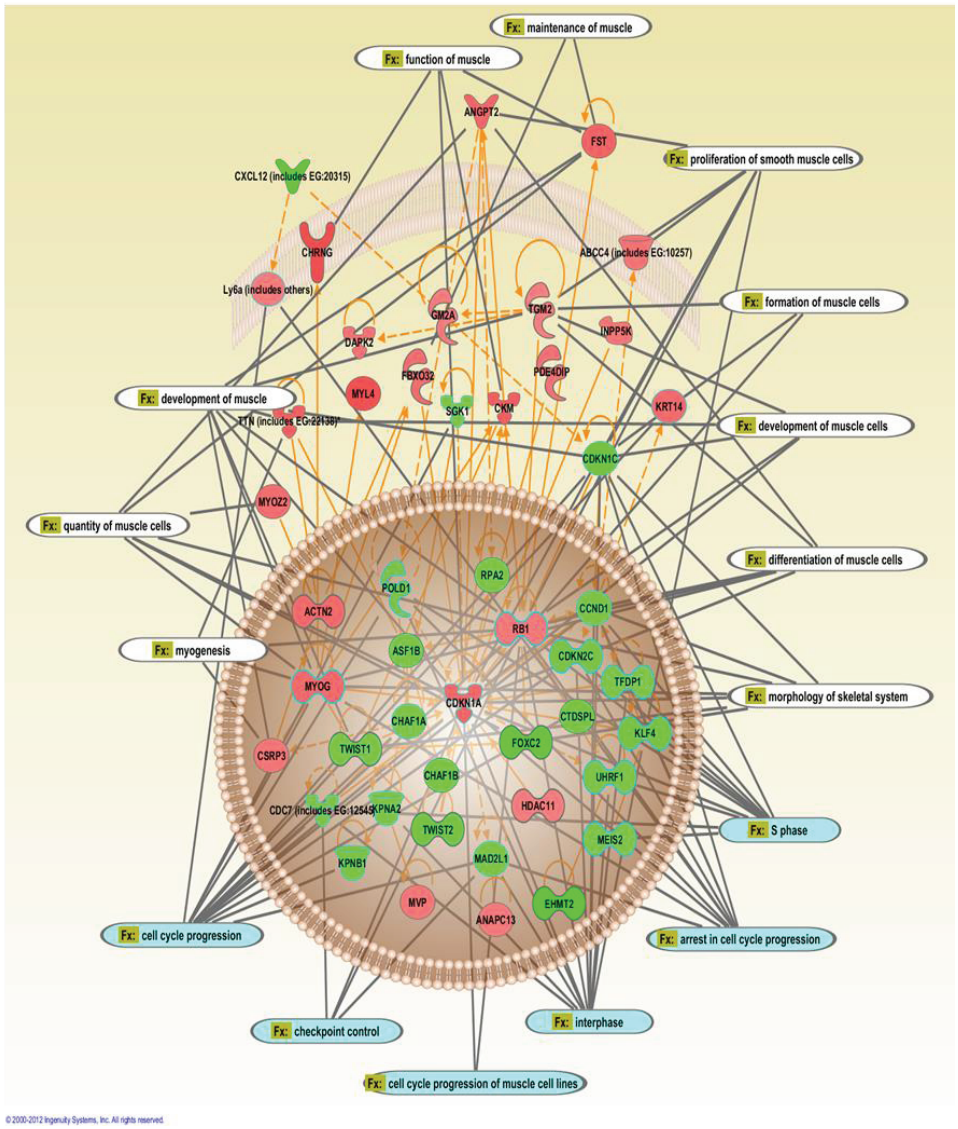


Figure 3.2.1F Gene expression studies using microarray. To understand the biological significance of G9a target genes, Gene ontologies were used to create the network - Molecular function ontology (indicates function of gene at molecular level) and Cellular component ontology (refers to the place where gene product is found). (F) Biological interaction network identifying possible interactions between G9a target genes related to cell cycle control and differentiation. Orange line indicates direct interaction. Dotted line indicates indirect interaction. Grey line indicates function altered by the genes.

Overall, results from the global gene expression analysis indicated that G9a targets both MyoD and E2F1 responsive genes in myoblasts. Since G9a knockdown up regulated MyoD target genes, it indicated that these may be direct transcriptional targets of G9a. On the other hand, since E2F1 targets were down regulated, G9a could be directly or indirectly involved in promoting expression of these genes.

3.2.2 Validation of microarray results

To validate the microarray results, we performed qRT-PCR on a few selected G9a target genes. RNA was isolated from control and siG9a cells and converted to cDNA and qRT-PCR was performed. Consistent with the microarray results, siG9a cells showed significant up regulation of MyoD target genes p21 and Rb1 whereas expression of E2F1 target genes such as Cyclin D1 and Cyclin E was down regulated (Fig 3.2.2 A&B), highlighting the reliability of the microarray results.

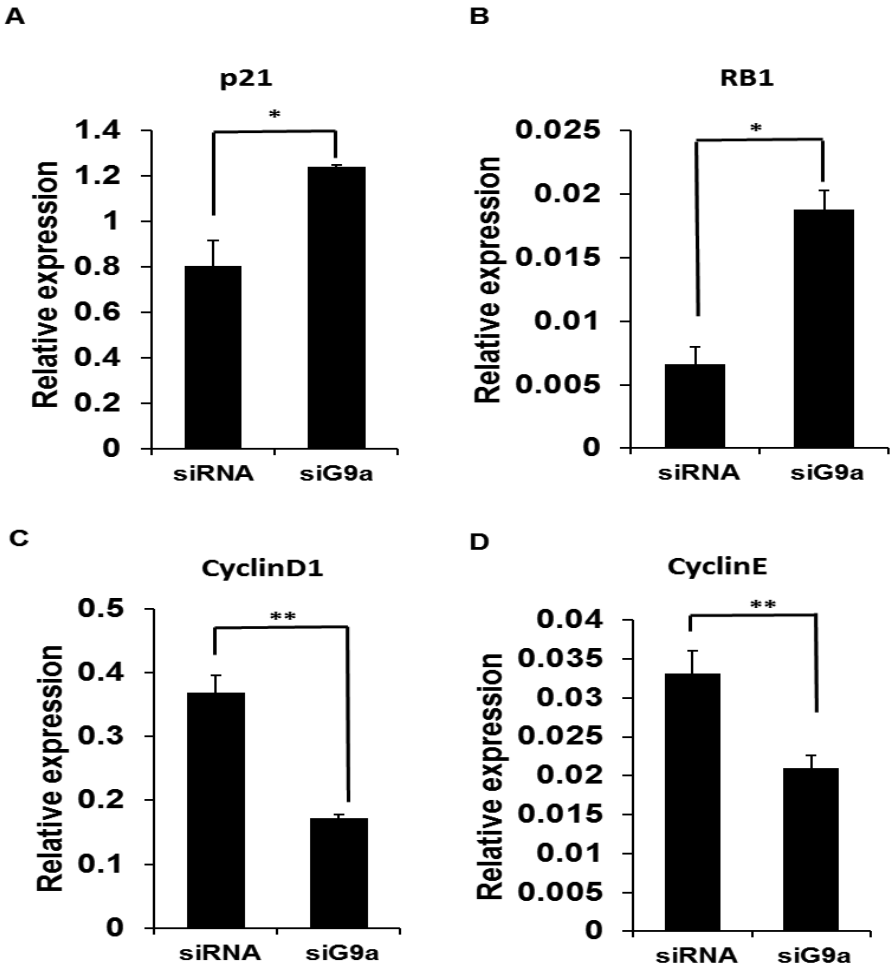


Figure 3.2.2 Validation of microarray results. G9a targets such as (A & B) p21, Rb1, (C & D) CyclinD1 and CyclinE expression were analyzed by qRT-PCR in control and siG9a C2C12 cells. Error bars indicate mean ± SD. * indicates *p*-value <0.05 ***p*-value <0.01.

3.3 G9a regulates differentiation and proliferation genes in mouse primary myoblasts

Next, we went on to validate if G9a regulates MyoD and E2F1 target genes in mouse primary myoblasts. Myoblasts were isolated and cultured from hind limbs of wild type mice. G9a knockdown was performed using siG9a for 72hr. Scrambled siRNA was used as control. RNA was isolated from the cells, cDNA was synthesized and qRT-PCR was performed to check the G9a knockdown efficiency (Fig 3.3A). Consistent with the microarray data, G9a knockdown cells showed significant up regulation of MyoD target genes p21 and Rb1 (Fig 3.3B&C) whereas E2F1 target genes CyclinD1, DHFR and CyclinE were significantly down regulated (Fig 3.3 D, E & F). Results from the microarray, C2C12 cells and primary myoblasts indicated that G9a regulate genes involved in cell cycle control and therefore it could be involved in regulating proliferation of cells.

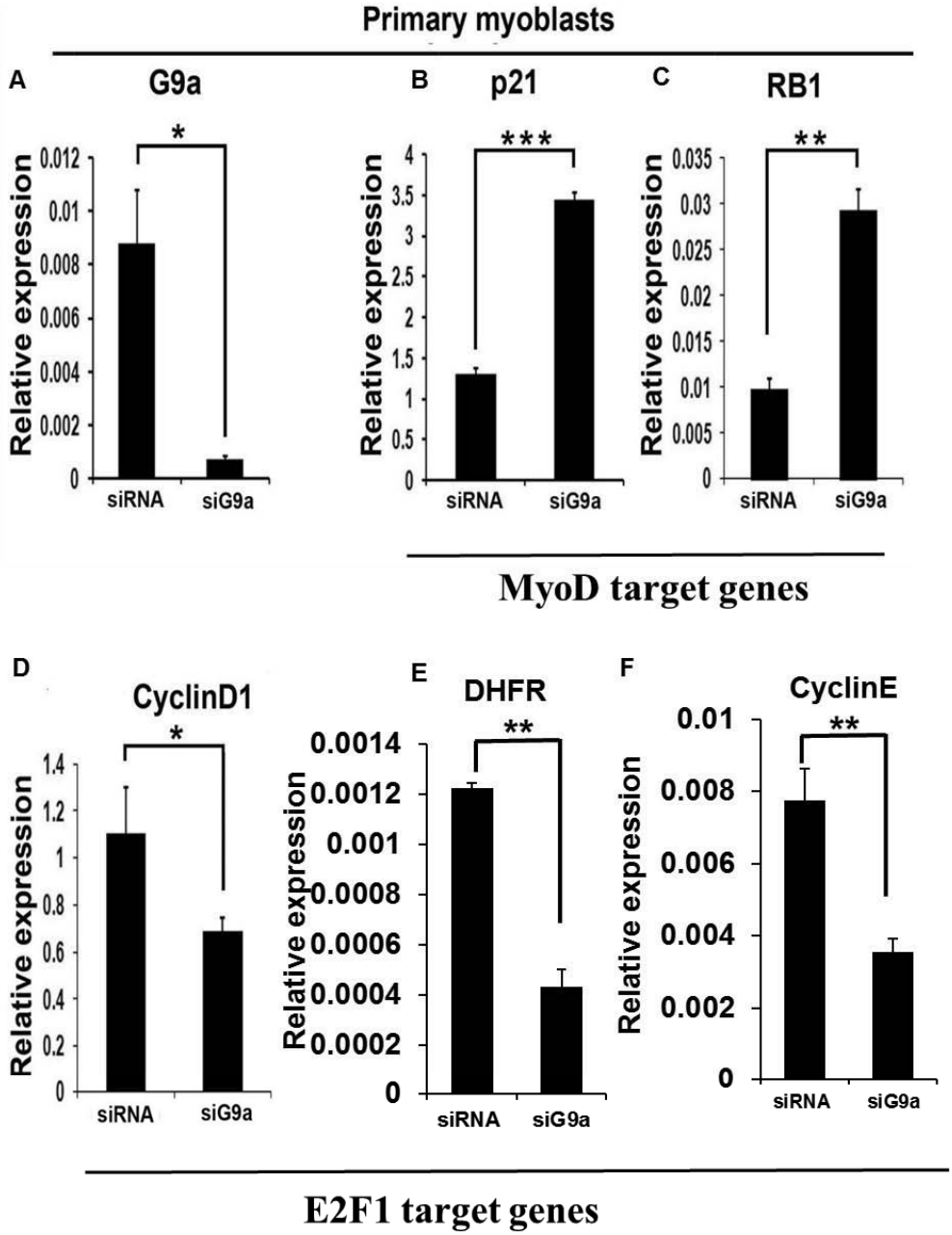


Figure 3.3 G9a regulates MyoD and E2F1 target genes in primary myoblasts. (A) G9a knockdown was analyzed by qRT-PCR in siG9a primary myoblasts. (B & C) p21, Rb1, (D, E & F) CyclinD1, DHFR and CyclinE expressions were analyzed by qRT-PCR in control and siG9a mouse primary myoblasts. Error bars indicate mean ± SD. *** indicates *p*-value <0.001, ** indicates *p*-value <0.01, * indicates *p*-value <0.05

3.4 Role of G9a in proliferation

3.4.1 G9a knockdown reduces proliferation of C2C12 cells

To examine if G9a indeed has any role in regulating proliferation of cells, we performed loss of function studies by carrying out G9a knockdown in C2C12 cells (siG9a cells) (Fig 3.4.1A). Both control and G9a knockdown cells were pulsed with BrdU for 30min, fixed and stained with anti-BrdU antibody. siG9a cells showed significantly lesser BrdU incorporation compared to control cells. This indicated that G9a knockdown led to reduced proliferation of cells (Fig 3.4.1B & C).

3.4.2 G9a knockdown reduces proliferation of primary myoblasts

Next, we confirmed the above results in siG9a mouse primary myoblasts. Knockdown of G9a was performed in primary myoblasts and confirmed by qRTPCR (Fig 3.4.2A). Both control and G9a knockdown cells were pulsed with BrdU for 30min, fixed and stained with anti-BrdU antibody. BrdU incorporation in siG9a cells was lesser compared to controls, indicating reduced proliferation in siG9a cells (Fig 3.4.2 B&C). Further, we also performed propidium iodide staining and analyzed cell cycle profiles by flow cytometry. Consistent with above results, siG9a cells showed lesser S phase cells compared to controls (Fig 3.4.2 D&E) and an arrest in the G1 phase of the cell cycle. Overall, our results suggested that G9a knockdown reduces proliferation of cells.

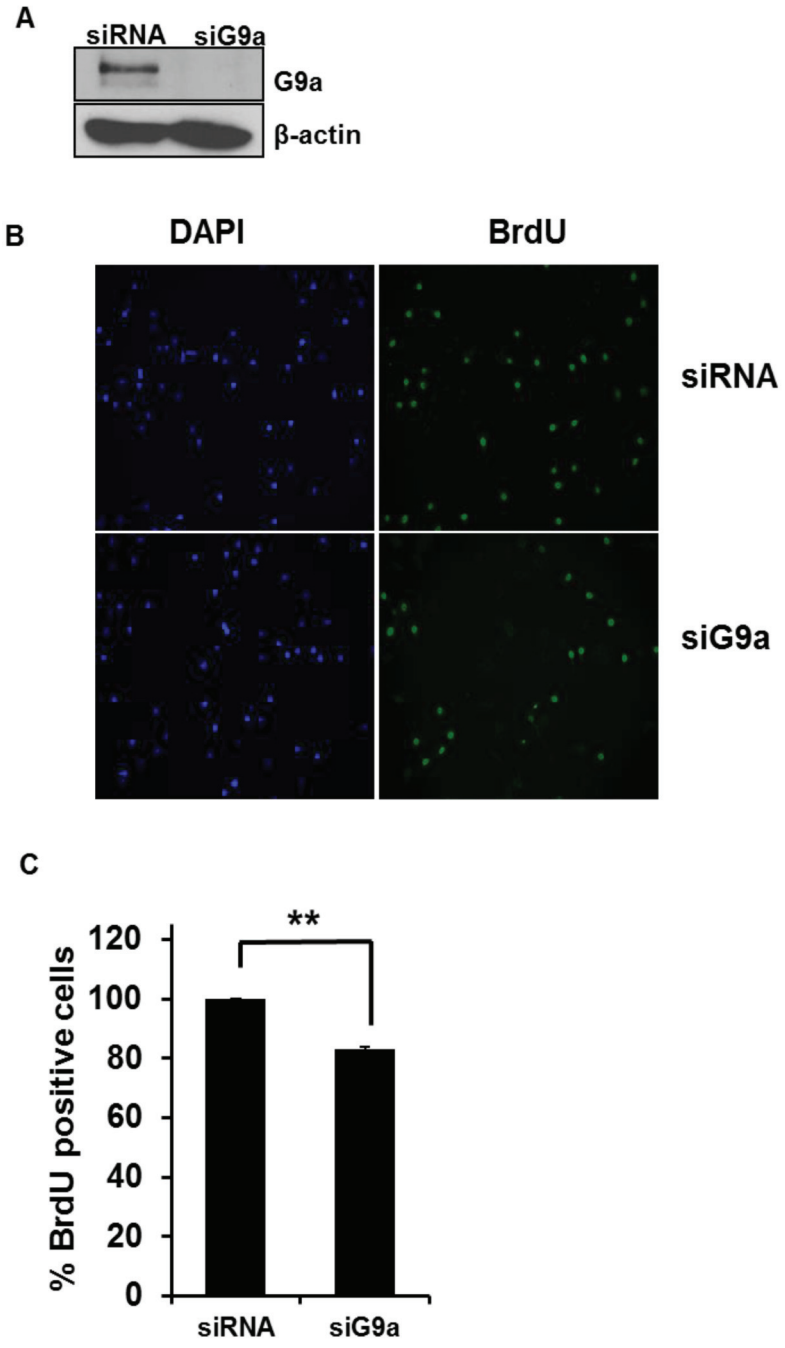


Figure 3.4.1 G9a knockdown reduces proliferation of C2C12 cells. (A) G9a down regulation in C2C12 cells shown by western blot. (B) BrdU positivity in control and siG9a C2C12 cells. Nuclei were counter stained with DAPI. (C) Quantification of BrdU positive cells. Error bars indicate mean ± SD. ** indicates *p*-value <0.01

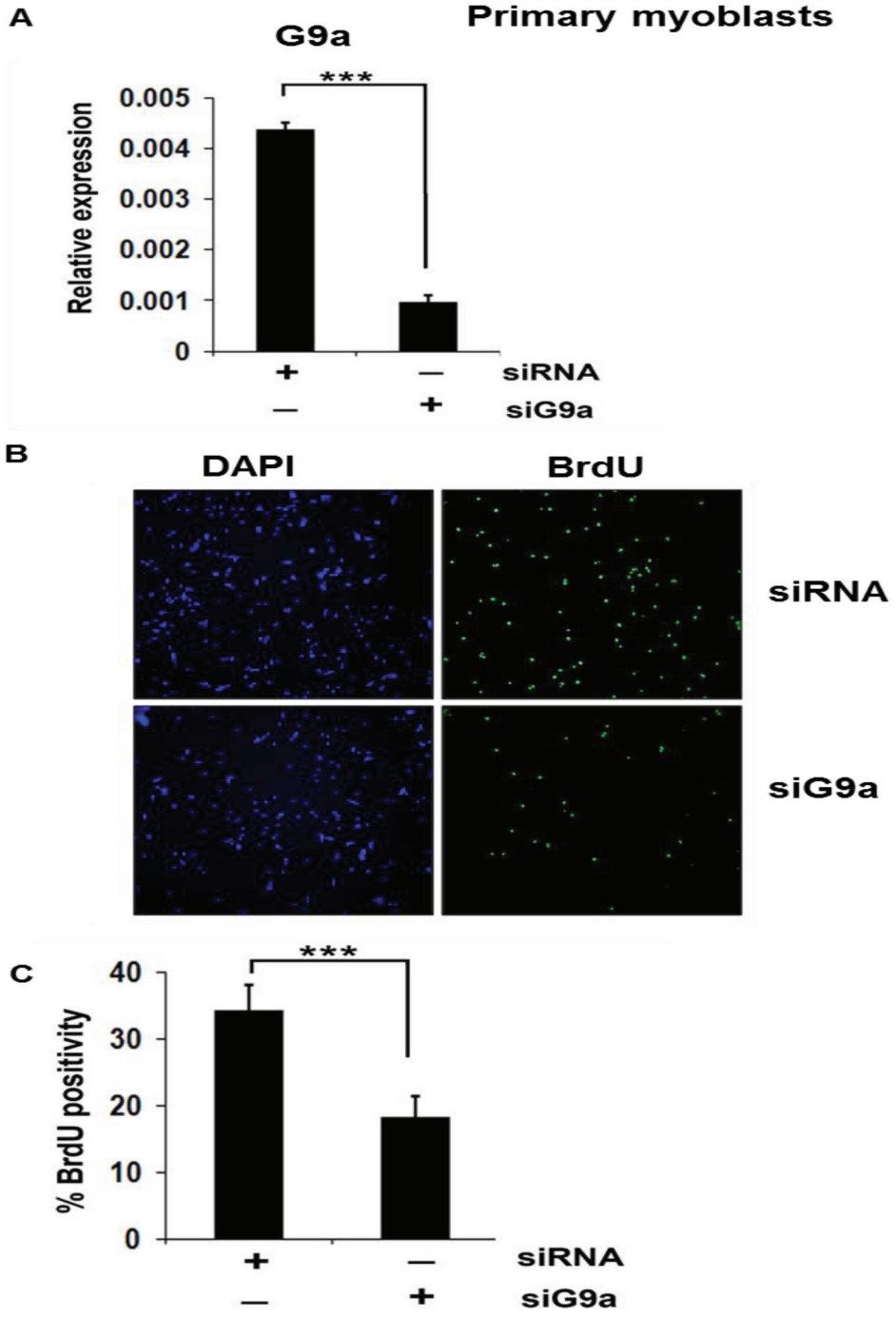


Figure 3.4.2 G9a knockdown reduces proliferation of primary myoblasts. (A) G9a knockdown in mouse primary myoblasts shown by qRT-PCR. (B) BrdU positive cells in control and siG9a primary myoblasts. Nuclei were counter stained with DAPI. (C) Quantification of BrdU positive cells. Error bars indicate mean ± SD. *** indicates *p*-value <0.001

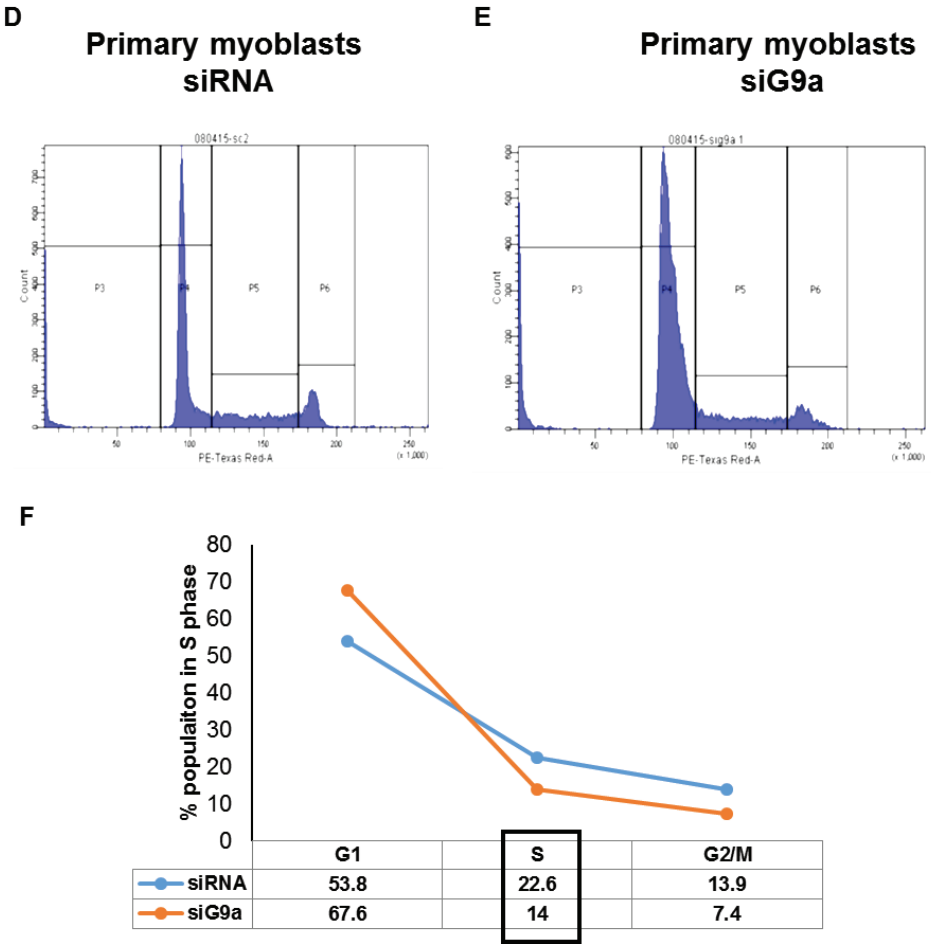


Figure 3.4.2 D,E&F. G9a knockdown reduces proliferation of primary myoblasts. (D & E) Flow cytometry analysis of PI stained control and siG9a primary myoblasts. (F) Graph indicating percentage population in S phase in control and siG9a cells. siG9a primary myoblasts had less S phase cells compared to control cells.

3.4.3 Inhibition of G9a methyltransferase activity reduces proliferation of cells

Since G9a knockdown led to decreased proliferation of cells, we went on to check if methyltransferase activity of G9a is required for its effect on proliferation. We treated both C2C12 cells and primary myoblasts with UNC0638 (a selective inhibitor of G9a methyltransferase activity) and as a control, cells were treated with DMSO (vehicle). Both control and UNC0638 treated cells were pulsed with BrdU for 30min, fixed and stained with anti-BrdU antibody. UNC0638 treated cells incorporated lesser BrdU compared to control, both in C2C12 cells (Fig 3.4.3A) and primary myoblasts (Fig 3.4.3B&C). This indicated that inhibition of G9a methyltransferase activity reduces proliferation of cells and G9a effect on proliferation is partially dependent on its enzymatic activity.

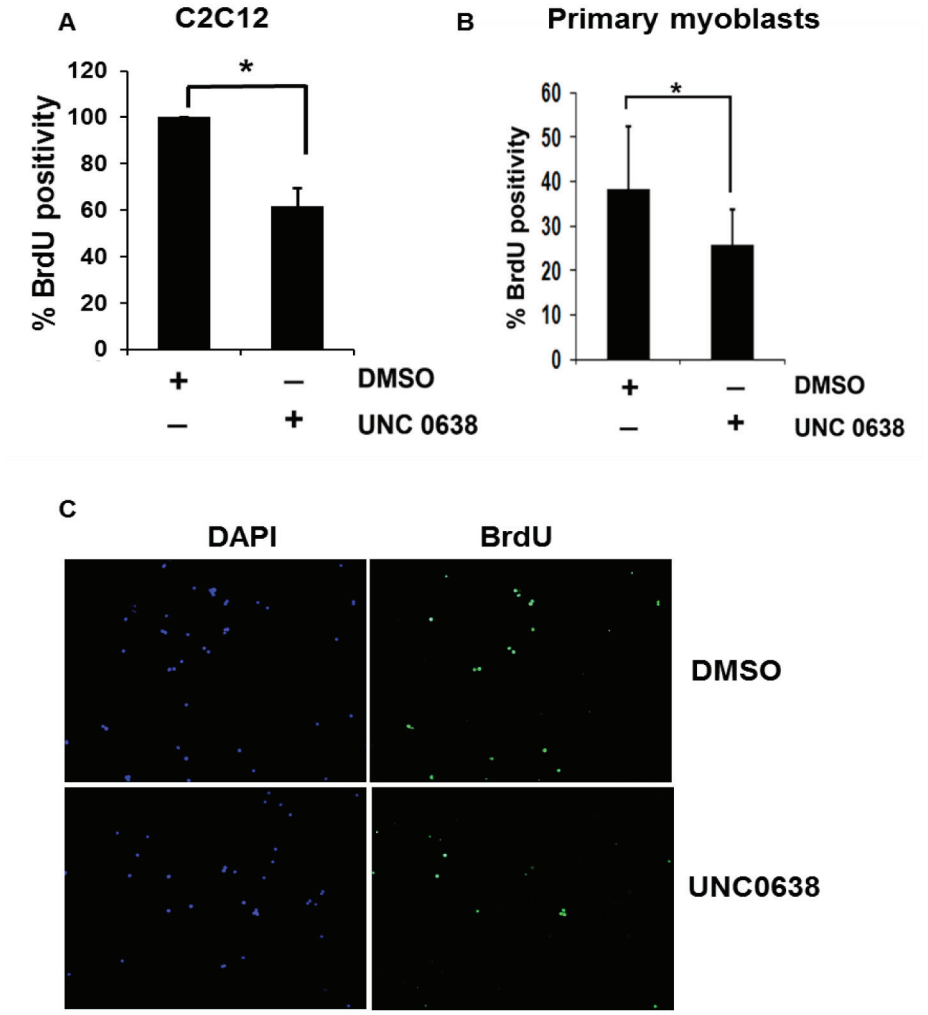


Figure 3.4.3 Inhibition of G9a methyltransferase activity reduces proliferation of cells. (A) BrdU positive cells were counted in DMSO and UNC0638 treated C2C12 cells. UNC0638 (0.25μM) was treated for 24hr. (B) BrdU positive cells were counted in DMSO and UNC0638 treated primary myoblasts. (C) Images of BrdU positive cells in primary myoblasts. Nuclei were counter stained with DAPI. Error bars indicate mean ± SD. * indicates *p*-value <0.05

3.4.4 G9a over expression increases proliferation of myoblasts

Next, we performed gain of function studies to examine the role of G9a in proliferation. Stable G9a over expression was done in C2C12 cells using a retroviral vector (pBabe-G9a cells) and over expression was confirmed by western blot (Fig 3.4.4A). To check the effect of G9a over expression on proliferation of cells, we performed immunofluorescence on BrdU pulsed control (pBabe) and pBabe-G9a cells. Both pBabe and pBabe-G9a cells were pulsed with BrdU for 30min, fixed and stained with BrdU antibody. G9a over expressing cells displayed higher BrdU positivity compared to control cells, indicating higher proliferation of pBabe-G9a cells (Fig 3.4.4A). Further, we performed propidium iodide staining and subjected the cells to flow cytometry analysis. Consistent with the above results, we found higher S phase cells in pBabe-G9a cells compared to controls (Fig3.4.4B & C).

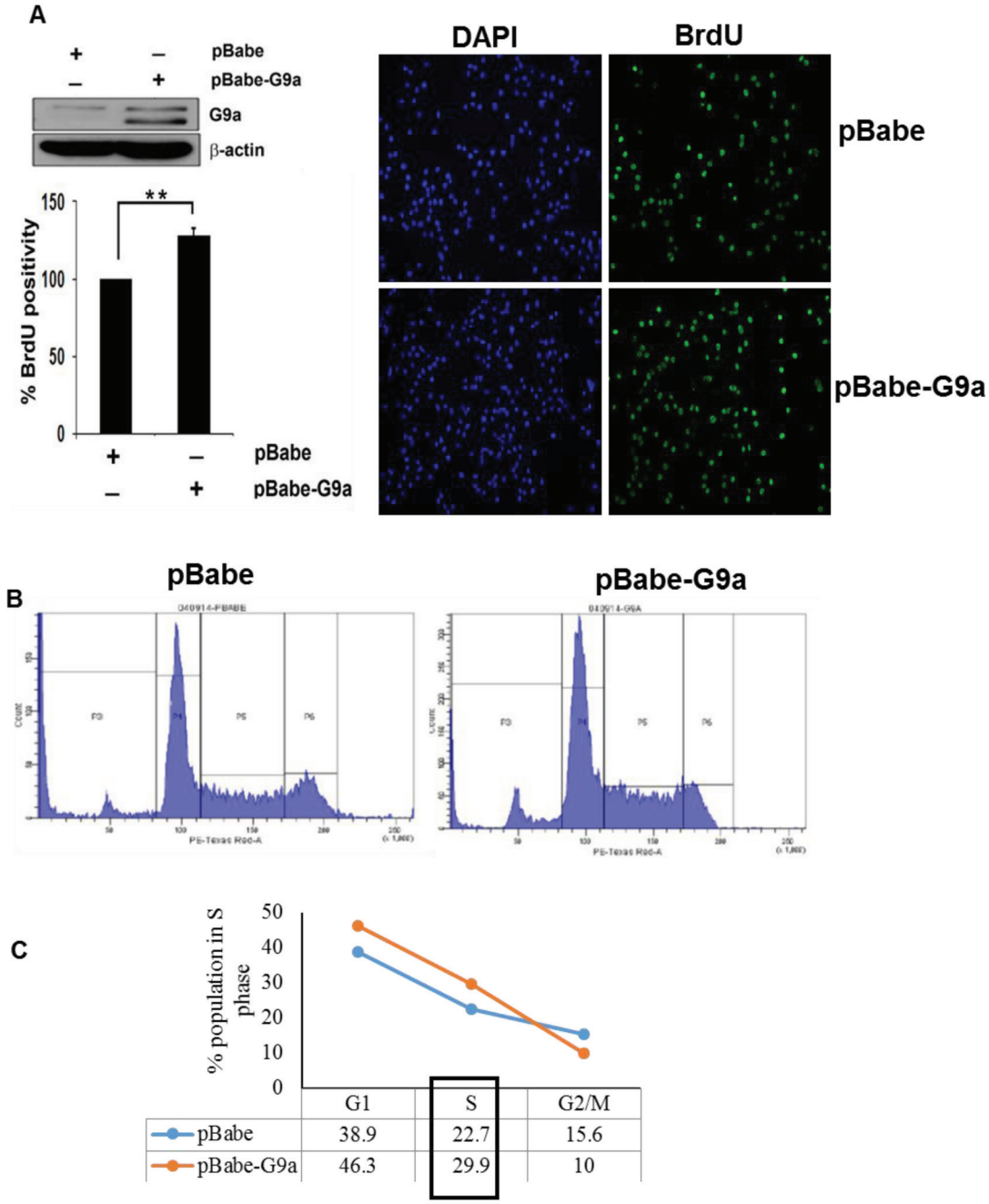


Figure 3.4.4 G9a over expression increases proliferation of cells. (A) Upper panel shows G9a over expression in C2C12 cells by western blot. BrdU positive cells were counted in pBabe and pBabe-G9a cells. (B) Flow cytometry analysis of PI stained control and pBabe-G9a cells. (C) Graph indicating percentage population of S phase cells in pBabe and pBabe-G9a cells. pBabe-G9a cells show higher S phase cells compared to controls. Error bars indicate mean ± SD. ** indicates *p*-values <0.01

3.5 Role of G9a in regulating cell cycle exit during differentiation

Since G9a knockdown reduced proliferation of cells we questioned whether it altered cell cycle exit process during differentiation. Among the G9a target genes involved in cell cycle control from the array, we found a cluster of genes that were up regulated in siG9a cells. Interestingly, MyoD target genes p21 and Rb1, which are involved in irreversible cell cycle exit during differentiation were among these genes (Fig 3.5A). Therefore, we looked into the role of G9a in regulating cell cycle exit of myoblasts during differentiation.

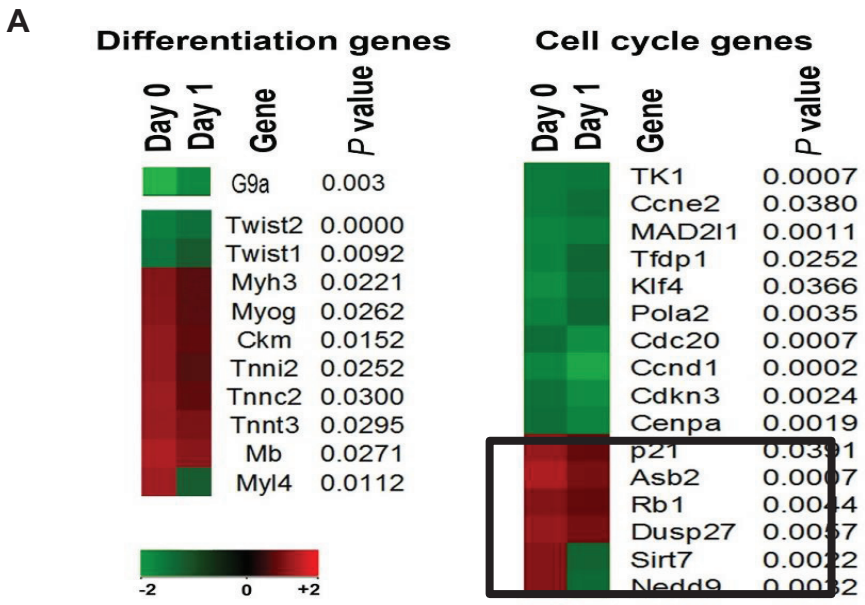


Figure 3.5 Role of G9a in regulating cell cycle exit during differentiation. (A) List of G9a target genes involved in differentiation and cell cycle control. Highlighted box indicates up regulated cell cycle genes.

3.5.1 G9a inhibits p21 and Rb1 expression during myoblasts differentiation

Since p21 and Rb1 which are required for irreversible cell cycle exit of myoblast are upregulated in siG9a cells, we examined if G9a alters cell cycle exit during differentiation. pBabe and pBabe-G9a cells were differentiated for indicated time points and p21 and Rb1 expression was analyzed at RNA and protein level by qRT-PCR and western blotting. G9a over expressing cells showed reduced p21 and Rb1 mRNA levels during differentiation (Fig 3.5.1A&C). Similarly, p21 and Rb1 protein levels were also reduced in G9a over expressing cells during differentiation. Reduced p21 and Rb1 expression could result in inhibition of myoblast differentiation as evidenced by decreased troponin-T expression (Fig 3.5.1B&D). These results suggested that G9a may be involved in regulating irreversible cell cycle exit required for differentiation.

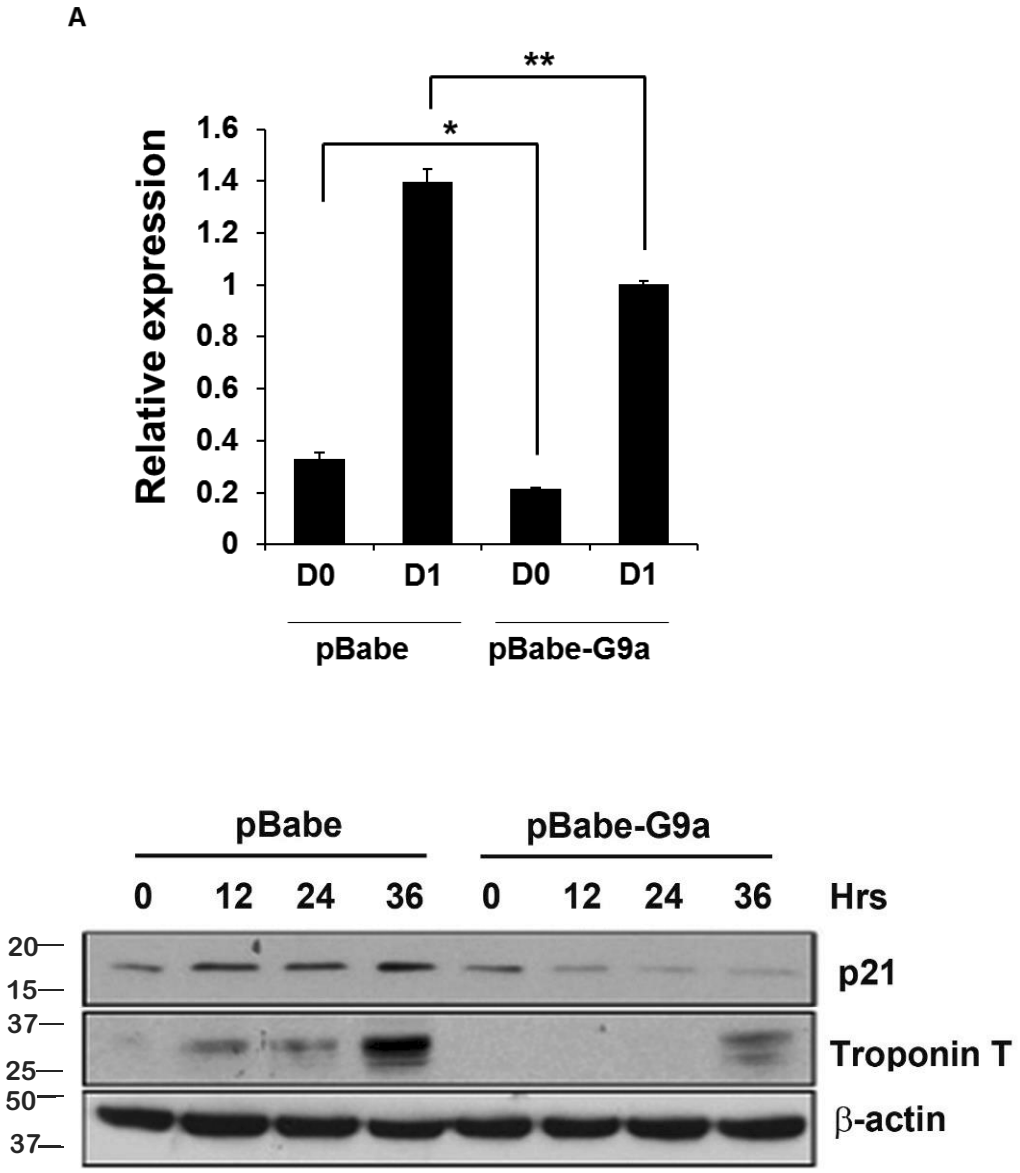


Figure 3.5.1 A&B. G9a inhibits p21 and Rb1 expression during differentiation. (A) p21 mRNA expression was analyzed in control and pBabe-G9a cells during proliferation (D0) and day 1 differentiation (D1) condition. (B) pBabe and pBabe-G9a cells were differentiated for 0 to 36hrs and analyzed for p21 and troponin-T expression by western blot. β-actin was used as internal control. Error bars indicate mean ± SD. ** indicates *p*-value <0.01, * indicates *p*-value <0.05

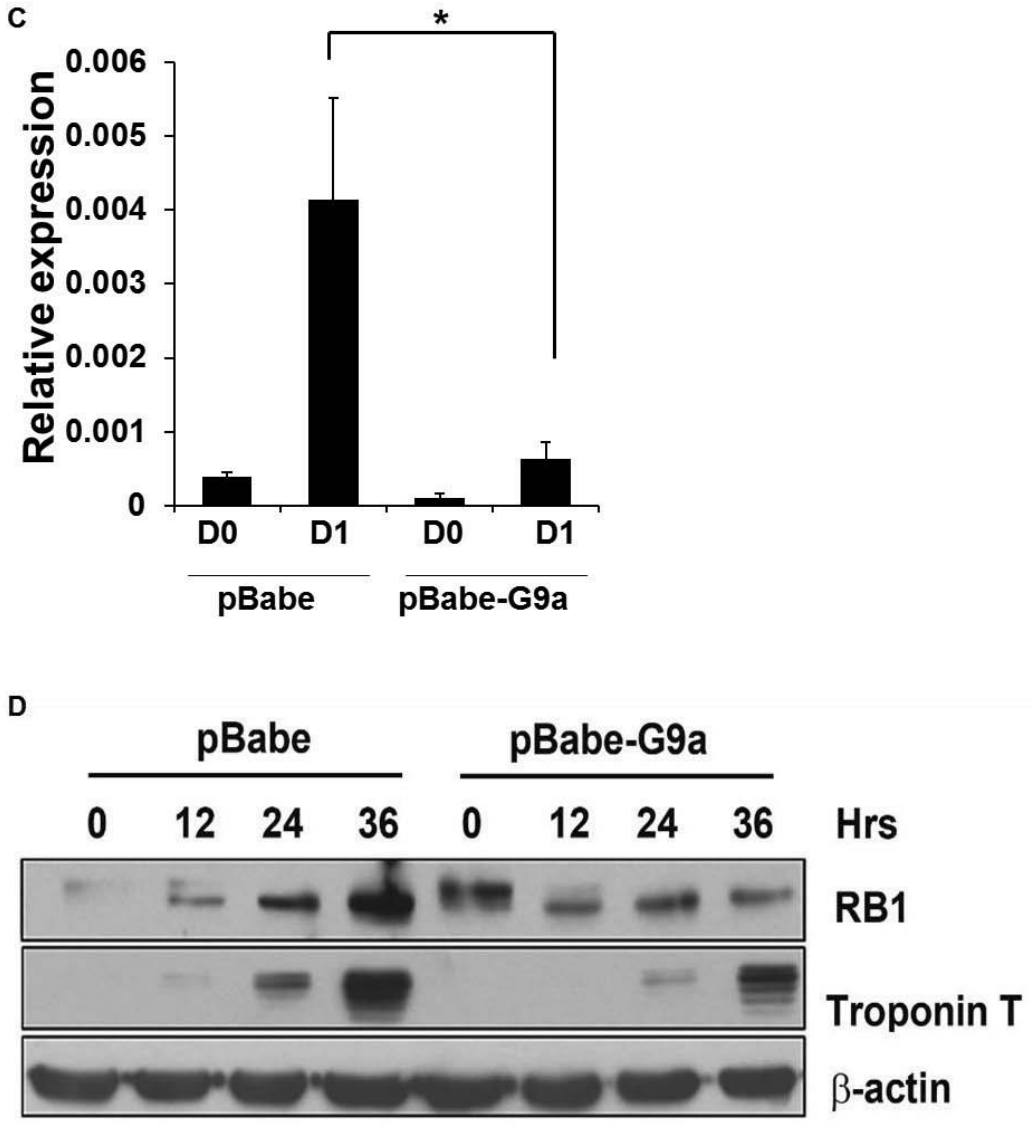


Figure 3.5.1 C&D. G9a inhibits p21 and Rb1 expression during differentiation. (C) Rb1 mRNA expression was analyzed in control and pBabe-G9a cells during proliferation (D0) and day 1 differentiation (D1) condition. (D) pBabe and pBabe-G9a cells were differentiated for 0 to 36hrs and analyzed for Rb1 and troponin-T expression by western blot. β-actin was used as internal control. Error bars indicate mean ± SD. * indicates *p*-value <0.05

3.5.2 G9a inhibition of p21 and Rb1 is dependent on its methyltransferase activity

To confirm if G9a methyltransferase activity is required for cell cycle exit, we blocked endogenous methyltransferase activity of G9a by treating cells with UNC0638. Control cells were treated with DMSO (vehicle). Cells were differentiated for indicated time points. Western blot analysis showed increased p21 and Rb1 expression in UNC0638 treated cells with increased differentiation as evidenced by higher troponin-T expression (Fig 3.5.2 A&B). This indicated that G9a regulation of cell cycle exit is dependent on its methyltransferase activity.

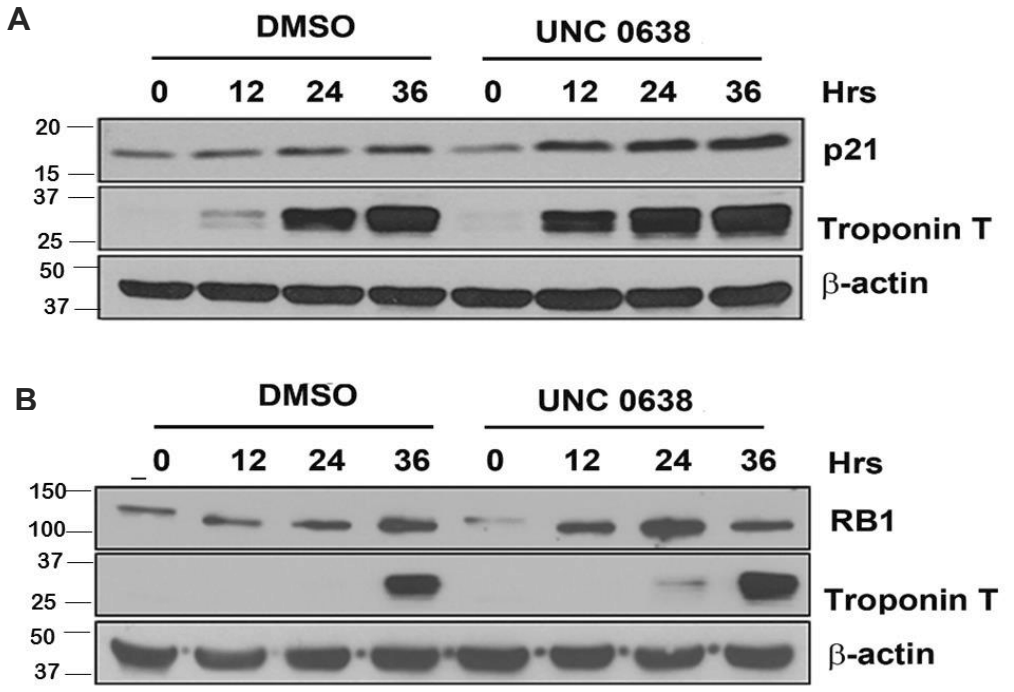


Figure 3.5.2 G9a inhibition of p21 and Rb1 is dependent on its methyltransferase activity. Control (DMSO treated) and UNC0638 treated (0.25uM) C2C12 cells were differentiated for 0 to 36hrs and protein lysates was analyzed for (A) p21, troponin-T or (B) Rb1 and troponin-T by western blot. β-actin was used as internal control. (Data provided by Shilpa Rani Shankar)

3.6 G9a mediates repressive H3K9me2 on MyoD target genes

G9a is generally known to function as a repressor by mediating repressive H3K9me2 on its target genes. Since over-expression of G9a inhibited p21 and Rb1 expression during differentiation, we checked if G9a mediates repression on both these promoters. Chromatin immunoprecipitation assay was performed with pBabe and pBabe-G9a cells under both proliferation (D0) and differentiation (D1) conditions. Cells were fixed using formaldehyde and lysed using SDS buffer. Lysates were subjected to chromatin immunoprecipitation assay using anti-H3K9me2 antibody. A higher level of H3K9me2 enrichment on p21 and Rb1 promoters was seen in pBabe-G9a cells compared to control cells (Fig 3.6.A & B). This result confirms that G9a mediates repressive marks on p21 and Rb1 promoters and inhibits their expression during cell cycle exit.

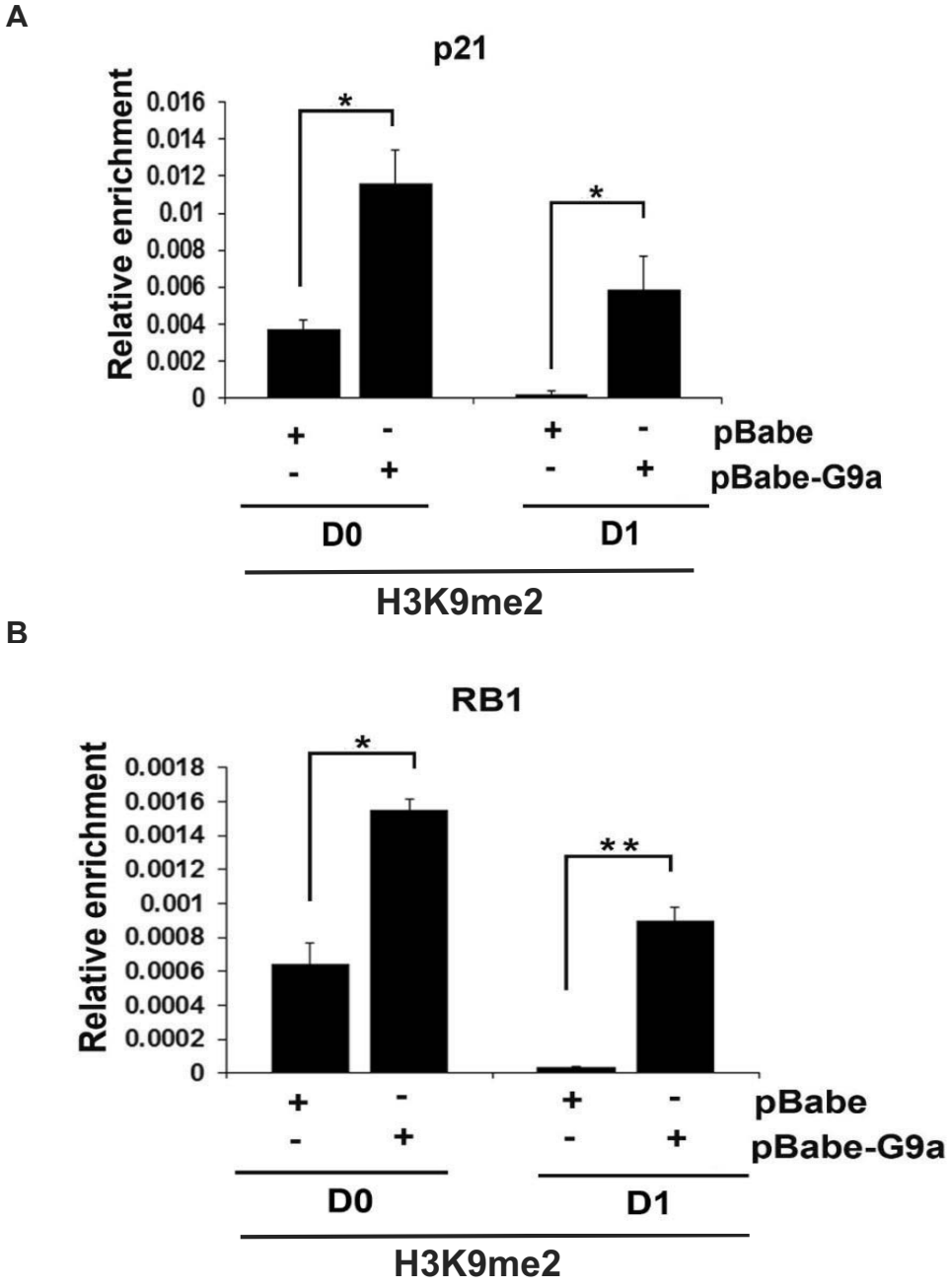


Figure 3.6 G9a mediates repressive H3K9me2 on p21 and Rb1 promoters. (A & B) ChIP assay was performed in pBabe and pBabe-G9a cells under proliferation day 0 (D0) and differentiation day 1 (D1) conditions. H3K9me2 enrichment was analyzed at the p21 and Rb1 promoters. Error bars indicate mean ± SD. ** indicates *p*-value <0.01, * indicates *p*-value <0.05

3.7 p21 and Rb1 rescue differentiation inhibition in G9a over expressing cells

Since G9a over expression inhibited both p21 and Rb1 required for cell cycle exit, we questioned if over expression of p21 or Rb1 could rescue the differentiation defect imposed by G9a. To this end, we performed a rescue experiment by transfecting p21 and Rb1 expression vector in control and pBabe-G9a cells (Fig 3.7A). Cells were differentiated for indicated time points and protein lysate was analyzed by western blot. Consistent with our previous results (Ling et al., 2012b), G9a over expression inhibited differentiation. p21 and Rb1 over expressing cells showed increased expression of differentiation markers compared to pBabe-G9a cells as evidenced by higher myogenin and troponin-T expression (Fig 3.7B).

We also performed immunofluorescence assay with MHC antibody which is expressed in differentiated myotubes. Consistent with the above results we found higher MHC positive myotubes in p21 and Rb1 transfected cells compared to pBabe-G9a cells (Fig 3.7C). Further, myogenic index was higher in p21 and Rb1 transfected cells compared to pBabe-G9a cells (Fig 3.7D).

Overall, all these results confirm the role of G9a in regulating irreversible cell cycle exit during myogenic differentiation. G9a inhibits p21 and Rb1 expression required for cell cycle exit and thus G9a acts as a master regulator of muscle differentiation program by exerting an additional layer of control over myoblast differentiation through regulation of cell cycle exit.

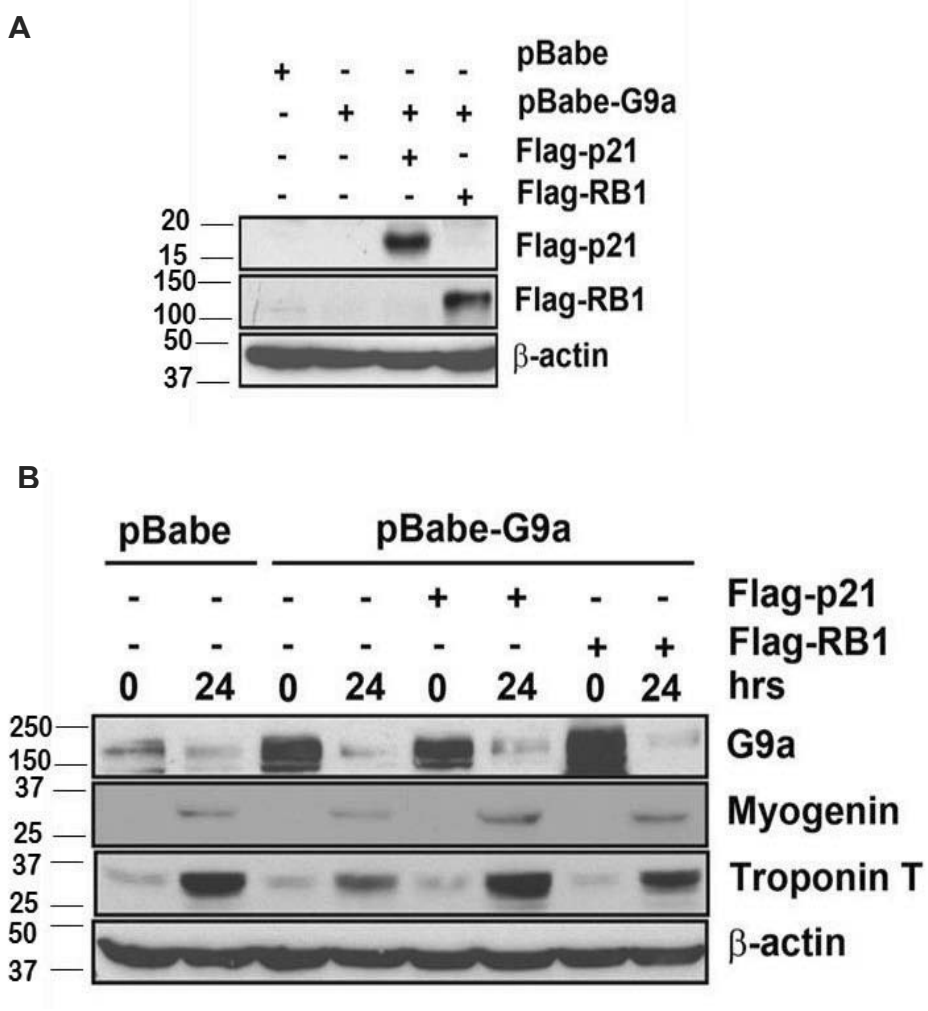


Figure 3.7 A&B p21 and Rb1 rescue differentiation inhibition in G9a over expressing cells (A) Flag-p21 and Flag-Rb1 over expression in control and G9a over expressing cells was checked using western blot. (B) pBabe and pBabe-G9a cells were differentiated for 0 to 24hrs and lysates were analyzed for myogenin and troponin-T by western blot. β-actin was used as internal control.

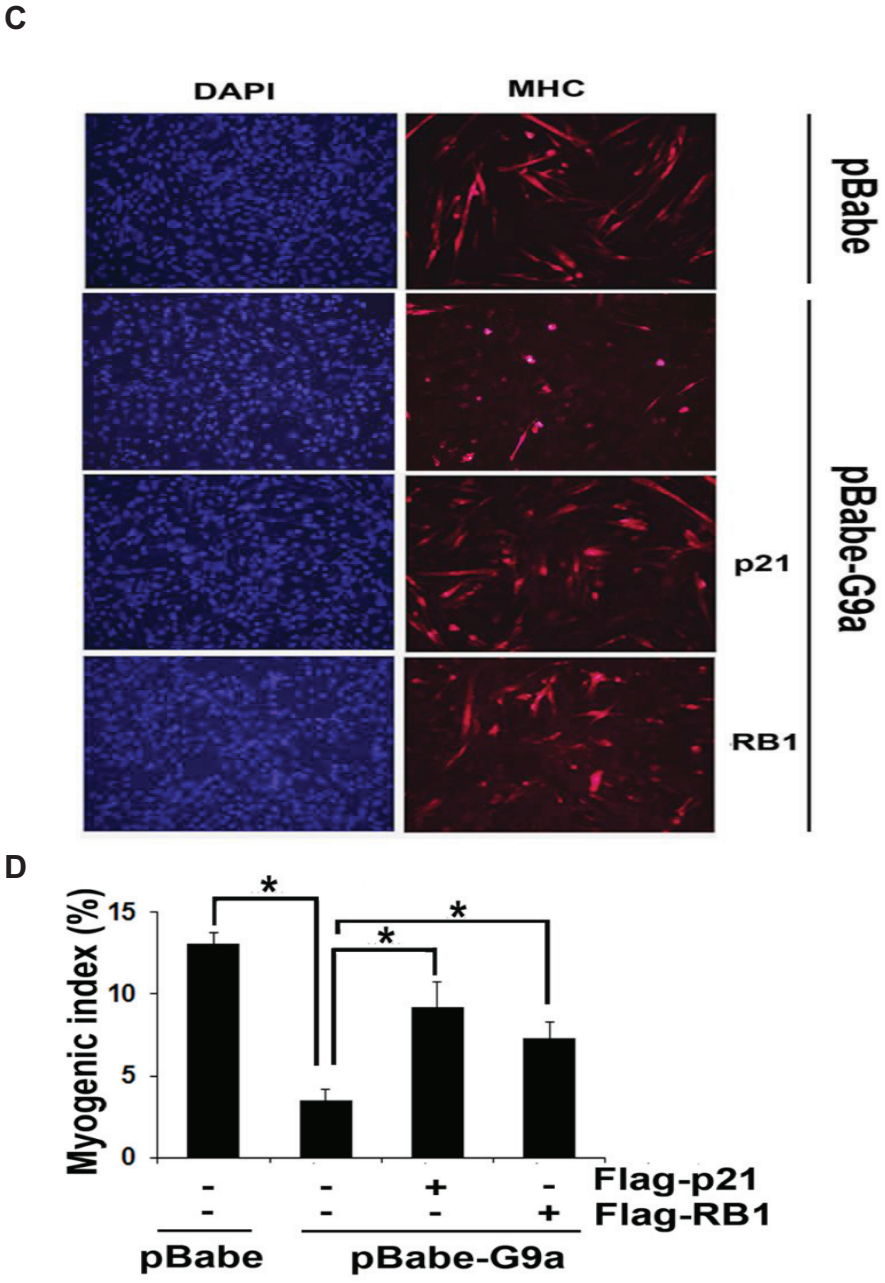


Figure 3.7 C&D p21 and Rb1 rescue differentiation inhibition in G9a overexpressing cells (C) pBabe, pBabe-G9a and pBabe-G9a cells transfected with p21 and Rb1 were differentiated for 0-36hrs and immunostained with myosin heavy chain antibody. (D) Reduction in myogenic index was observed in G9a over expressing cells whereas myogenic differentiation rescue was observed in cells transfected with p21 and Rb1. Error bars indicate mean ± SD. * indicates *p*-value <0.05

3.8 Role of G9a in activating E2F1 target gene expression

From the microarray results, we also found a cluster of cell cycle genes that were significantly down regulated upon G9a knockdown (Fig 3.8). Interestingly, most of these genes are E2F1 targets that are required for proliferation of cells. Although G9a is widely regarded as repressor of gene expression, emerging evidence suggests that G9a could act as an activator of gene expression as well. However, activator function of G9a is known to be independent of its methyltransferase activity (Bittencourt et al., 2012). Since G9a promotes proliferation of cells, we determined whether G9a directly activates expression of these E2F1 target genes, or indirectly regulates their expression.

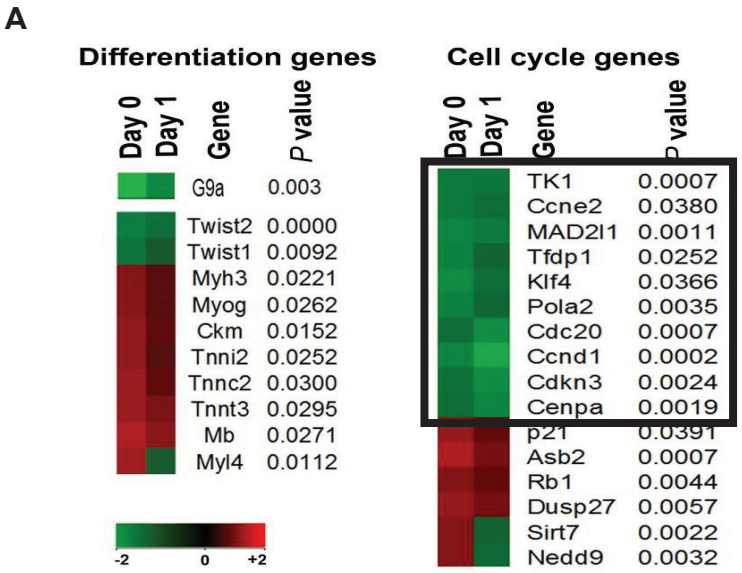


Figure 3.8 Role of G9a in activating E2F1 target gene expression. (A) List of G9a target genes involved in differentiation and cell cycle control. Highlighted box indicates down regulated cell cycle genes.

3.8.1 G9a promotes E2F1 target gene expression

pBabe and pBabe-G9a cells were differentiated for indicated time points and protein lysates were analyzed by western blot for E2F1 target gene expression. We found that the expression of Cyclins such as CyclinD1 and CyclinE were higher in G9a over expressing cells and sustained during differentiation (Fig 3.8.1A&B). Moreover, Rb1 was hyper phosphorylated in G9a over expressing cells which could in turn free E2F1. Thus in G9a over expressing cells, E2F1 would be free to transcribe its target genes such as cyclins required for cell proliferation. This suggested a positive role for G9a in promoting their expression

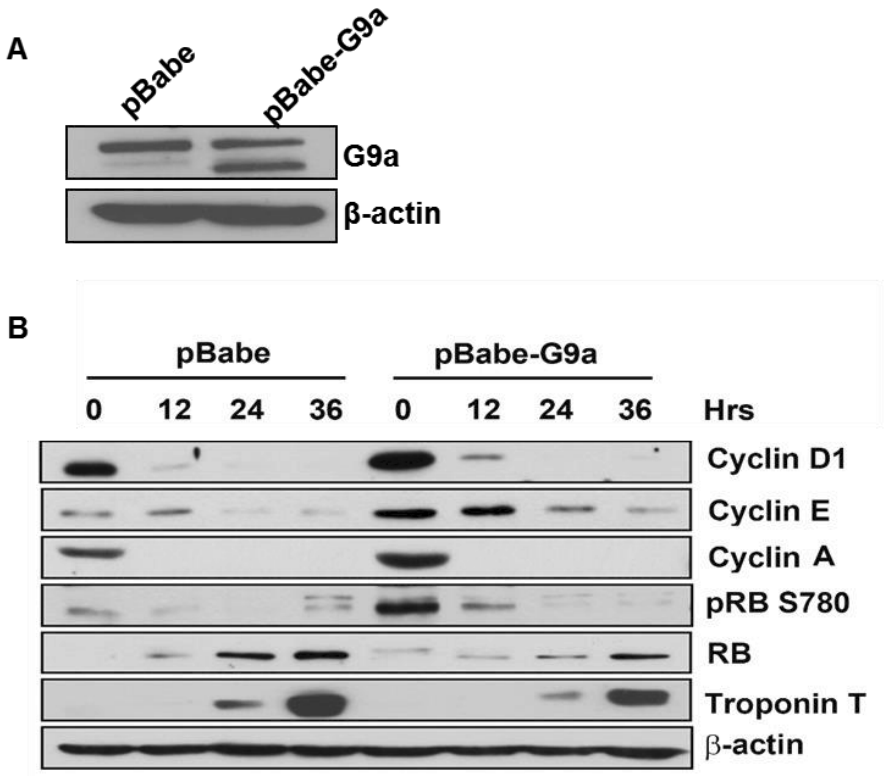


Figure 3.8.1 G9a promote E2F1 target gene expression. (A) G9a over expression shown in C2C12 cells by western blot. (B) Control and G9a over expressing cells were differentiated for 0 to 36hr and protein lysate was analyzed to check Cyclin-D,-E,-A, Rb1 and Troponin-T by western blot. β-actin was used as internal control. (Data provided by Shilpa Rani Shankar)

3.8.2 G9a regulation of E2F1 target genes is independent of its methyltransferase activity

Several studies suggest that activator function of G9a is independent of its methyltransferase activity. Therefore, we inhibited endogenous methyltransferase activity of G9a and checked its effect on E2F1 target gene expression. RNA was isolated from mouse primary myoblasts treated with UNC0638 or DMSO, and was analyzed for E2F1 target gene expression. Interestingly, we found no significant change in the expression of E2F1 target genes upon UNC0638 treatment (Fig 3.8.2A). However, unlike its effect on E2F1 target genes, the expression of MyoD target genes, p21 and Rb1, were upregulated in UNC0638 treated cells (Fig 3.8.2 B).

Next, we checked effect of UNC0638 treatment on CyclinD1 protein expression. C2C12 cells were differentiated with or without UNC0638 for indicated time points and protein lysates were analyzed for CyclinD1 expression. Consistent with the mRNA results, no change in the expression of CyclinD1 upon UNC0638 treatment (Fig 3.8.2C). During proliferation, hyper phosphorylated Rb1 levels remained unchanged, whereas consistent with earlier results, the hypo phosphorylated form of Rb1 increased during differentiation. These results suggest that G9a regulation of E2F1 target genes is independent of its methyltransferase activity.

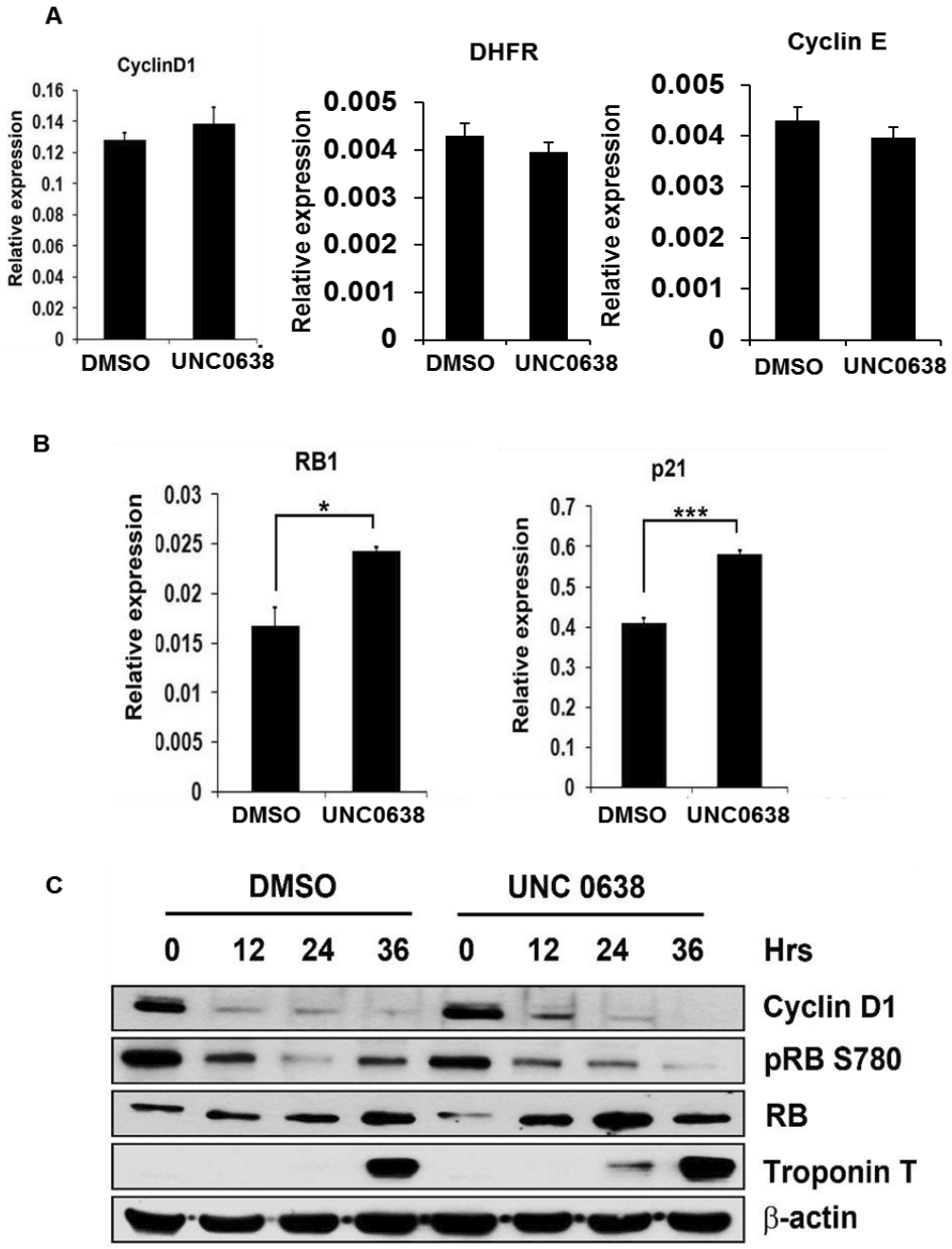
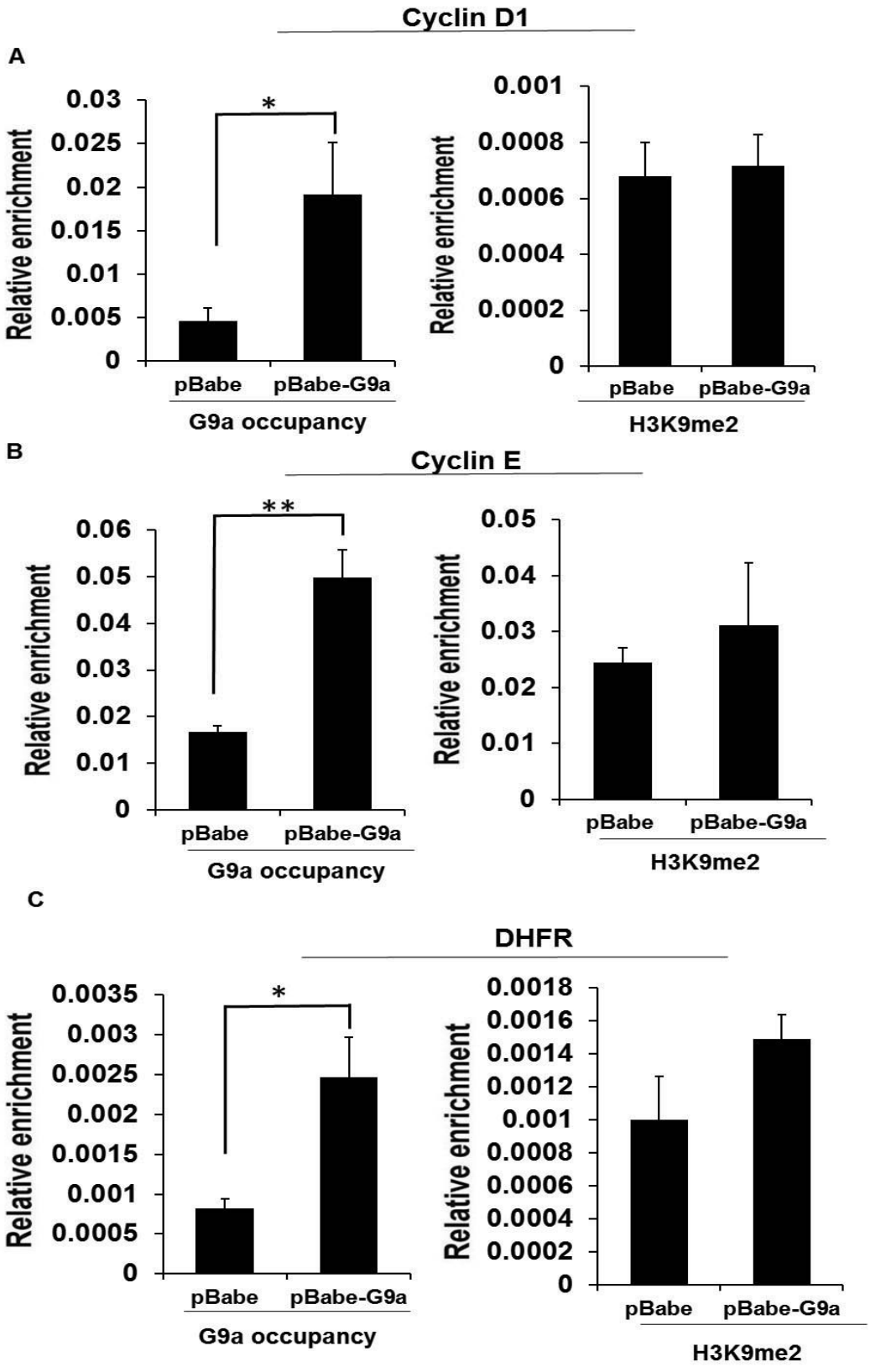


Figure 3.8.2 G9a regulation of E2F1 target genes is independent of its methyltransferase activity (A) CyclinD1,DHFR and CyclinE mRNA expression was analyzed by qRT-PCR in control and UNC0638 treated primary myoblasts. (B) p21 and Rb1 expression analyzed by qRT-PCR (C) C2C12 cells were treated with either DMSO or UNC0638 (0.25um) and differentiated for 0-36hrs. Protein lysate was analyzed for CyclinD1, pRb S780, total Rb and Troponin-T by western blot. β-actin was used as internal control. Error bars indicate mean ± SD. *** indicates *p*-value <0.001, * indicates *p*-value <0.05

3.9 G9a does not mediate H3K9me2 on E2F1 target genes

Since the activator function of G9a does not involve its methyltransferase activity and also the fact that inhibition of G9a activity did not affect E2F1 target gene expression, we looked at the H3K9me2 levels at the promoters of E2F1 target genes in G9a over expressing cells. We performed chromatin immunoprecipitation with pBabe and pBabe-G9a cells and looked at G9a occupancy as well as repressive H3K9me2 on the promoter of CyclinD1, CyclinE (involved in cell cycle control) and DHFR (involved in DNA synthesis). If G9a activates E2F1 target gene expression, we would expect G9a occupancy on these promoters with no corresponding repressive H3K9me2. If G9a indirectly regulates E2F1 target genes, then we do not expect to see G9a occupancy on their promoters. As expected, increased G9a occupancy on E2F1 target gene promoters was seen in pBabe-G9a cells compared to control cells. However, there was no corresponding increase in H3K9me2 enrichment (Fig 3.9A, B & C). In contrast, G9a occupancy was correlated with increased H3K9me2 enrichment on MyoD target gene promoters (Fig 3.9 D & E). These results indicate that G9a may actively promote E2F1 target genes by acting as an activator of gene expression.


Cyclin D1
A
Relative enrichment
0.03
0.025
0.02
0.015
0.01
0.005
0
*
pBabe
pBabe-G9a
G9a occupancy
0.001
0.0008
0.0006
0.0004
0.0002
0
pBabe
pBabe-G9a
H3K9me2
B
Cyclin E
**
0.06
0.05
0.04
0.03
0.02
0.01
0
pBabe
pBabe-G9a
G9a occupancy
0.05
0.04
0.03
0.02
0.01
0
pBabe
pBabe-G9a
H3K9me2
C
DHFR
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
*
pBabe
pBabe-G9a
G9a occupancy
0.0018
0.0016
0.0014
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H3K9me2

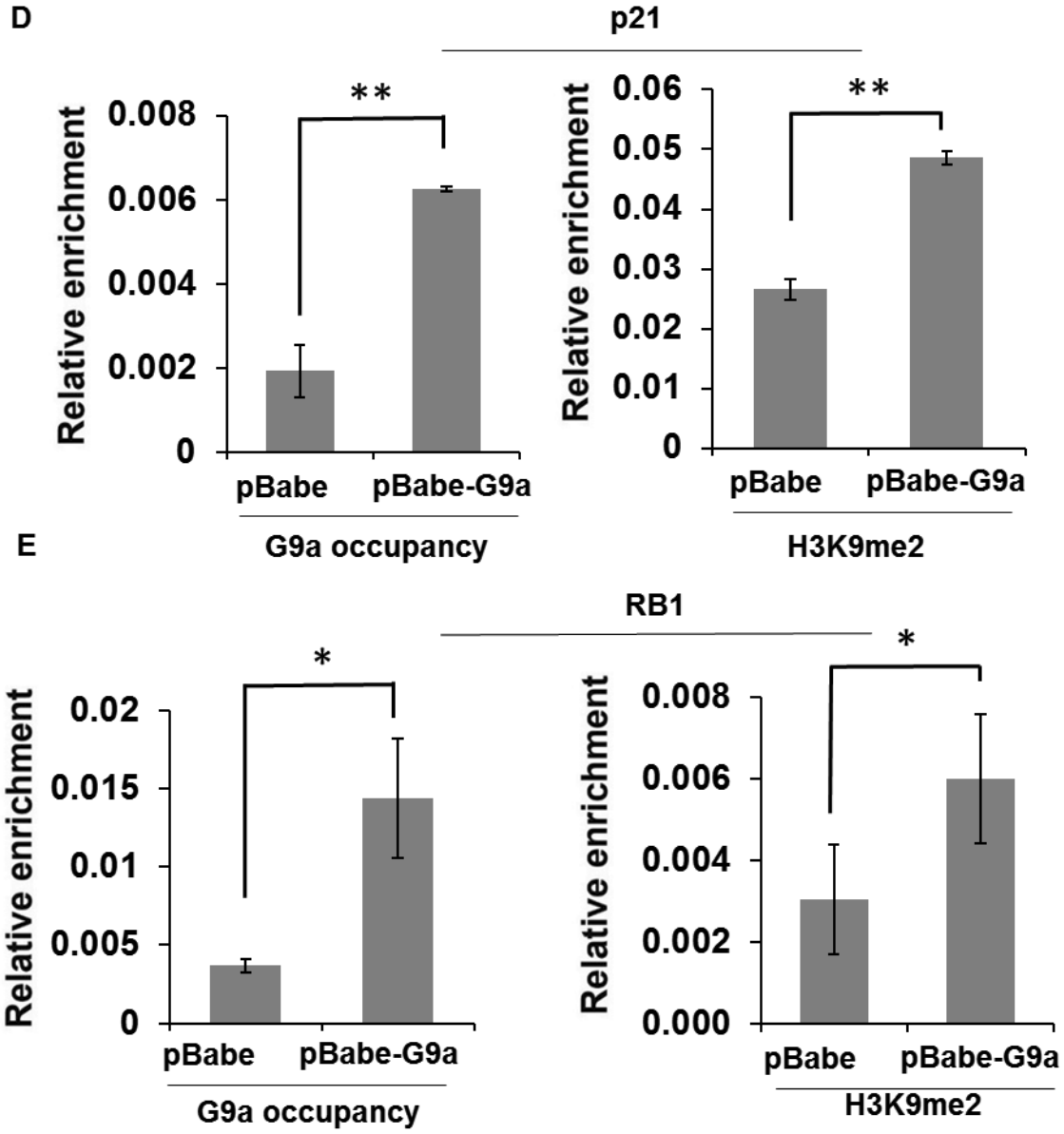


Figure 3.9 G9a does not mediate repressive H3K9me2 on E2F1 target gene promoters. ChIP assay was performed in pBabe and pBabe-G9a cells with G9a and H3K9me2 antibody. (A,B & C) G9a occupancy and corresponding H3K9me2 enrichment was analyzed at the CyclinD1, CyclinE and DHFR promoters, and (D & E) at p21 and Rb1 promoters. Error bars indicate mean ± SD. ** indicates *p*-value <0.01, * indicates *p*-value <0.05

3.10 Inhibition of G9a methyltransferase activity does not alter H3K9me2 on E2F1 target promoters

To further test the methyltransferase independent role of G9a in activating E2F1 target genes, we performed ChIP assays with cells treated with either DMSO or UNC0638 to block endogenous methyltransferase activity of G9a. If G9a functions in a methyltransferase independent manner, we would expect no change in H3K9me2 levels on E2F1 target gene promoters in UNC0638 treated cells, whereas a reduction in repression should be apparent on MyoD target gene promoters.

Consistent with our hypothesis, UNC0638 treatment did not significantly alter H3K9me2 levels on CyclinD1 and DHFR promoters (Fig 3.10A & B). However G9a repression on MyoD target genes p21 and Rb1 was significantly reduced upon UNC0638. This result indicates that G9a functions in methyltransferase independent manner while regulating E2F1 target genes.

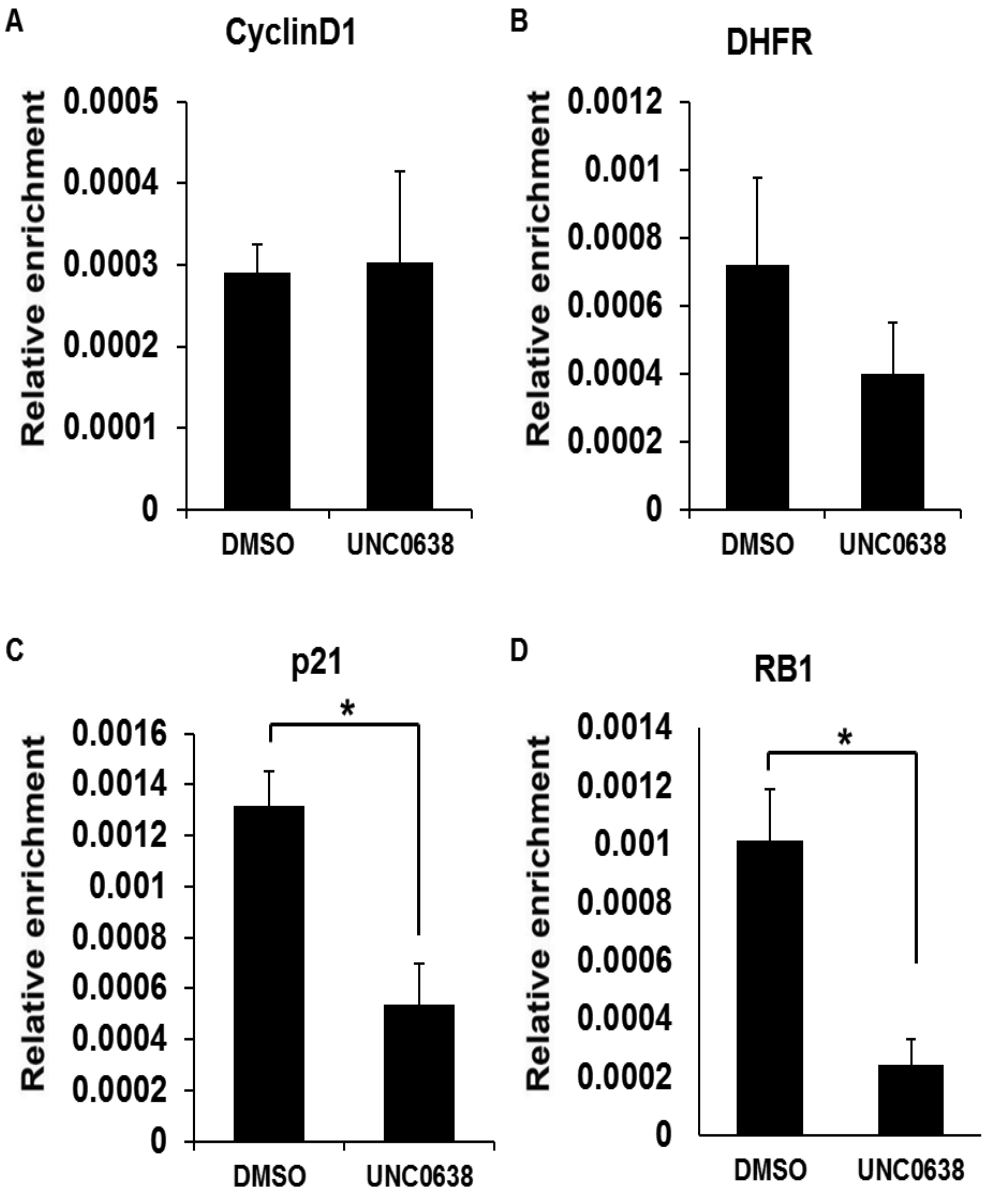
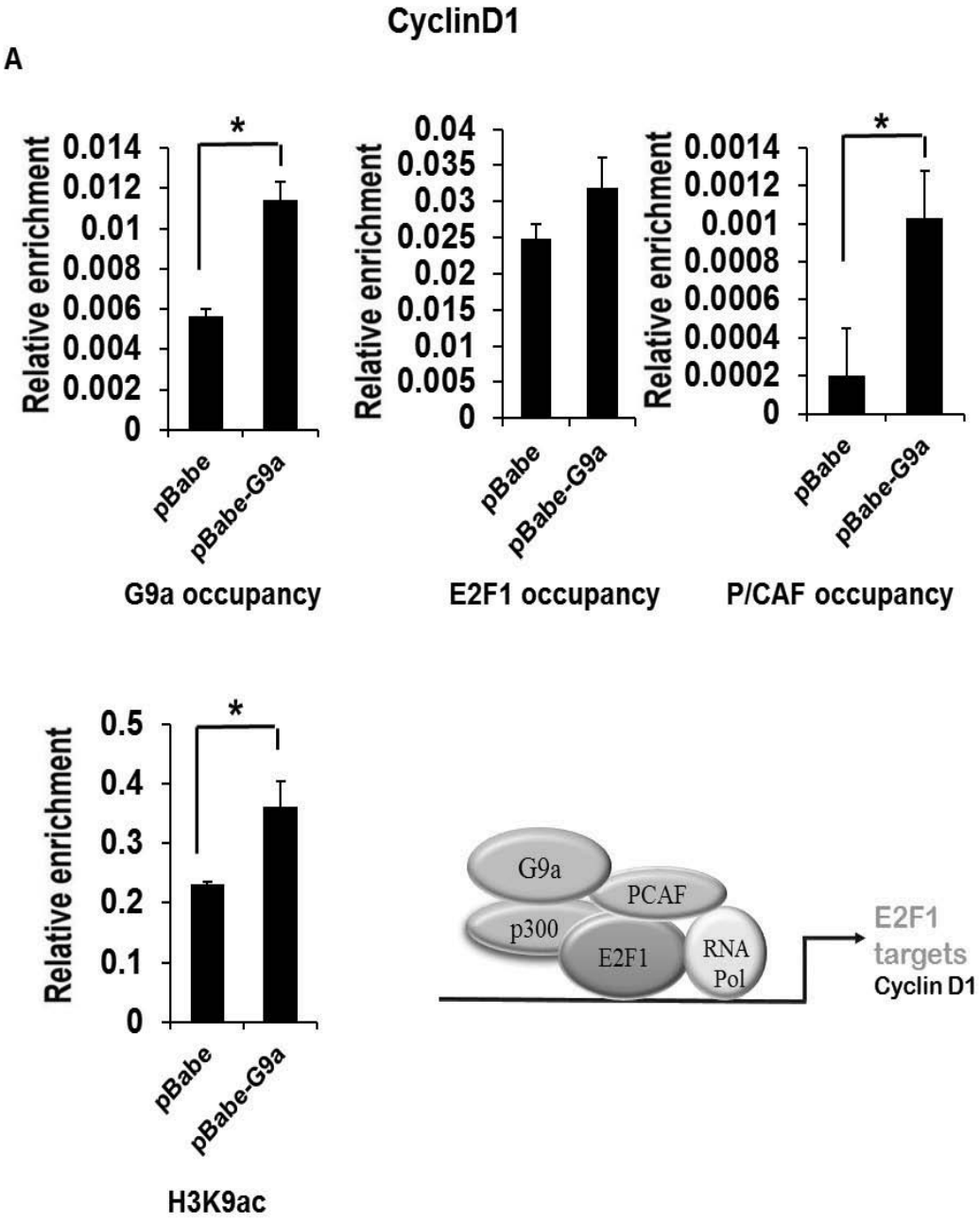


Figure 3.10 Inhibition of G9a methyltransferase activity does not alter H3K9me2 on E2F1 target promoters. ChIP assays were performed in C2C12 cells treated with either DMSO or UNC0638 with H3K9me2 antibody. (A & B) No significant changes in H3K9me2 enrichment on CyclinD1 and DHFR promoters (C & D) Significant down regulation of H3K9me2 on both the p21 and Rb1 promoters. Error bars indicate mean ± SD. * indicates *p*-value <0.05 (Data provided by Ow Jin Rong)

3.11 G9a occupancy along with E2F1 and P/CAF correlated with increased H3K9ac on E2F1 target promoters

Next, we examined if G9a occupancy on E2F1 target promoters is associated with H3K9ac (activation mark). To this end, we performed chromatin immunoprecipitation assay with G9a, E2F1 and P/CAF antibodies in pBabe and pBabe-G9a cells. Consistent with our earlier results we found G9a occupancy on E2F1 target genes. Furthermore, E2F1 and P/CAF occupancy was also evident on E2F1 target genes (Fig 3.11 A&B). Interestingly, G9a occupancy along with P/CAF correlated with acetylation status of E2F1 target genes. We found higher H3K9 acetylation, a mark of transcriptional activation, on E2F1 target promoters in G9a over expressing cells compared to control cells. Therefore, our results suggested that in myoblasts, G9a could be in complex with P/CAF on E2F1 target genes and help in promoting their expression


CyclinD1
A
Relative enrichment
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
*
pBabe
pBabe-G9a
G9a occupancy
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
E2F1 occupancy
0.0014
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
P/CAF occupancy
0.5
0.4
0.3
0.2
0.1
H3K9ac
G9a
PCAF
p300
E2F1
RNA Pol
E2F1 targets
Cyclin D1

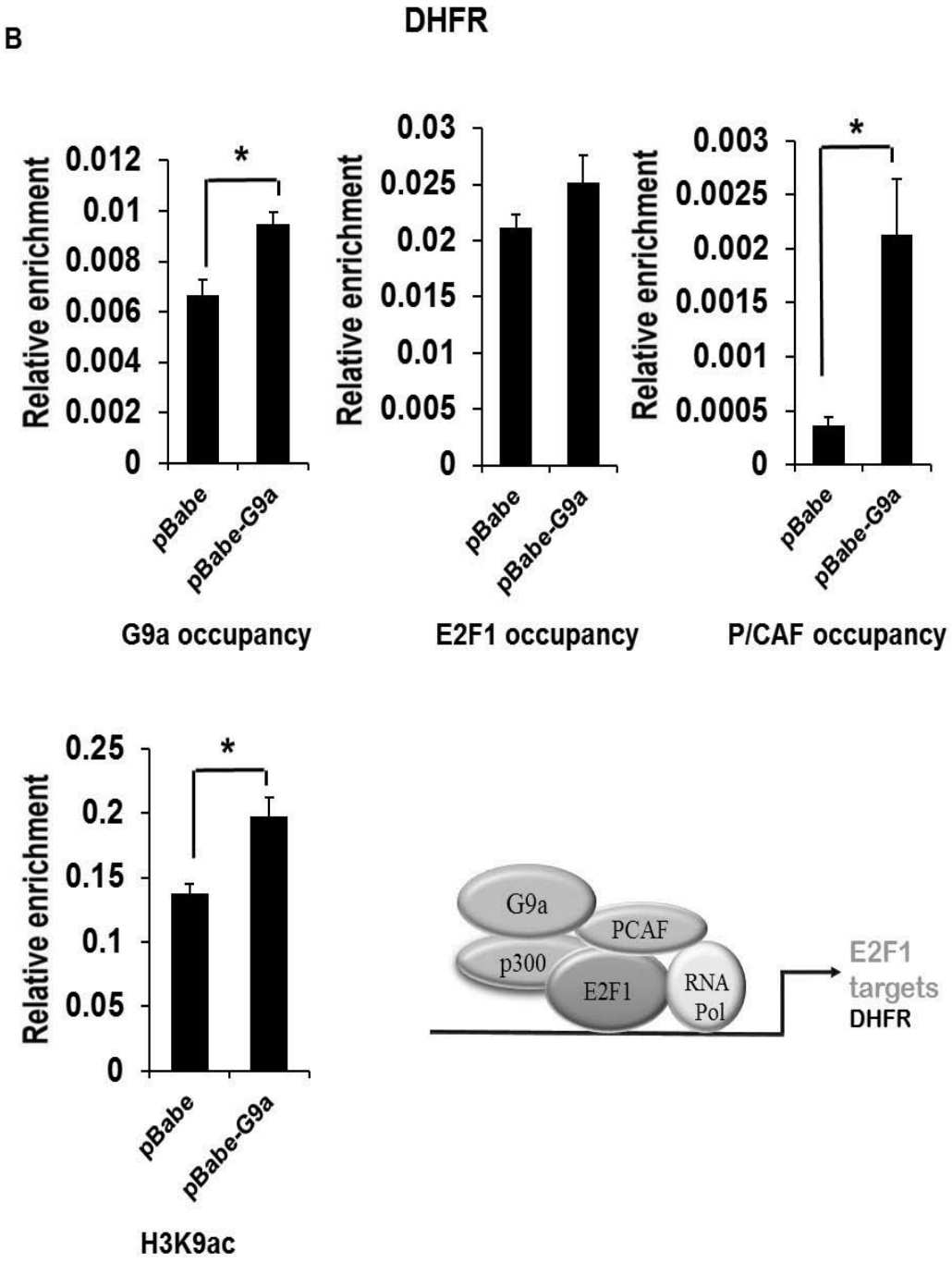


Figure 3.11 G9a occupancy along with E2F1 correlated with increased H3K9ac on E2F1 target promoters. ChIP assay was performed in control and G9a over expressing cells with anti G9a, anti E2F1, anti P/CAF and anti H3K9ac antibodies. (A & B) H3K9ac enrichment was apparent along with G9a, E2F1 and P/CAF occupancy on both CyclinD1 and DHFR promoters. Error bars indicate mean ± SD. * indicates *p*-value <0.05

3.12 G9a interacting partners in myoblasts

In myoblasts, MyoD dependent genes are silenced whereas E2F1 dependent genes are activated. MyoD is known to be in association with repressor complex including Suv39h1, HDAC1 and G9a on muscle promoters leading to the block in differentiation. In contrast, E2F1 is associated with activator complex including p300 and PCAF on cell cycle gene promoters (Fig 1.7).

3.12.1 G9a complexes with E2F1 and P/CAF in myoblasts

Since we found G9a occupancy on E2F1 target gene promoters, we would expect G9a to be in complex along with E2F1 and P/CAF in proliferating myoblasts. To examine this, we performed endogenous co-immunoprecipitation assay using nuclear extracts from proliferating myoblasts. Co-immunoprecipitation was performed using anti-G9a antibody to check for its interaction with E2F1 and P/CAF. Rabbit IgG pulldown was used as negative control. We found G9a interaction with E2F1 and P/CAF (Fig 3.12.1A). Alternatively, we over expressed Flag-G9a in C2C12 cells and performed Co-IP with anti-Flag antibody. From the western blot analysis, we found G9a interaction with E2F1 and P/CAF (Fig 3.12.1B). Similarly, we also checked if G9a is present in a repressor complex with MyoD. We performed endogenous Co-IP with MyoD antibody. Consistent with the earlier studies, we found MyoD interaction with G9a and HDAC (Fig 3.12.1C).

Taken together our results suggest that G9a is in distinct complex with E2F1 and MyoD in myoblasts and this could potentially explain G9a role as both an activator and repressor of gene expression.

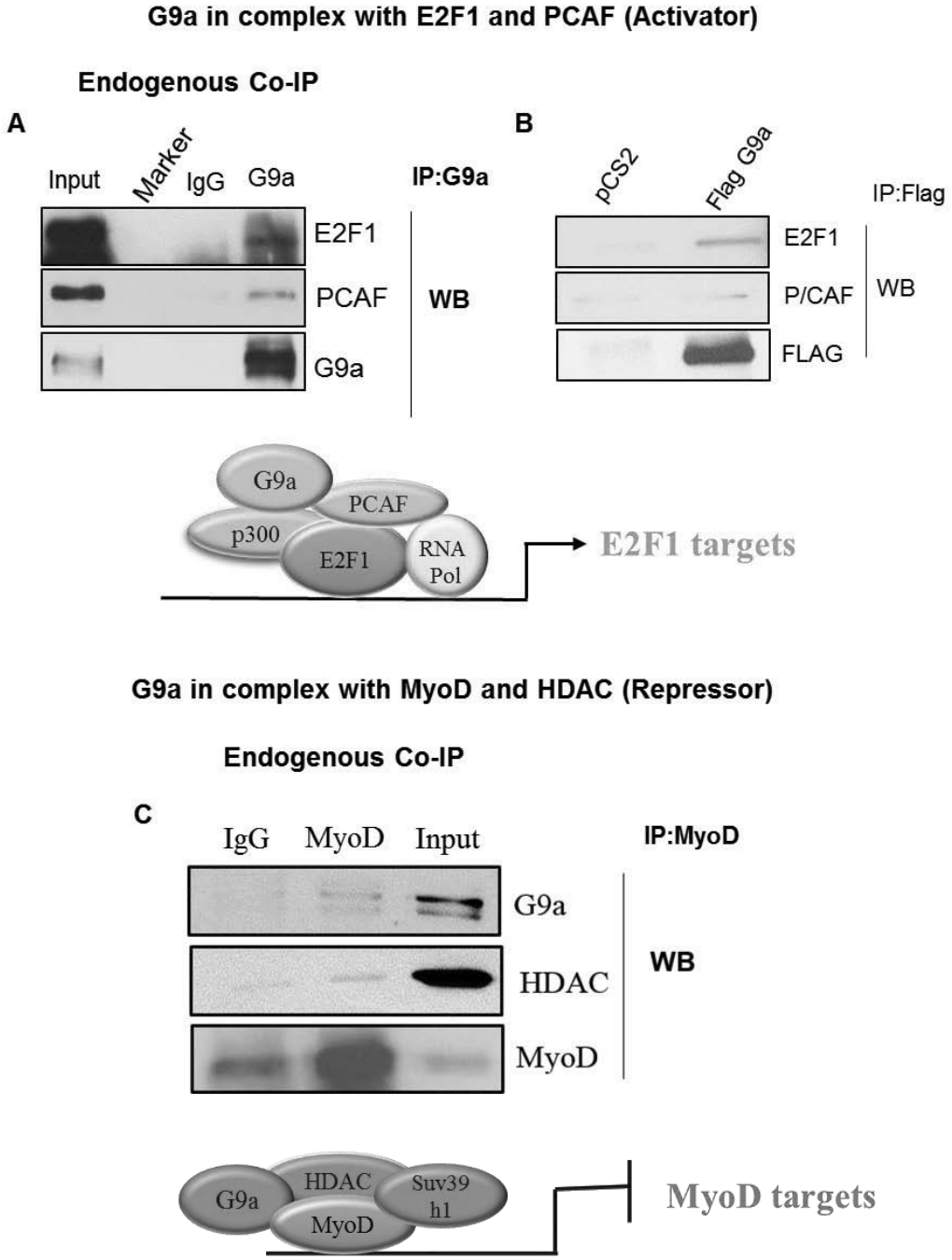


Figure 3.12.1 G9a complex with E2F1 and P/CAF in myoblasts (A) G9a pulldown performed with anti-G9a antibody in C2C12 nuclear extracts. IgG pull down was performed as control. Interaction with E2F1 and P/CAF was confirmed by western blot. (B) Pull down was performed with Flag beads using lysate from Flag-G9a over expressed C2C12 cells and interaction with E2F1 and P/CAF was checked by western blot. (C) Endogenous MyoD pull down was performed and interaction with HDAC and G9a was checked using western blot.

3.13 G9a over expressing cells display higher CyclinD1 promoter activity

We found G9a in complex with E2F1 and P/CAF and could possibly act as activator of E2F1 target genes. Moreover, from our earlier results G9a overexpressing cells displayed hyper phosphorylated Rb1, which results in free E2F1. Therefore, we questioned if G9a over expressing cells display enhanced E2F1 activity. To test this, we performed luciferase reporter assay with cyclinD1 reporter construct. We transfected cyclinD1 luciferase reporter construct into control pBabe and pBabe-G9a cells. We found that pBabe-G9a cells displayed higher cyclinD1 promoter activity compared to control cells (Fig 3.13A). This indicated that G9a over expressing cells display higher E2F1 activity.

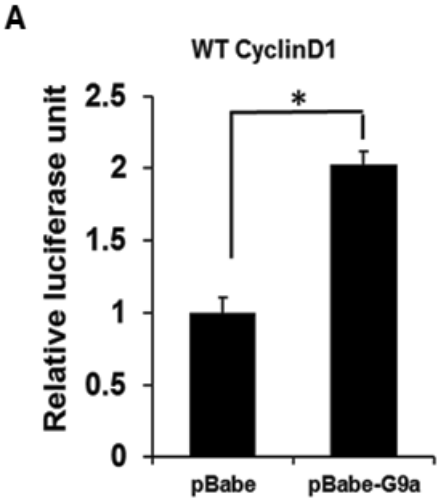


Figure 3.13A G9a overexpressing cells display higher cyclinD1 promoter activity and is E2F1 dependent. (A) Control pBabe and pBabe-G9a cells were transfected with pD1luc reporter construct. Luciferase activity was measured using dual luciferase reporter system. (Data provided by Ow Jin Rong)

Next, in order to find out if the higher cyclinD1 promoter activity in pBabe-G9a cells is E2F1 dependent, we transfected control and G9a over expressing cells with either WT cyclinD1 reporter or with point mutation at E2F1 binding site (Fig 3.13B). Interestingly, we found no significant changes in the activity of mutant cyclinD1 reporter (Fig 3.13B). However, consistent with our earlier results WT CyclinD1 promoter activity was higher in pBabe-G9a cells. This confirms that G9a over expressing cells display higher E2F1 activity and this is due to free E2F1 in pBabe-G9a cells.

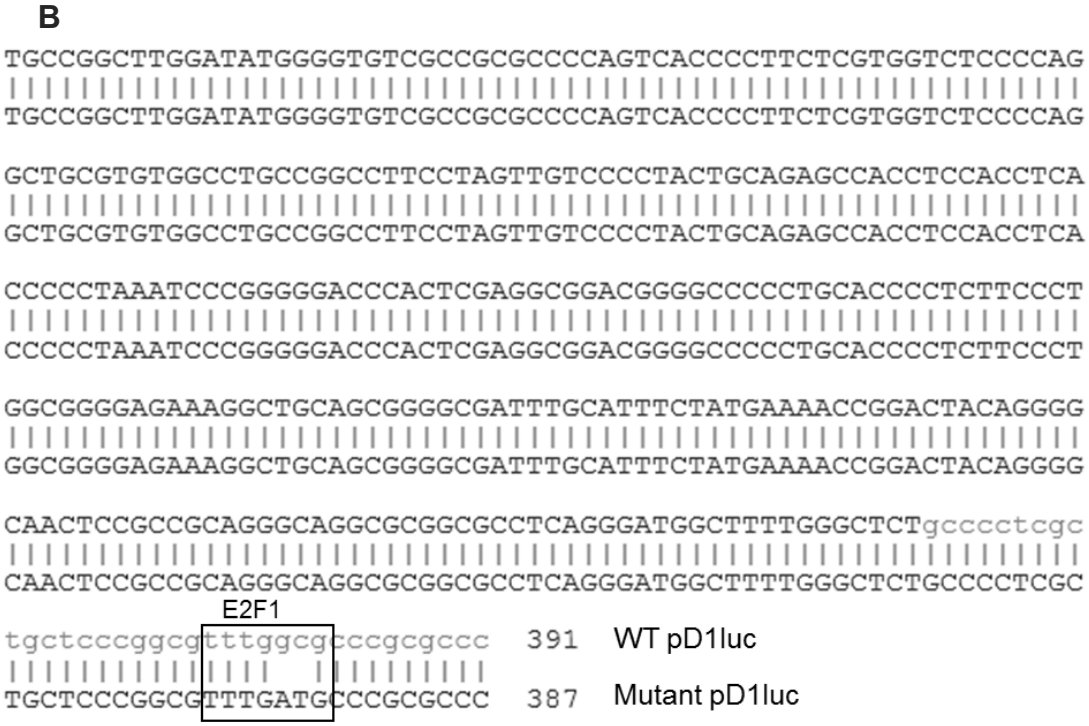


Figure 3.13B G9a overexpressing cells display higher cyclinD1 promoter activity and is E2F1 dependent (B) Sequencing result from wild type pD1luc and point mutant at E2F1 binding site constructs. E2F1 binding site is shown in box with mutation. (Point mutant construct was generated by Wang Yaju)

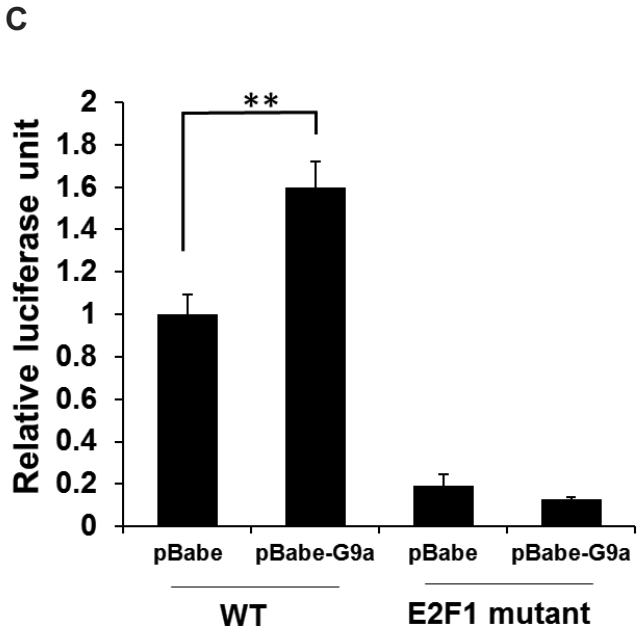


Figure 3.13C G9a overexpressing cells display higher cyclinD1 promoter activity and is E2F1 dependent (C) Control pBabe and pBabe-G9a cells were transfected with either WT or point mutant (at E2F1 binding site) pD1luc reporter construct. Luciferase activity was measured using dual luciferase reporter system. (Data provided by Ow Jin Rong)

3.14 Myoblasts from G9a knockout mice display reduced proliferation and decreased expression of proliferation genes

Finally, to validate the physiological *in vivo* relevance of our findings, we isolated myoblasts from G9a knockout mice. Since G9a knockout is embryonically lethal (Tachibana et al., 2002), G9a was knocked out specifically in muscle satellite cells of $G9a^{fl/fl}$ $Pax7^{Cre\ ERT2\ /+}$ mice using tamoxifen. As a control same genotype mice were injected with corn oil (vehicle). Primary myoblasts was isolated and cultured from control (vehicle) and tamoxifen treated mice (Fig 3.14A). Pure myoblasts population were obtained by pre-plating technique and verified using PAX7 staining. Almost 97% of cells were positive for PAX7 (Fig 3.14B). Next, G9a knockout was confirmed by performing real time PCR using RNA isolated from control and G9a null myoblasts (Fig 3.14C). These cells were used for further experiments. It is important to note that significant amount of cell death was observed in G9a null myoblasts. This observation is consistent with the other reports indicating apoptosis in $G9a^{-/-}$ embryonic stem cells (Tachibana et al., 2002)

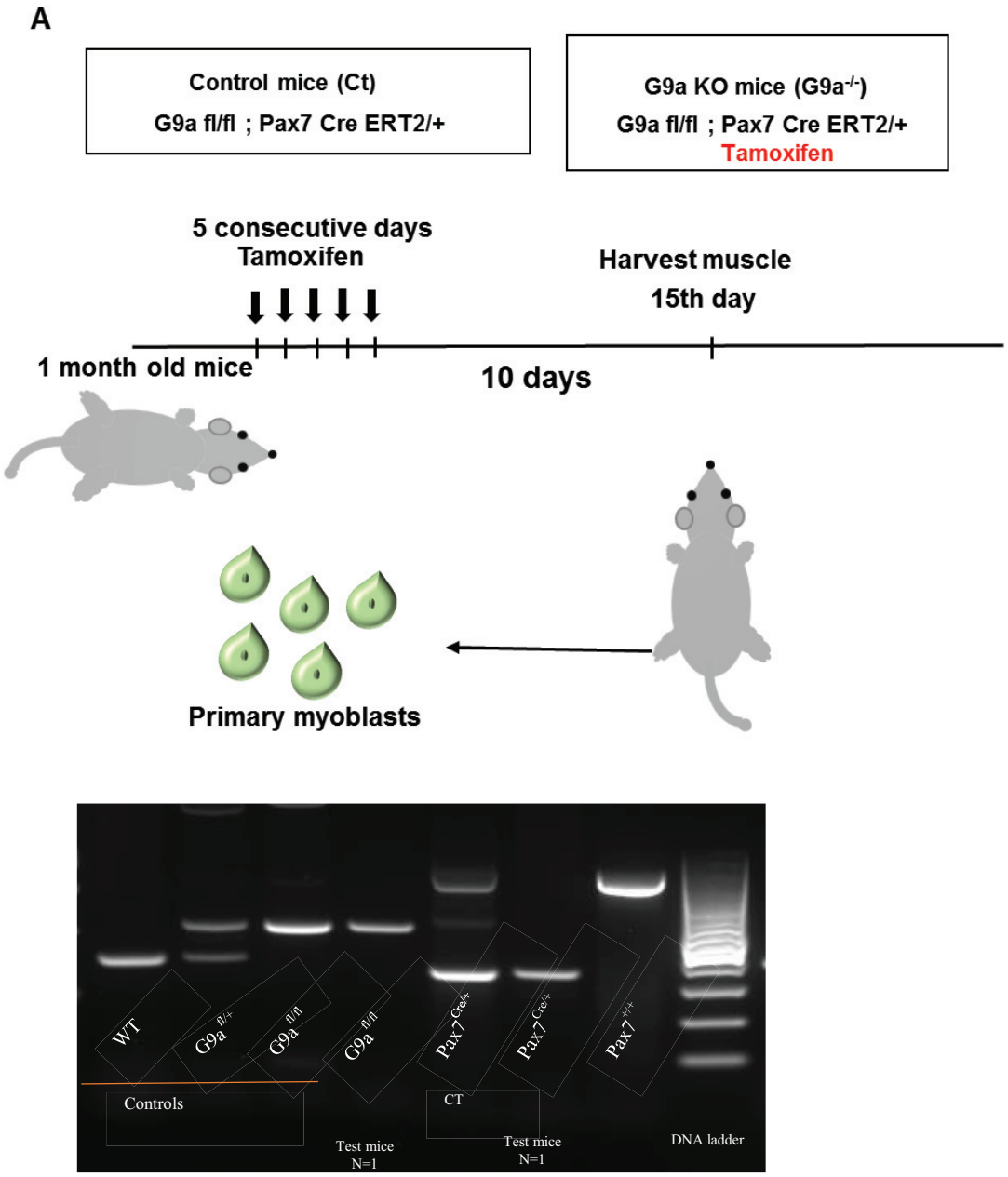


Figure 3.14A Myoblasts from G9a knockout mice display reduced proliferation and decreased expression of proliferation genes (A) Schematic showing tamoxifen injection regime. Mice with genotypes $G9a^{fl/fl}$; $Pax7^{Cre\ ERT2/+}$ were injected with tamoxifen (1mg/10g of mice) for 5 consecutive days and muscles were harvested on 15th day. Control (ct) mice with vehicle control (corn oil). Myoblasts were isolated from both control and tamoxifen injected mice. Below panel shows genotype PCR results for G9a conditional knockout mice. Mice (Test mice N=1) with genotype $G9a^{fl/fl}$ $Pax7^{cre/+}$were taken for the experiments. First 3 lanes are positive controls are shown for $G9a^{fl/fl}$ and fifth lane for $Pax7^{Cre}$ are shown.

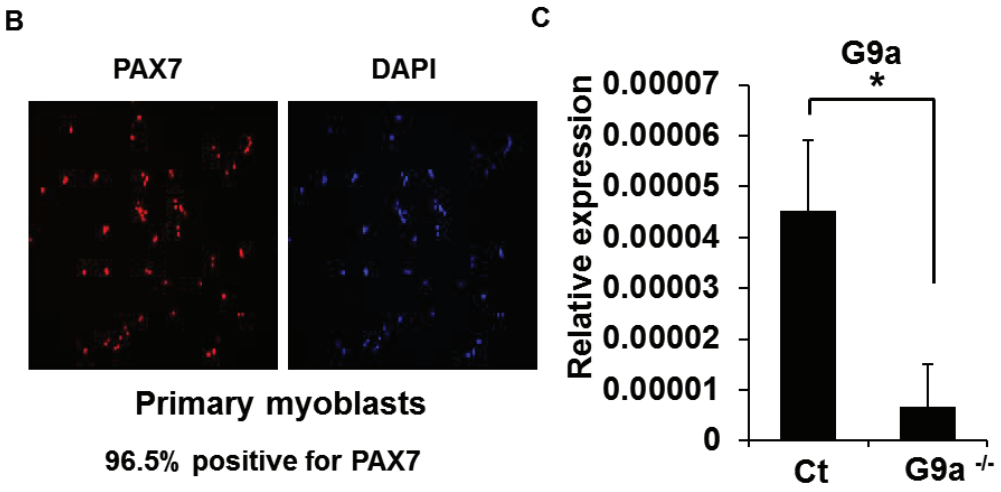


Figure 3.14 B&C. Myoblasts from G9a knockout mice display reduced proliferation and decreased expression of proliferation genes (B) Pax7 staining for primary myoblasts isolated from G9a $^{fl/fl}$; Pax7 Cre ERT2/+ mice. (C) G9a mRNA levels in control (Ct) and G9a null myoblasts (G9a$^{-/-}$) by real time PCR.

To validate G9a role in proliferation, BrdU incorporation assay was performed with BrdU pulsed control and G9a null myoblasts. Consistent with our knockdown results, G9a null myoblasts displayed reduced proliferation as evidenced by lesser BrdU incorporating cells (Fig 3.14D).

Further, control and G9a null myoblasts were analyzed for the expression of proliferation and differentiation genes. We found up regulation of p21 and Rb1 while cyclinD1, DHFR and CyclinE expression was down regulated (Fig 3.14E). These results recapitulate the findings from the G9a knockdown studies indicating the importance of G9a for proliferation and cell cycle exit of myoblasts. However, since these results are from single mice (N=1), these experiments have to be validated with G9a null myoblasts isolated from a few more G9a conditional knockout mice.

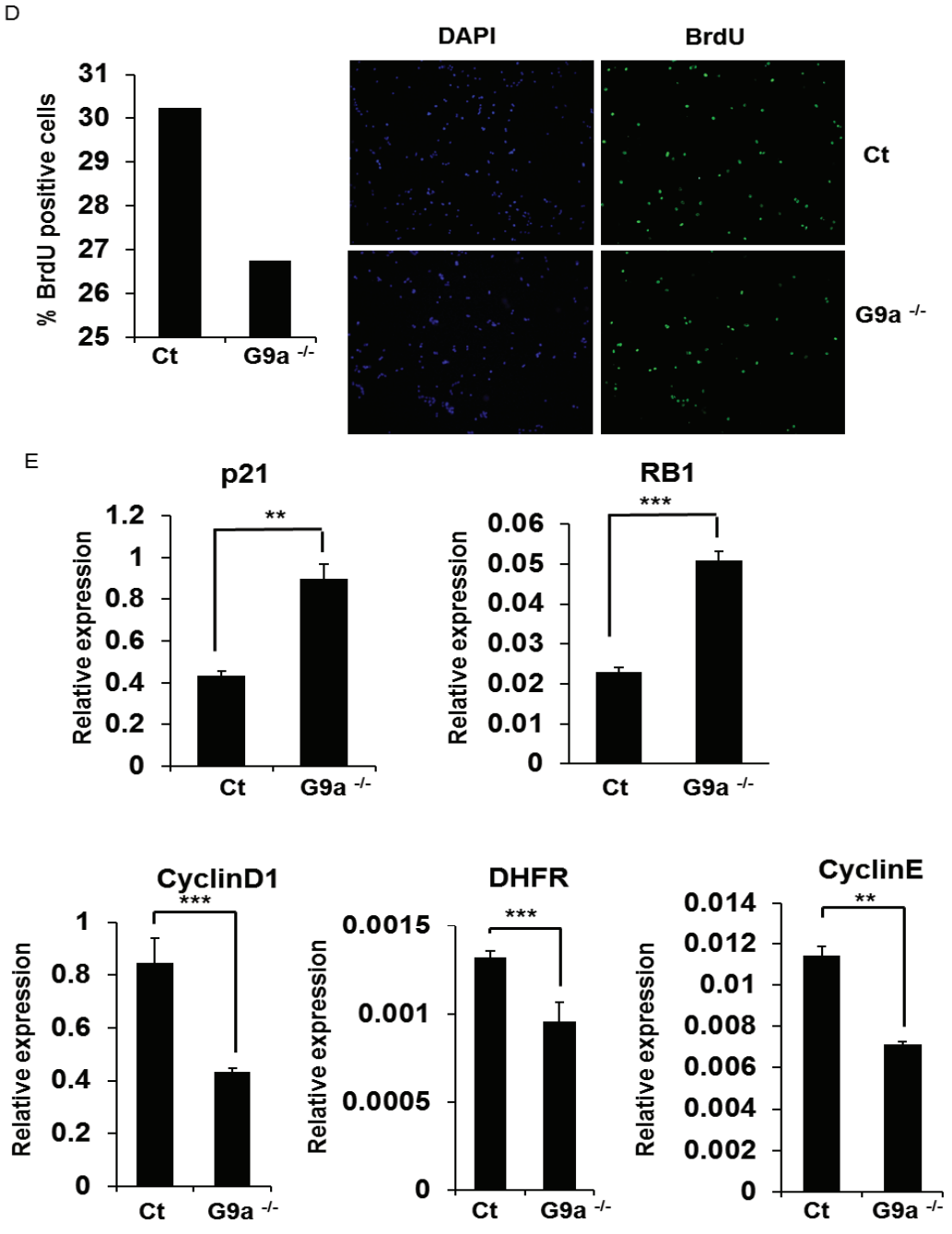


Figure 3.14 D&E. Myoblasts from G9a knockout mice display reduced proliferation and decreased expression of proliferation genes (D) BrdU positive cells were counted in control (Ct) and G9a null myoblasts ($G9a^{-/-}$) (N=1). Atleast 500 cells were counted. BrdU and DAPI staining of cells from control and $G9a^{-/-}$ myoblasts. (E) mRNA levels of p21, Rb1, CyclinD1, DHFR and CyclinE were analyzed using real time PCR.

4 Discussion

Previous studies from our lab have identified a role for G9a in negatively regulating myogenic differentiation (Ling et al., 2012a, 2012b). To further understand the mechanisms by which G9a regulates muscle differentiation, in the present thesis work, we first aimed to identify the genome wide targets of G9a in skeletal muscle cells. To this end, we performed global gene expression analysis using microarray. Interestingly, we found that several cell cycle control genes were de-regulated in G9a knockdown cells (Fig 3.2.1). This prompted us to find out if G9a plays a role in proliferation of cells. Using gain-of-function and loss-of-function studies G9a was found to promote proliferation of cells (Fig 3.4.1 - 3.4.4). Among the cell cycle target genes that we identified from the microarray, MyoD target genes (p21 and RB1) required for cell cycle exit were up regulated suggesting that G9a represses their expression. On the other hand, E2F1 target genes (cyclins, DHFR, TK) required for proliferation were significantly down regulated indicating that G9a may directly or indirectly regulate their expression. We therefore examined the mechanisms by which G9a differentially regulates their expression. G9a mediated repressive H3K9me2 on both p21 and Rb1 promoters in a methyltransferase-dependent manner (Fig 3.6). More importantly, G9a-mediated inhibition of differentiation was rescued by re-expression of p21 and Rb1 indicating that repression of cell cycle exit contributes to myogenic differentiation defect imposed by G9a (Fig 3.7).

Next, we examined if G9a actively promotes the expression of E2F1 target genes that are important for cell cycle progression. G9a overexpression increased the expression of E2F1 target genes (Fig 3.8.1). Interestingly, G9a occupancy was not correlated with repressive histone methylation marks on E2F1-target genes unlike on p21 and Rb1 promoters (Fig 3.9). Interestingly, G9a occupancy was associated with H3K9ac marks instead (Fig 3.11). Furthermore, protein-protein interaction studies indicated that G9a is in complex with P/CAF and E2F1 in myoblasts, which could possibly explain G9a association with H3K9ac marks on E2F1 target promoters (Fig 3.12.1). Consistently, we also found that G9a overexpressing cells displayed higher E2F1-dependent CyclinD1 promoter activity than control cells (Fig 3.13). Finally, experiments with G9a null myoblasts isolated from G9a conditional knockout mice recapitulated our findings *ex vivo* (Fig 3.14). G9a null myoblasts displayed reduced proliferation. Cell cycle exit genes were upregulated and E2F1 target genes were down regulated validating our *in vitro* findings. Overall, our data support a model in which G9a both promotes proliferation and prevents cell cycle exit of muscle cells to block differentiation.

4.1 G9a orchestration of myoblast cell cycle

Our results demonstrate that knockdown of G9a reduced proliferation whereas it's over expression increased proliferation of cells. This finding is consistent with the published studies which suggest that inhibition of G9a activity reduces proliferation of smooth muscle cells (Yang et al., 2012). siG9a cells up regulated p21 and Rb1 expression required for cell cycle exit while down regulated E2F1 target genes required for proliferation. Hence we went on to examine the possibilities of G9a

regulation of muscle cell cycle through a) indirectly promoting proliferation by repressing the repressors p21 and Rb1 thus avoiding cell cycle exit or b) directly promoting the expression on E2F1 target genes required for proliferation.

4.1.1 G9a regulation of cell cycle exit genes

Several chromatin modifiers are shown to inhibit myogenic differentiation. For instance Suv39h1 and EZH2 are known to mediate repressive marks on early and late muscle gene promoters respectively and inhibit their expression (Caretti et al., 2004; Mal, 2006). However, G9a not only mediates repressive marks on myogenin promoter but also methylates MyoD and inhibits its transcriptional activity (Ling et al., 2012b). Cell cycle exit is a pre-requisite step during myogenic differentiation. In this study we provide evidence for a role of G9a in inhibiting MyoD mediated irreversible cell cycle exit. G9a mediates H3K9me2 on p21 and Rb1 promoters. This finding is consistent with the studies in other cell lines indicating the of involvement of G9a in suppressing p21 expression (Kim et al., 2009; Nishio and Walsh, 2004). In our ChIP assays although we amplified the MyoD binding regions on both p21 and Rb1 promoters, it would be meaningful to observe MyoD binding on their promoters upon G9a over expression.

Up regulation of p21 during differentiation also help to reduce kinase activity of Cyclins/CDK complexes and as a consequence, Rb1 is hypo phosphorylated leading to the inhibition of cell cycle progression (Guo et al., 1995). p21 and hypo phosphorylated Rb1 are not only required to block proliferation, but both these proteins play an important role in maintaining the permanent cell cycle arrest of myotubes. In other words, p21 and Rb1 are involved in maintenance of post mitotic

state of myotubes (Mal et al., 2000). Unlike quiescent cells, differentiated myotubes neither can enter cell cycle nor initiate DNA synthesis upon growth factor stimulation. This is achieved by high expression of p21 and hypo phosphorylated Rb1. Although Rb1 null myoblasts differentiate, they cannot maintain permanent exit state. Higher frequency of apoptosis has been observed in Rb1 null myotubes due to endoreduplication (Zacksenhaus et al., 1996). Absence of both p21 and Rb1 has been shown to be capable of initiating DNA synthesis in myotubes (Andrés and Walsh, 1996). Indeed inactivation of p21 and Rb1 by E1A proteins leads to synthesis of DNA and restoration of Cyclin kinases activity in myotubes (Mal et al., 2000). Moreover, Suv39h1 is shown to be involved in permanent silencing E2F1 target genes in differentiated myoblasts (Ait-Si-Ali et al., 2004). Differentiated cells express low levels CyclinD1, CyclinA and DHFR. Given the low expression of G9a during differentiation it is unlikely that G9a is involved in suppressing the expression of proliferation genes. Our findings demonstrate that G9a overexpression promotes expression of E2F1 target genes. However, the possibility of G9a over expressing myotubes re-synthesizing DNA upon addition of growth media needs to be checked. It could be possible that G9a over expressing cells may not be able to maintain the post mitotic arrest.

It is well established that, in skeletal muscle cells, p21 up regulation is dependent on MyoD rather than p53 (Halevy et al., 1995). Since p21 expression is MyoD dependent and the fact that G9a restrains MyoD activity (Ling et al., 2012b), it is possible that reduced p21 expression in G9a over expressing cells might be a result of reduced MyoD activity. Luciferase reporter experiments involving mutant

MyoD (K104R) which is resistant to MyoD methylation and p21 reporter construct would help to us in better understanding the underlying mechanisms. Overall, we conclude that G9a acts as a master regulator controlling myogenic differentiation program by controlling proliferation and cell cycle exit of myoblasts in addition to its role in suppressing muscle specific genes.

4.1.2 G9a regulation of E2F1 target genes

In order to find out if G9a actively promotes the expression of proliferation genes, we examined G9a occupancy on E2F1 target gene promoters. Remarkably, unlike p21 and Rb1 promoters, G9a occupancy was not correlated with repressive H3K9me2 marks on E2F1-target genes. These findings were particularly interesting because G9a is widely regarded as a repressor, nonetheless in this case its binding was not associated with repression. These results are consistent with the observation from other groups that H3K9me2 mediated by Suv39h1 was apparent on Rb/E2F1 target promoters in differentiating cells but not in proliferating cells (Ait-Si-Ali et al., 2004). Therefore, our findings prompted us to investigate if G9a could function as an activator of gene expression.

Increasing number of evidences suggest G9a can act as an activator of gene expression and this is independent of its SET domain activity (Bittencourt et al., 2012). Therefore, to substantiate this possibility, we pharmacologically blocked endogenous methyltransferase activity of G9a and examined the expression of E2F1 target genes. Since activator function of G9a is independent of methyltransferase activity, we expected no changes in the expression of proliferation genes. Consistent with our hypothesis we found no apparent changes

in the mRNA levels of E2F1 target genes. In addition, unlike on p21 and Rb1 promoters, H3K9me2 on E2F1 target promoters were unaltered upon inhibition of G9a activity. Intriguingly, G9a occupancy was associated with H3K9ac marks, which are generally associated with activation of gene expression. A recent study indicated that G9a can interact with acetyl-transferase P/CAF (Oh et al., 2014). Therefore, we looked into the possibility of G9a interaction with activators.

From our Co-IP interaction studies it appears that G9a is in complex with P/CAF and E2F1 in myoblasts and this could possibly explain G9a association with H3K9ac marks on E2F1 target promoters. Our results indicate that G9a is in complex with P/CAF, however the possibility of other co-activators such as p300 and CARM1 needs to be investigated. Further ChIP experiments testing the occupancy of co-activators in the absence of G9a (siG9a cells) would give more insights on the involvement of G9a in recruiting co-activators to the target gene promoters.

In addition, from luciferase experiments we found that G9a overexpressing cells displayed higher activity of CyclinD1 promoter and this is E2F1 dependent. Additional luciferase experiments involving other target genes promoters such as DHFR and CyclinE will help substantiate the G9a role as an activator of E2F1 target genes.

4.2 G9a promotes proliferation and inhibits cell cycle exit of myoblasts: implications in rhabdomyosarcoma

Given its role in proliferation of cells, it is not surprising to find several publications reporting G9a over expression in various cancers (Chen et al., 2010). Rhabdomyosarcoma is a common pediatric sarcoma arising due to unlimited proliferation of myogenic precursor cells which fail to undergo cell cycle exit required for myogenic differentiation (Keller and Guttridge, 2013). Data from our studies indicate that G9a over expression increased proliferation of cells and inhibited p21 and Rb1 expression required for cell cycle exit. Therefore, it is tempting to speculate the involvement of G9a in conditions such as rhabdomyosarcoma where cell cycle is de-regulated. It will be meaningful to test if G9a is indeed deregulated in rhabdomyosarcoma and whether it can be targeted as an advanced therapeutic molecule to treat these cancers.

4.3 Future studies and conclusion

Skeletal muscle has a remarkable ability to regenerate. During muscle regeneration the quiescent satellite cells are activated forming proliferating myoblasts which subsequently exit cell cycle and fuses to repair the injured myofibers. Our *in vitro* findings established a role for G9a in proliferation and cell cycle exit, therefore it is sensible to explore its role in skeletal muscle regeneration *in vivo*. We predict a possible role for G9a during muscle regeneration. Since we have G9a conditional knockout mice, it would be meaningful to test if G9a null satellite cells are able to activate and proliferate during muscle regeneration. It is noteworthy that G9a is found as a hetero-dimer with another lysine methyltransferase GLP, yet they are also known to play different roles in a number of tissue types. Either G9a/GLP both work together to regulate the same target genes, or they may have different cellular targets thus play non-overlapping cellular functions. It is possible that GLP may compensate for the loss of G9a in G9a conditional knockout mice. Further work needs to be done to understand the role of GLP in regulating muscle differentiation.

Along similar lines, it will be interesting to explore if G9a has a role in muscle pathologies such as Duchene muscular dystrophy (DMD) and its mice model *mdx* where muscle is undergoing constant regeneration and degeneration process. Muscle integrity is compromised in muscular dystrophies due to loss of several genes involved in formation of sarcomere structure and maintenance of structural integrity. From our microarray results Myozenin (Myoz2), Myomesin (Myom3) and Sarcoglycans (Sgc-α,-β,-γ) genes which are involved in muscle structural integrity maintenance were found to be direct targets of G9a. It is well established

that most of these genes are driven by MEF2 transcription factors and de-regulation of their expression is implicated in DMD. Hence G9a could be involved in repression of their expression in case of muscular dystrophies. It is also possible that G9a regulates MEF2 dependent transcription of these genes. Indeed, recent evidence suggest that G9a methylates MEF2 and regulates its activity (Choi et al., 2014).

Furthermore, from the microarray results calcium signaling was found to be one of the top pathways altered upon G9a knockdown. Calcium binding protein Calcequestrin 2 (Casq2), calcium release channel Ryanodine receptor1 (Ryr1) and calcium transporting ATPase (Atp2a1) which are involved in maintaining calcium homeostasis in muscle cells were found to be significantly upregulated in siG9a cells. Altered expression of these genes is associated with muscle pathologies including DMD, where intracellular calcium is overloaded in the muscle (Kunert-Keil et al., 2014). Taken together our findings implicate a potential role of G9a in muscle pathologies where calcium signaling and muscle integrity is affected.

4.4 Conclusion

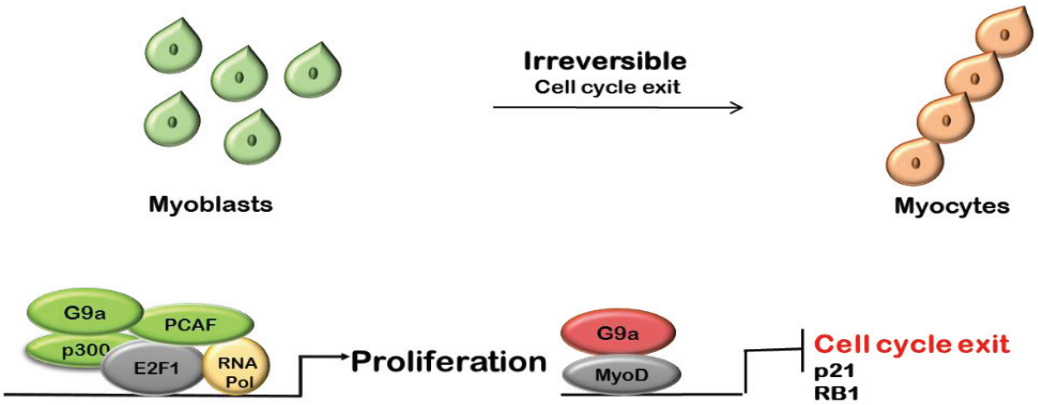


Figure 4.4 Schematic model summarizing the mechanisms by which G9a regulates proliferation and cell cycle exit. G9a is present in activator complex and promotes proliferation of cells and also inhibits cell cycle exit by repressing p21 and Rb1.

My thesis work provides evidence for a role of G9a in promoting proliferation of cells and inhibiting cells from exiting the cell cycle (Fig 4.4). Apart from G9a, other epigenetic modifiers could also be involved in regulating cell cycle in myoblasts. In the future, it will be interesting to examine by ChiP-Seq global binding of G9a; as well as identification of its interacting partners in myoblasts by mass spectrometry. Our findings indicate that G9a exists in activator complex as well as repressor complex in myoblasts, however, more work needs to be done to completely understand G9a functioning as an activator driving E2F1 target gene expression. Also, increasing evidence suggest possible role of epigenetic modifiers in pathologies involving deregulation of cell cycle such as cancer. I believe that my work sheds new light and identified mechanisms by which G9a regulates the cell cycle in muscle cells. These studies raise the possibility that its expression may be altered in myopathies associated with an imbalance of proliferation and differentiation of muscle precursor cells.

5 References

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