CARDIAC LINEAGE PROTEIN 1 (CLP-1), AN INHIBITOR OF POSITIVE TRANSCRIPTION ELONGATION FACTOR B (P-TEFB), PLAYS A REGULATORY ROLE IN SKELETAL MUSCLE CELL DIFFERENTIATION

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By

Josephine Galatioto

Thesis advisor: M.A.Q. Siddiqui, Ph.D.

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Department of Cell Biology
State University of New York
Downstate Medical Center
Brooklyn, New York

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2. CLP-1 Associates with MyoD and HDAC to Regulate Skeletal Muscle Differentiation. (1-27)
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ABBREVIATIONS

a.a.	amino acid
BCDIN3/MEPCE	Bicoid-Interacting Protein 3 homolog/ Methylphosphate Capping Enzyme
bHLH	basic Helix-loop-Helix
Brd4	Bromodomain protein
CBP	CREB Binding Protein
Cdk9	Cyclin dependent kinase 9
ChIP	Chromatin Immunoprecipitation
CLP-1	Cardiac Lineage Protein-1
Co-IP	Co-immunoprecipitation
CREB	Cyclic Adenosine Monophosphate Response Element Binding protein
CTD	Carboxyl Terminal Domain
DM	Differentiation Medium
DRB	5,6-dichloro-1-6-D-ribofuranosyl benzimidazole riboside
DSIF	DRB Sensitivity Inducing Factor
EDTA	Ethylenediaminetetra acetic acid
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM	Growth Medium
HBSS	Hank’s Buffered Salt Solution
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase

HeLa	Henrietta Lacks cervical cancer cells
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
HEXIM1	Hexamethylene bis-acetamide inducible protein 1
HMBA	Hexamethylene bis-acetamide
HRP	Horse Radish Peroxidase
IF	Immunofluorescence
IP	Immunoprecipitation
MEF2	Myocyte Enhancer Factor 2
MRF	Muscle Regulatory Factor
NAD +	Nicotinamide Adenine Dinucleotide
NELF	Negative Elongation Factor
NLS	Nuclear Localization Sequence
PBS	Phosphate Buffered Saline
PCAF	p300/CBP-Associated Factor
PCR	Polymerase Chain Reaction
PI3/Akt	Phosphoinositide 3/Akt Kinases
PMSF	Phenyl Methyl Sulfonyl Fluoride
Pol II	RNA Polymerase II
P-TEFb	Positive Transcription Elongation Factor b
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
Shh	Sonic hedge hog
snRNA	small nuclear RNA

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ABSTRACT

Emerging evidence suggests that one level of transcriptional regulation in eukaryotic cells is at the stage of transcription elongation in part by the Positive-Transcription Elongation Factor b (P-TEFb) complex. The P-TEFb complex consists of cyclin dependent kinase 9 (cdk9) and its cyclin partner, cyclin T and is dynamically partitioned between the active versus inactive states. The kinase activity is inactivated when P-TEFb associates with Cardiac Lineage Protein-1 (CLP-1), the mouse ortholog of human Hexamethylene bis-acetamide inducible protein 1 (HEXIM1). Recent reports implicate a link between P-TEFb and skeletal muscle in which P-TEFb associates with the skeletal muscle specific transcription factor MyoD. In this study, I present evidence that CLP-1 protein is critical for the regulation of skeletal muscle cell differentiation. I examined the expression of CLP-1 in the mouse myoblast cell line, C2C12, and found that there was a marked increase in CLP-1 association to P-TEFb at the onset of differentiation. I also observed that CLP-1 associates with MyoD, as well as with histone-deacetylases (HDACs), and that this association was maximal in the early stage of C2C12 differentiation. To further investigate the role of CLP-1 in skeletal muscle cell differentiation, I generated CLP-1 +/- knock-down C2C12 cells through homologous recombination. The deficiency in CLP-1 resulted in an inability of C2C12 heterozygote cells to transition into myotubes. I next examined the possibility that the inhibitory complex consisting of CLP-1, MyoD and HDACs targets the promoter of proliferative genes, such as cyclin D1. Chromatin immunoprecipitation (ChIP) analysis confirmed CLP-1/MyoD/HDAC binding to the cyclin D1 gene promoter during differentiation. These data suggest that CLP-1 plays a synergistic role in conjunction with MyoD and

HDAC to control the genetic program that ensues in the transition of skeletal myoblasts from proliferative to terminally differentiated cells.

INTRODUCTION

Transcriptional Elongation: Eukaryotic gene transcription mediated by RNA polymerase II (Pol II) can be subdivided into distinct stages: pre-initiation, initiation, promoter clearance, elongation and termination (Roeder, 1996). The elongation stage of transcription is highly regulated by the Positive Transcription Elongation Factor b (P-TEFb) complex which is composed of cdk9, a serine/threonine kinase, and its regulatory partner cyclin T1 or T2 (Peng et al., 1998b; Wei et al., 1998; Zhu et al., 1997). T-type cyclins share a conserved amino-terminus with 81% identity, while the carboxyl terminus has only 46% homology (Peng et al., 1998b). There are two isoforms of cdk9, 42 KDa and 55 KDa, which are molecularly similar. The cdk9-55 KDa isoform is the result of an additional upstream transcription start-site (Shore et al., 2003). Cdk9 kinase activity is dependent upon its phosphorylation at conserved Threonine-186 on the T-loop (Chen et al., 2004; Li et al., 2005). This phosphorylation induces a conformational change, which allows the entry of the substrate and ATP into the catalytic pocket (Chen et al., 2004). One of the substrates of P-TEFb is the carboxyl-terminal domain (CTD) of the largest subunit, RPB1, of RNA Pol II, which contains a heptapeptide sequence of amino acids Tyr^1-Ser^2-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7 that is repeated 53 times (Marshall et al., 1996; Ramanathan et al., 2001). Before initiation of transcription, unphosphorylated Pol II is recruited to promoters along with general transcription factors to form the pre-initiation complex (Sims et al., 2004). For promoter clearance, the CTD is phosphorylated at Serine 5 of the heptapeptide repeat by Transcription Factor II H (TFIIH), a member of the general transcription machinery, particularly Cdk7 the kinase component of this multi-protein complex (Conaway et al., 2000; Goodrich and Tjian, 1994; Lu et al., 1992; Roy et

al., 1994; Serizawa et al., 1995; Svejstrup et al., 1996). Soon after promoter clearance, Pol II stalls due to the activity of negative elongation factors (Reines et al., 1996; Wada et al., 2000; Yamaguchi et al., 2002). The P-TEFb complex is recruited to the stalled Pol II and phosphorylates the CTD at Serine 2 of the heptapeptide repeat, allowing for efficient transcription (Marshall and Price, 1992; Marshall and Price, 1995; Peng et al., 1998a; Price, 2000; Taube et al., 2002). In addition to phosphorylating Pol II, P-TEFb also phosphorylates negative elongation factors, DRB (5,6-dichloro-1-6-D-ribofuranosylbenzimidazole riboside) Sensitivity-Inducing Factor, DSIF, and Negative Elongation Factor, NELF, which results in conversion of DSIF into a positive elongation factor and dissociation of NELF (Fujinaga et al., 2004; Hartzog et al., 1998; Ivanov et al., 2000; Price, 2000; Wada et al., 2000; Yamaguchi et al., 1999). The positively acting Bromodomain protein (Brd4) also interacts with the P-TEFb complex to promote its kinase activity (Jang et al., 2005). Brd4 protein is ubiquitously expressed and has the ability to bind and acetylate histones (Dey et al., 2000). Experiments using specific cdk inhibitors and RNAi suggest that P-TEFb plays a role in regulating expression of most protein coding genes (Chao and Price, 2001; Shim et al., 2002). Recent reports suggest that shifts in the equilibrium between inactive and active P-TEFb influence activation of alternative pathways that lead cells toward either unrestrained growth or terminal differentiation (He et al., 2006; Sano et al., 2002; Turano et al., 2006; Wittmann et al., 2003).

Regulation of P-TEFb by CLP-1: CLP-1, the mouse ortholog of human HEXIM1, was isolated, cloned and knocked out in mice in our laboratory (Huang et al., 2002; Huang et al., 2004). Also, we have shown that CLP-1 is expressed in early cardiac

muscle cells and has enhanced expression in the left ventricle during early heart formation (Ghatpande et al., 1999; Huang et al., 2002; Huang et al., 2004). The phenotype of the CLP-1 -/- mouse is lethal in late fetal stages, E17/18, due to pronounced cellular growth and hypertrophy of the cardiomyocytes (Huang et al., 2004). The heterozygote CLP-1 mice survive and appear phenotypically similar to wild-type mice. Our laboratory has shown that upon crossing CLP-1 +/- mice with mice with cardiac-specific over-expression of cyclin T1 (MHC-cyclin T1) (Sano et al., 2002) that the previously reported hypertrophic phenotype of the MHC-cyclin T1 hearts (Sano et al., 2002), was exacerbated in MHC-cyclin T1/CLP-1 +/- mice (Espinoza-Derout et al., 2009; Espinoza-Derout et al., 2007). Our laboratory has also shown that CLP-1 dissociates from the P-TEFb complex in response to stress via mechanical stretch or hypertrophic agonists phenylephrine and endothelin-1 and the dissociation activates P-TEFb, resulting in an increase in global RNA and protein synthesis, features suggestive of cardiac hypertrophy (Espinoza-Derout et al., 2007).

The role of 7SK has been investigated in several laboratories and it appears that CLP-1/HEXIM1 associates first with the scaffold 7SK small nuclear RNA (7SK) and then with the P-TEFb complex by binding directly to cyclin T1 **(Fig. 1A)** (Barboric et al., 2005; Egloff et al., 2006; Michels et al., 2004; Michels et al., 2003; Schulte et al., 2005; Yik et al., 2003; Yik et al., 2004). When CLP-1 is bound to P-TEFb, it inactivates cdk9 kinase which inhibits the transcriptional elongation activity of P-TEFb. Reversible phosphorylation of cdk9 at Threonine-186 is required for *in vitro* association of P-TEFb with CLP-1 (Chen et al., 2004). 7SK is an abundant ($2x10^5$ molecules per cell), 332 nucleotide long non-coding nuclear RNA (Wassarman and Steitz, 1991). The 3' and 5'

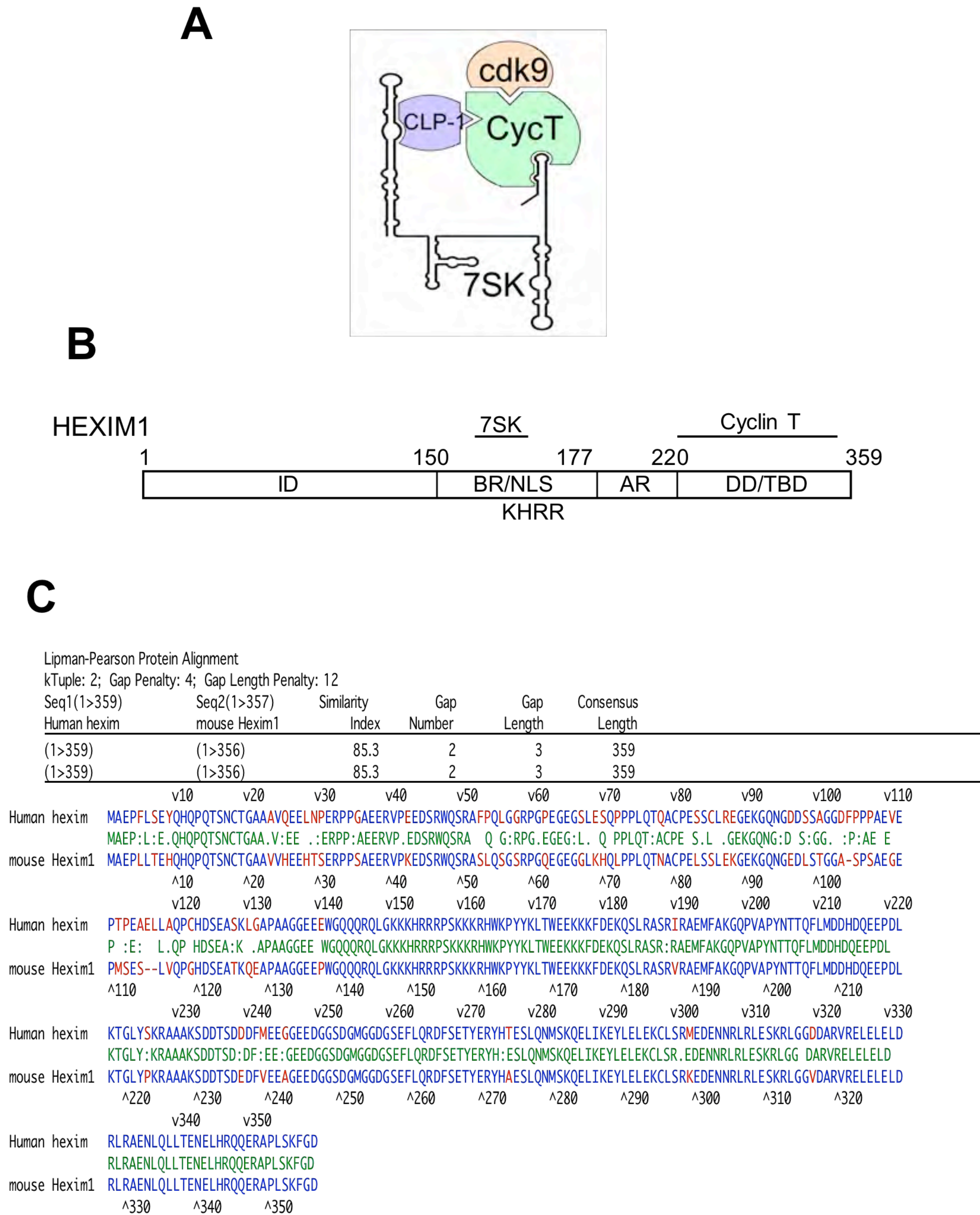


Figure 1: Model of CLP-1 associated to P-TEFb and sequence homology between HEXIM1 and CLP-1. *A*, CLP-1 associates with P-TEFb (cdk9/cyclin T) by binding directly to cyclin T. 7SK snRNA acts as a scaffold. *B*, HEXIM1 protein domain model *(see text)*. ID, Inhibitory domain, BR/NLS, basic region/nuclear localization sequence, AR, adjacent/acidic region, DD/TBD, dimizerization domain/cyclin T binding domain. *C*, Lipman-Pearson Protein Alignment between human HEXIM1 and mouse HEXIM1 (CLP-1).

ends of 7SK are protected by specific capping enzymes, BCDIN3/MEPCE and PIP7S/LARP7 (Jeronimo et al., 2007; Krueger et al., 2008). 7SK RNA binds to the "basic region" of CLP-1/HEXIM1 amino acids (a.a.) 150-177, and leads to the exposure of the cyclin T binding domain in its carboxyl terminus, allowing CLP-1/HEXIM1 to bind cyclin T1 (Li et al., 2007). CLP-1/HEXIM1 proteins are ubiquitously expressed. More recently HEXIM2, a homolog of HEXIM1, was described (Blazek et al., 2005; Dulac et al., 2005; Egloff et al., 2006; Yik et al., 2005). The HEXIM2 gene is located 10,000 bp downstream to HEXIM1 and the highest homology (47%) between HEXIM1 and HEXIM2 is in the carboxyl termini, while the amino termini are very dissimilar (Byers et al., 2005). HEXIM2 protein can dimerize with HEXIM1 protein and can also act to functionally inhibit P-TEFb kinase activity (Byers et al., 2005).

CLP-1/HEXIM1 proteins have been implicated as inhibitors of cell proliferation and as enhancers of the cell differentiation program. HEXIM1 was first cloned from vascular smooth muscle cells, in which it was the only transcript up-regulated upon treatment with hexamethylene-bis-acetamide (HMBA), a compound which causes suppression of proliferation (Kusuhara, 1999). Similarly, in breast epithelial cells it was reported that over-expression of HEXIM1 caused down-regulation of cell proliferation genes and activation of cell differentiation genes (Wittmann et al., 2003). Also, ectopic over-expression of HEXIM1 in neuronal cells was shown to cause growth inhibition and promote terminal differentiation (Turano et al., 2006).

HEXIM1/CLP-1 protein can be subdivided into four domains: the variable proline rich amino terminus, amino acids (a.a.) 1-150 called the "inhibitory domain"; the central "basic region", which is lysine and arginine rich a.a. 150-177; the "adjacent region" a.a.

185-220; and the carboxyl terminal “cyclin T binding domain” (TBD) a.a. 220-359 which is enriched in aspartic and glutamic acids **(Fig. 1B)**. The “inhibitory domain” prevents HEXIM1/CLP-1 from interacting with P-TEFb, upon deletion of the domain inhibition by HEXIM1 is enhanced (Li et al., 2005; Michels et al., 2004; Yik et al., 2003). The “basic region” contains the nuclear localization sequence (NLS), amino acids 152 to155, KHRR, and the 7SK binding domain (Barboric et al., 2005; Michels et al., 2004; Michels et al., 2003; Ouchida et al., 2003; Yik et al., 2004). The “adjacent region” is the region of highest homology between species and contains negatively charged clusters. This “acidic region” binds the “basic region” in absence of 7SK RNA (Barboric et al., 2005). The “cyclin T binding domain” a.a. 202-205 PYNT binds cyclin T and contains the site of dimerization between HEXIM1 proteins (Li et al., 2005; Schulte et al., 2005). CLP-1 and HEXIM1 proteins are 85% homologous **(Fig. 1C)**. The HEXIM1 gene is on human chromosome number 17 and the CLP-1 gene is on mouse chromosome number 11 (Byers et al., 2005; Huang et al., 2004; Michels et al., 2003).

Post-Translational Modifications of P-TEFb: More recently, the role of post-translational modifications of P-TEFb components has been implicated in its regulation. Cdk9 has been reported to be acetylated at Lysine 44 by p300, which led to positive regulation of P-TEFb (Fu et al., 2007). A conflicting report showed that acetylation of cdk9 at Lysines 44 and 48 by PCAF reduced kinase function and transcriptional activity of P-TEFb (Sabo et al., 2008). Most recently, acetylation of cyclin T1 by p300 on Lysines 380, 86, 90 and 404 has been shown to cause dissociation of HEXIM1 and activation of P-TEFb (Cho et al., 2009). The only reported post-translational modification of HEXIM1 is phosphorylation at Threonine 270 and Serine 278 by the

PI3/Akt kinase pathway activated by HMBA which resulted in the release of HEXIM1 from P-TEFb (Contreras et al., 2007). These post-translational modifications, therefore, appear to be important in regulation of P-TEFb activity and, potentially, in the regulation of specific gene programs.

Skeletal Muscle Cell Development (a) Determination: During embryonic development, skeletal muscle cells are derived from mesodermal precursor cells originating from the somites (Miller et al., 1999; Stockdale, 1992). Somites are transient epithelial spheres which pinch off the paraxial mesoderm which lines both sides of the neural tube (Buckingham et al., 2003). The dermamyotome compartment of the somite expresses transcription factors paired-box gene 3 and 7 (pax3, pax7), which respond to early positive signaling molecules emanating from the notochord, neural tube and epidermis (Pownall et al., 2002; Relaix et al., 2005; Ridgeway and Skerjanc, 2001; Seale et al., 2000). These signaling molecules include, Wnt, Sonic hedge hog (Shh) and Noggin. The ventral part of the somite, the sclerotome, contributes to formation of cartilage, and bone of the vertebral column and ribs. The dorsal part, the dermamyotome, gives rise to the overlying dermis of the back and to the skeletal muscle of the body and limbs (Ben-Yair and Kalcheim, 2005; Dale and Pourquie, 2000; Daubas et al., 2000; Gros et al., 2005; Ordahl and Le Douarin, 1992). When the mesodermal precursors commit to the myogenic lineage and become myoblasts, they down-regulate pax3 and pax7, delaminate from the dermamyotome and give rise to the myotome, which expresses muscle specific transcription factors (Braun and Arnold, 1996; Cinnamon et al., 1999; Denetclaw and Ordahl, 2000; Huang et al., 2000; Kahane and Kalcheim, 1998; Relaix et

al., 2005; Tajbakhsh et al., 1998). Subsequently, the cells undergo fusion and maturation into multi-nucleated skeletal myotubes.

Skeletal Muscle Cell Development (b) Differentiation: Skeletal muscle cell differentiation begins when proliferating myoblast cells exit from the cell cycle through down-regulation of proliferative gene expression (Andres and Walsh, 1996; Franklin and Xiong, 1996). This is followed by fusion of myoblasts into multi-nucleate myotubes and up-regulation of skeletal muscle specific genes necessary for the contractile function of the fibers (Bassel-Duby and Olson, 2006; Charge and Rudnicki, 2004; Lassar et al., 1994; Olson, 1992; Perry and Rudnick, 2000; Yun and Wold, 1996). The first fibers within the mouse limbs, primary fibers, appear during embryonic days 11 to 14; secondary fiber formation and innervation occur at embryonic days 14 to 16 (Ontell and Kozeka, 1984).

There are two families of transcription factors that act cooperatively to promote skeletal muscle development and differentiation. These are the Muscle Regulatory Factors (MRFs) (Sabourin and Rudnicki, 2000; Yun and Wold, 1996), which include MyoD, Myf5, myogenin and MRF4; and the Myocyte Enhancer Factor 2 (MEF2) factors (Gossett et al., 1989), which include MEF2A, B, C and D. The MRFs contain a conserved DNA binding domain, the basic Helix-loop-Helix (bHLH) domain, which is required to form heterodimers with ubiquitously expressed E-proteins, which bind target E-box sequences present in the promoters of muscle-specific genes (Bailey et al., 2001; Buskin and Hauschka, 1989; Davis et al., 1987; French et al., 1991; Lassar et al., 1994; Miller et al., 1999; Murre et al., 1989; Sabourin and Rudnicki, 2000; Weintraub et al., 1990). MEF2 factors are not skeletal muscle specific, but they potentiate the transcription-inducing activity of the MRFs. Forced expression of MyoD in non-muscle

cell lines activates expression of the MRFs and directs a transition into the myogenic phenotype (Davis et al., 1987; Weintraub et al., 1989).

Several reports implicate the dual role played by MyoD in myogenesis. MyoD acts to down-regulate proliferation genes and, at the same time, it activates muscle specific gene transcription (Bergstrom et al., 2002; Chu et al., 1997; Crescenzi et al., 1990; Guo et al., 1995; Halevy et al., 1995; Kitzmann et al., 1998; Sorrentino et al., 1990; Tintignac et al., 2000). Based on chromatin immunoprecipitation (ChIP) followed by microarray, "ChIP-on-chip" assays, about 200 genes were shown to be targeted by MyoD in C2C12 cells (Blais et al., 2005). Of these, approximately 100 are present in undifferentiated cells and the other half in differentiated cells. In gene ablation studies it was shown that skeletal muscle develops relatively normal without either MyoD or Myf5 (Braun et al., 1992; Rudnicki et al., 1992), however, when both genes were ablated, skeletal muscle fibers failed to develop and the myoblasts remained multi-potent (Rudnicki et al., 1993). Similarly, in the myogenin knock-out mouse, the skeletal muscle lineage was appropriately specified but the cells failed to terminally differentiate (Hasty et al., 1993; Nabeshima et al., 1993). These genetic studies have demonstrated that MyoD and myf5 are required for myogenic determination, while myogenin is required for differentiation.

Signaling Pathways in Skeletal Myogenesis: The p38 Mitogen-activated protein kinase (MAPK) pathway acts as an upstream regulator of myogenic transcription. At the onset of muscle cell differentiation p38 MAPK is activated and phosphorylates E protein E47, promoting heterodimerization of MyoD with E47 (Lluis et al., 2005). It has been reported that treatment of myoblasts with p38 alpha and beta inhibitor SB203580

prevented fusion into myotubes (Cuenda and Cohen, 1999; Li et al., 2000; Wu et al., 2000). Oppositely, forced expression of constitutively active p38 MAPK is able to induce expression of differentiation markers and induce differentiation of myoblasts (Suelves et al., 2004; Wu et al., 2000). The Calciniurin-NFAT signaling pathway has also been implicated in skeletal myogenesis, upon differentiation MyoD/NFATc2/c3 to activate transcription from the myogenin gene promoter (Armand et al., 2008).

C2C12 Cells as a Model of Skeletal Muscle Differentiation: The mouse myoblast cell line, C2C12 cells, are a widely used model of skeletal muscle differentiation. The proliferating myoblasts cells are maintained in high (10%) fetal bovine serum growth medium. For induction of differentiation confluent cells are switched to low (2%) horse serum differentiation medium. Mitogens within the fetal bovine serum act to promote proliferation thereby inhibiting differentiation. These mitogens may include basic Fibroblast Growth Factor (bFGF) (Johnson and Williams, 1993), Transforming Growth Factor beta (TGF-beta) (Hannon and Beach, 1994), interferon-B (Multhauf and Lough, 1986), Platelet-derived Growth Factor-BB (PDGF) (Jin et al., 1991; Yablonka-Reuveni and Rivera, 1997), and Leukemia inhibitor factor (Austin et al., 1992). Serum mitogens inhibit differentiation in part by down regulating myogenic determination factors (Coleman et al., 1995; Multhauf and Lough, 1986; Tapscott et al., 1988; Tollefsen et al., 1989a; Tollefsen et al., 1989b). Removal of serum mitogens induces the expression of endogenous Insulin-like Growth Factors (IGF's) in C2C12 cells (Bartoccioni et al., 1994) and inhibits bFGF and TGF-beta (Johnson and Williams, 1993; Milasincic et al., 1996).

HATs and HDACs: Skeletal muscle specific gene expression, like other tissue specific gene expression, is dependent on the ability of transcription factors, such as MRFs and MEF2, to access their cognate DNA binding sites (Jenuwein and Allis, 2001). Histone acetylase proteins, which include p300, its paralog CBP (Cyclic Adenosine Monophosphate Response Element Binding protein-Binding protein) and p300/CBP-associated factor (PCAF), acetylate lysine residues by transferring an acetyl group from Acetyl Coenzyme A to the sigma-amino group of lysine (Kouzarides, 2000; Shahbazian and Grunstein, 2007; Sterner and Berger, 2000; Strahl and Allis, 2000). In the context of chromatin structure, HATs acetylate histone tails to relax condensed chromatin and expose DNA binding sites for transcription factors (Strahl and Allis, 2000).

The stimulatory effect of HATs on gene expression is antagonized by histone deacetylases (HDACs), which promote chromatin condensation and thereby prevent transcription (Johnson and Turner, 1999; Ng and Bird, 2000). HDACs are divided into three classes (Gray and Ekstrom, 2001): Class I, HDAC1, 2, 3 and 8 are expressed ubiquitously; Class II, HDAC4, 5, 6, 7, 9 and 10 are enriched in the heart, skeletal muscle and brain (Fischle et al., 1999; Grozinger et al., 1999); and Class III, SIRT1 to 7, are NAD+ dependent. It is well established that these chromatin-modifying enzymes are essential regulators of cell-type specific gene expression and differentiation (Timmermann et al., 2001).

HDACs and HATs have been implicated in the control of muscle development and differentiation through their association with myogenic transcription factors (McKinsey et al., 2001). Studies *in vivo* showed that mouse embryos exposed to HDAC inhibitors displayed increased formation of somites (Iezzi et al., 2002). Transcription

assays in cell culture have shown that HDAC1 suppresses MyoD transcriptional activity on muscle gene promoters in growth conditions (Mal et al., 2001). Similarly, Class I HDAC3 and Class II, HDAC4, 5, 6 and 9 have been reported to associate with MEF2 and inhibit skeletal muscle specific gene transcription in proliferating myoblasts (Gregoire et al., 2007; Haberland et al., 2007; Lemercier et al., 2000; Mal et al., 2001; Miska et al., 1999). Conversely, HATs promote MyoD mediated transcription in differentiated skeletal muscle. Histone acetylase protein p300 stimulates MyoD-dependent transcription by directly binding to MyoD and then recruiting PCAF (Puri et al., 1997). PCAF and CBP acetylate MyoD, in turn facilitating MyoD's dimerization with E-proteins and thus increasing MyoD's affinity for target-promoter DNA (Polesskaya et al., 2000; Puri et al., 1997; Sartorelli et al., 1999). Acetylated MyoD was not only in differentiated cells, but in proliferating myoblasts as well (Polesskaya et al., 2000). HATs also activate MEF2 promoter-binding, addition of p300/CBP complex to transcription assays enhanced myogenic transcriptional activity (Sartorelli et al., 1997). Regulation by HATs and HDACs has been reported to be restricted to the cell state, acting to keep MyoD mediated transcription off in myoblasts and facilitating the up-regulation of skeletal muscle specific gene expression in differentiation conditions (Mal and Harter, 2003).

P-TEFb in Skeletal Muscle: The P-TEFb complex has been implicated in regulation of skeletal muscle specific transcription. Cdk9 has been reported to bind skeletal muscle regulator MyoD (Simone et al., 2002). This association with the P-TEFb complex was seen in C2C12 cells both in proliferation conditions and in differentiated myotubes (Simone et al., 2002). Through gene reporter assays, P-TEFb was shown to

enhance MyoD mediated transcription whereas, inhibition of cdk9 kinase activity prevented activation of myogenic promoters (Giacinti et al., 2006; Simone et al., 2002). The P-TEFb complex along with p300, acetylated histones and phosphorylated Pol II, were also localized on a muscle-specific gene promoter (Giacinti et al., 2006). Thus MyoD mediated recruitment of P-TEFb on muscle-specific regions activates transcription upon skeletal muscle cell differentiation.

The data presented above provide strong mechanistic support to my hypothesis that CLP-1 association/dissociation from P-TEFb dictates the transition of skeletal myoblasts to myotubes. I used C2C12 mouse myoblasts, which uniformly initiate differentiation following deprivation of serum in the medium. The prior work in our laboratory offered a method to manipulate the level of CLP-1 in C2C12 cells and examine the potential of CLP-1 as a regulator of myogenesis. I examine the association between CLP-1 and proteins known to be critical to skeletal myogenesis. Finally, I test my hypothesis by localizing CLP-1 and its associated protein complexes on the promoter DNA of a cell cycle gene. I expect that my results will unequivocally portray the role of CLP-1 in the regulation of skeletal muscle differentiation and allow me to postulate a model mechanism for cell differentiation.

EXPERIMENTAL PROCEDURES

Antibodies: Polyclonal rabbit anti-CLP-1 antibody was generated to the peptide HRQQERAPLSKFGD (Proteintech Group, Inc.). Anti-cdk9 (C-20, D-7), cyclin T1 (H-245), GAPDH (FL-335), HDAC1 (H-11), HDAC3 (B-12), HDAC5 (H-714), HEXIM2 (M-90), PCAF (E-8), actin (C-2), Pol II (N-20), myogenin (F5D), MyoD (5.8A, C-20), and PCNA (PC10) antibodies were from Santa Cruz Biotechnology. Anti-cyclin D1 antibody was from Abcam Inc. Anti-Myosin Heavy Chain (MF20) antibody was from Developmental Studies Hybridoma Bank. HDAC antibody sampler kit from Cell Signaling Technology. ChIP Grade antibodies; anti-HEXIM1 and anti-MyoD (C-20x) were from Abcam Inc., and Santa Cruz Biotechnology respectively. Monoclonal antibody against Serine 2 phosphorylated Pol II CTD was from Covance.

Cell Culture: Mouse derived C2C12 myoblast cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin and 100 μg/ml streptomycin (referred to as growth medium). To induce differentiation, growth medium was substituted with differentiation medium (DMEM supplemented with 2% horse serum, 100 U/ml penicillin and 100 μg/ml streptomycin). Cells were incubated at 37°C with 5% CO_2.

Whole-Cell Lysis: C2C12 cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100) supplemented with 1 mM dithiothreitol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail (P-8340 Sigma). Protein concentration was calculated and equal concentrations were analyzed by western blot.

Western blot Analysis: Proteins were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane in electroblotting buffer (20 mM Tris, 150 mM Glycine, 20% methanol) for 1 hr. The membrane was blocked in Tris-Buffered Saline - Tween 20 (TBS-T) (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 5% milk, for 1 hr at room temperature. The membrane was probed with primary antibody overnight at 4°C with shaking. The membrane was then washed with TBS-T and incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Amersham) for 1 hr at room temperature. After three washes with TBS-T, proteins were visualized using an enhanced chemiluminescence reagent (Pierce).

Protein Concentration: Protein concentrations were calculated by spectrophotometry. The absorbance was read versus a blank control (H_2O) at 280 and 260 nm and applied to the following formula: $((1.55 \times A_{280}) - (.76 \times A_{260})) \times 50 \times$ dilution factor = concentration (mg/ml).

Co-Immunoprecipitation: C2C12 cells were lysed in Buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 0.5% NP-40) (Michels et al., 2004) supplemented with 1 mM dithiothreitol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor cocktail (P-8340 Sigma) and RNasin (Promega). Lysate was subjected to "freeze" on dry ice, and "thaw" at 37°C, followed by centrifugation at 16,000 X *g*. Equal concentrations of lysate were incubated with antibody overnight at 4°C with rotation; for antibody-protein capture, protein A/G plus agarose beads (Santa Cruz Biotech) were added for 2 h at 4°C with rotation. After extensive washing with Buffer A, bound proteins were denatured by boiling in 1X SDS

loading buffer (63 nm Tris, pH 6.8, 10% glycerol, 2% SDS, 0.0025% Bromophenol blue, 5% 2-Mercaptoethanol) and then subjected to western blot analysis.

Stable Transfections: Vector DNA pKO (targeting vector) (Lexiocon Genetics, Inc.) and CLP-1 allele replacement vector (pKO-HR-CLP-1neo) (Huang et al., 2004), were transfected into C2C12 cells at 10% confluency using Fugene 6 transfection reagent (Roche). Neomycin (G418) antibiotic selection was used to isolate resistant clones. Transfected cells were treated with neomycin and fresh growth media every three days. Cells which had incorporated the vector were resistant to antibiotic and single cells grew into colonies within three weeks. Single cell colonies were demarcated using *cloning cylinders* (Bel Art), trypsinized (0.25% Trypsin, 0.1% EDTA in HBSS) and re-plated for amplification. C2C12 cells heterozygous for the targeted CLP-1 allele were determined by genotype analysis *(see below)*.

Genotype Analysis: CLP-1 wild-type, heterozygote and knock-out alleles were assessed by PCR. DNA was extracted (Stratagene) and genotype was determined by PCR (Huang et al., 2004). The CLP-1 primers were as follows:

Forward primer 5’-AACCTCCTCTCCTTGCGCAACCAACTC-3’

Reverse primer 5’-TACTGTCCTCCTTGGGCACCCGTTCC-3’

Neomycin resistance gene primer:

Reverse primer 5’-TACCGGTGGATGTGGAATGTGTGCGA-3

DNA was amplified for 30 cycles (denaturation: 30 sec at 94°C, annealing: 30 sec at 65°C, and elongation: 30 sec at 72°C). DNA was visualized on a 1.5% agarose gel with ethidium bromide stain.

Immunofluorescence Analysis: C2C12 cells were seeded on glass cover slips in 6 well tissue culture dishes. Cells were fixed with 4% paraformaldehyde in Phosphate-Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) for 15 min at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100 for 10 min. After blocking with 5% Fetal Bovine Serum in PBS for 1 hr at room temperature, cells were incubated with primary antibody overnight at 4°C, followed by washes in PBS and incubation with secondary antibody (1:200) conjugated to either Alexa-488 or Alexa-594 (Invitrogen) for 1 hr at room temperature. For actin staining, cells were incubated for 20 min with 2 units of Alexa-594 phalloidin (Invitrogen), diluted in blocking buffer. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Slides were mounted with Pro-long antifade solution (Invitrogen) and fluorescence was observed using a Zeiss Axiokop microscope at 40X magnification and digital imaging was done using Axio CamMRc camera and Axio vision software.

Cellular Fractionation: C2C12 cells were lysed in Cell Extraction Buffer A (10 mM HEPES, pH 7.4, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA) supplemented with 1 mM dithiothreitol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor cocktail (P-8340 Sigma) and RNasin (Promega). Lysate was homogenized using a Wheaton dounce homogenizer and monitored microscopically with trypan blue. Samples were centrifuged (1,500 X *g*) at 4°C for 5 min. Supernatant was collected as cytoplasmic extract. Pellet was incubated with Cell Fractionation Buffer B (10 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5% NP-40) supplemented with 1 mM dithiothreitol, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail (Sigma) and RNasin (Promega). Samples were subjected to "freeze" on dry ice and

"thaw" at 37°C, with vortexing. After centrifugation (16,000 X *g*) at 4°C for 10 min, the supernatant was collected as nuclear extract.

Chromatin Immunoprecipitation (ChIP) Assay: Proteins were cross-linked to DNA in cultured C2C12 cells with 1% formaldehyde (w/v) culture media (10 min, room temperature). Cells were lysed in cell lysis buffer (10 mM Tris, pH 8.1, 1.5 mM $MgCl_2$, 10 mM KCl) with homogenization using a Wheaton dounce homogenizer and monitored microscopically with trypan blue. Nuclei were sedimented by centrifugation (1,500 X *g*) for 5 min at 4°C. Pellet was resuspended in Nuclear Lysis Buffer (50 mM Tris-HCl, 10mM EDTA, 1% SDS) and subjected to "freeze", on dry ice and "thaw", at 37°C, with vortexing. Chromatin in the cell lysate was sonicated using a Sonic Dismembrator model 100 sonicator (Fisher Scientific), five times for 30 sec each, generating DNA fragments between 250 to 1,000 bp. DNA fragmentation was verified by agarose gel electrophoresis and ethidium bromide stain. DNA was isolated from a fraction of the cell lysate using a PCR purification kit (Qiagen) and concentration was calculated using spectrophotometry at 260 nm. Equal concentrations of DNA were used per immunoprecipitation. The lysates were diluted in ChIP dilution buffer (20 mM Tris, pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.01% SDS, 1% Triton X-100) and antibodies were added overnight at 4°C, with rotation. The immune complexes were collected by the addition of Protein G agarose/salmon sperm DNA (Millipore) for 2 hr at 4°C, with rotation. Sonicated salmon sperm DNA blocked non-specific DNA binding sites on protein G agarose. After extensive washes, the immune complexes were eluted with 1% SDS, 0.1 M $NaHCO_3$ solution. To reverse cross-linked protein-DNA complexes, the

eluted complexes were treated with 190 mM NaCl solution overnight. DNA was purified using a PCR purification kit (Qiagen) and amplified by PCR. Sequence of primers:

Cyclin D1 promoter

Forward primer 5'-GCGCGTACCCTGACACCA-3'

Reverse primer 5'-GTCTTCACGCTTCTCCTCCAG-3'

GAPDH primer control

Forward primer 5'-CGGTGCGTGCCCAGTTG-3'

Reverse primer 5'-GCGACGCAAAAGAAGATG -3'

DNA was amplified for 18 cycles (denaturation: 30 sec at 94°C, annealing: 30 sec at 60°C, and elongation: 30 sec at 72°C). PCR amplified products were run on 2% agarose gel, visualized by ethidium bromide staining. An 8-bit digital camera (Canon) captured the fluorescence.

Animals: CLP-1 knock-out mice have been described previously (Huang et al., 2004). All experiments were performed in accordance with the Guidelines of the National Institute of Health. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Skeletal Muscle Tissue Isolation: To obtain CLP-1 -/- embryos, CLP-1 +/- male and female mice were *time-mated*. Female mice were observed daily for the presence of a "vaginal plug" (Plug = Day 0). At day 12 post-coitum embryos were collected. The head was used for genotype analysis, internal organs were removed and the remaining tissue was collected as skeletal muscle. Tissue was lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1mM EDTA, 1% Triton X-100) supplemented with 1 mM dithiothreitol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and protease inhibitor cocktail

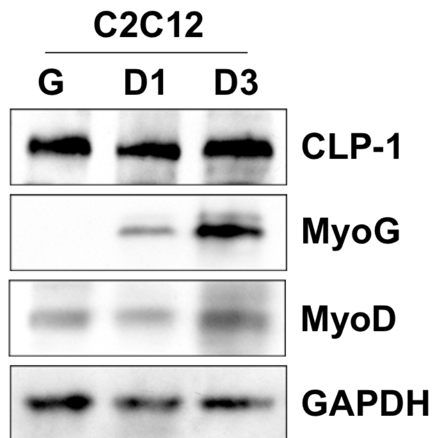
(P-8340 Sigma) and manually homogenized using a Wheaton dounce homogenizer. Protein concentrations were calculated and equal concentrations were analyzed by western blot.

Statistical Analysis: For quantitative western blot analysis, films were scanned and the band signal intensities determined using ImageJ software. The densitometry values were expressed as a fold level relative to the control, and standardized to corresponding total GAPDH densitometry values obtained from the same sample. Data were expressed as means $\pm$SE. To compare two means/groups for significance, a paired t-test was used and $p < 0.05$ were considered significant. Statistical analyses were performed with the Prism Software for Mac (GraphPad Inc.).

RESULTS

CLP-1 expression in C2C12 skeletal muscle cells: To investigate the role of CLP-1 during differentiation of skeletal muscle cells, I first determined the level of endogenous CLP-1 protein in the established mouse myoblast cell line, C2C12. This widely used cell line can be induced to differentiate in culture by lowering serum, which causes confluent cells to uniformly exit the cell cycle and fuse to form multi-nucleated myotubes. I performed western blot on cellular lysates from C2C12 cells that were proliferating in growth media (G) containing 10% fetal bovine serum, and cells induced to differentiate in media containing 2% horse serum for 24 hours (D1) and 72 hours (D3) **(Fig. 2)**. CLP-1 was present in C2C12 cells in both growth and differentiation conditions without marked changes in the protein level. Myogenin, a known marker for differentiation, is expressed in D1 and D3, but not in G. Skeletal muscle specific transcription factor MyoD is expressed in both myoblasts and myotubes, as expected. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

P-TEFb complex in skeletal muscle cell differentiation: Next, I analyzed CLP-1 association to the P-TEFb complex in C2C12 cells. Lysates from C2C12 myoblasts in growth media (G) and myotubes in differentiation media for 24 hours (D1) and 72 hours (D3) were immunoprecipitated (IP) with antibody for cdk9 and western blotted with anti-CLP-1 and anti-cdk9 antibodies. As shown in **Fig. 3**, there was an increase in CLP-1 association with P-TEFb in early differentiation (D1), which was low in both proliferating cells (G) and in the late differentiation (D3) stage. IP with antibody alone served as a control for non-specific immunoreactivity. The association of CLP-1 with the P-TEFb complex in C2C12 cell differentiation was distinct from the pattern of total



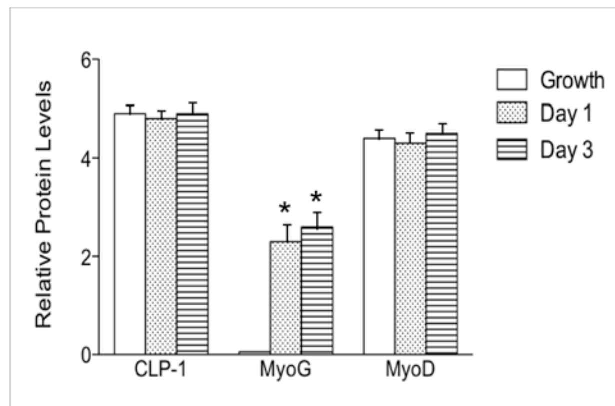


Fig. 2: Western blot analysis of CLP-1 protein expression in C2C12 cell differentiation. C2C12 cells were induced to differentiate and whole cell lysates were collected from growth medium (G), and differentiation medium at 24 hr (D1) and at 72 hr (D3). Equal concentrations of protein were subjected to SDS-PAGE followed by immunoblotting. Myogenin served as a marker of differentiation. GAPDH served as a loading control. Data shown represent one of three separate experiments. Relative CLP-1, myogenin (MyoG), and MyoD levels depicted graphically. GAPDH was used for normalization. Data are mean ± SE ($P<0.05$) as compared to G.

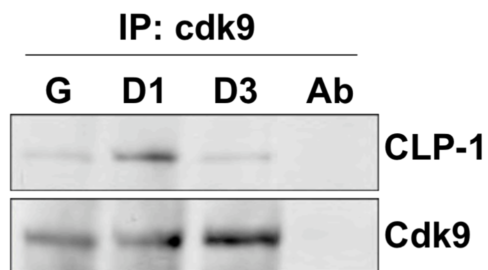


Fig. 3: P-TEFb complex association in C2C12 cell differentiation. Immunoprecipitation (IP) of C2C12 cell lysates from growth medium (G), and differentiation medium at 24 hr (D1) and at 72 hr (D3) with anti-cdk9 antibody and western blotting with anti-CLP-1 and anti-cdk9 antibodies. IP with antibody alone (Ab) served as a control. Data shown represent one of three separate experiments.

protein where the protein level remained the same in all three stages (*See Fig. 2*). The preferential increase of P-TEFb associated CLP-1 at the D1 stage suggests for the first time that perhaps CLP-1 regulates P-TEFb in the transition of cells from growth to differentiation.

Generation of CLP-1 gene knock-down C2C12 cells by homologous recombination: To explore the putative function of CLP-1 in early myogenic differentiation, I chose to knock-down CLP-1 in C2C12 cells by homologous recombination. C2C12 cells were stably transfected with a homologous recombination vector previously created in our laboratory (Huang et al., 2004) **(Fig. 4A)**. The vector contains a neomycin selection cassette in place of the CLP-1 gene, flanked on either side by the chromosomal sequences adjacent to the CLP-1 gene. The CLP-1 gene consists of only one exon. To assess if homologous recombination had occurred, DNA was isolated from stable cells and amplified by PCR using three primers, two that recognize the 5' and 3' ends of the wild-type allele and one that recognizes the 3' end of the recombinant allele. The DNA was visualized on an agarose gel with ethidium bromide staining **(Fig. 4B)**. Negative clones (+/+) in which homologous recombination had not occurred contained one band representative of two copies of the wild-type allele, while heterozygote (+/-) clones contain two bands, one representative of the homologous recombination allele and the other the wild-type allele. The C2C12 CLP-1 +/- cells were also assessed for CLP-1 protein levels by western blot analysis **(Fig. 4C)**. CLP-1 was lower in heterozygote cells as compared to wild-type cells. Levels were quantified (**Fig. 4C**, *right panel*), and the decrease in CLP-1 protein level was statistically significant. GAPDH served as a loading control.

A

Homologous Recombination Vector

Plasmid 5' CLP-1 1.4 kb Neo^r CLP-1 3' 3 kb Plasmid

B

bp M +/+ +/-

500 wild type

400 mutant

C

+/+ +/-

CLP-1

GAPDH

Relative CLP-1 Protein Level

0.0 0.2 0.4 0.6 0.8

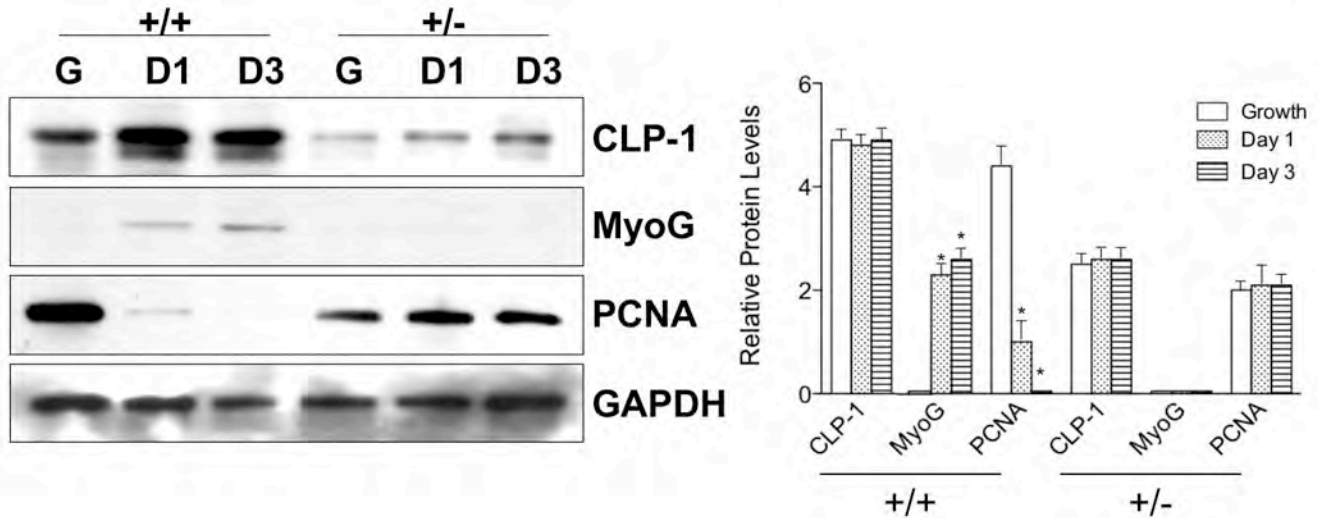
C2C12 CLP-1 +/+ C2C12 CLP-1 +/-

*

Fig. 4: Knock-down of CLP-1 protein through homologous recombination. *A*, Schematic representation of the homologous recombination vector, in which the CLP-1 exon was replaced by a neomycin resistance cassette (Huang et al., 2004). *B*, PCR for CLP-1 gene and mutant allele on DNA from C2C12 CLP-1 +/+ and +/- cells. Primers generate a 457 bp product for the CLP-1 gene and a 383 bp product for the mutated allele. (M) denotes DNA size ladder. Data shows one representative clone. *C*, Western blot of C2C12 CLP-1 +/+ cells and C2C12 CLP-1 +/- cells in growth medium probed with anti-CLP-1 antibody. GAPDH served as a loading control. Western blot shown is one representative clone. *Right panel*, the analysis was performed on three independently isolated C2C12 CLP-1 +/+ and +/- cell cultures and means are depicted graphically, GAPDH was used for normalization. Data are mean $\pm$ SE (*P<0.05).

I then examined whether the reduced level of CLP-1 in CLP-1 knock-down heterozygote C2C12 cells affects the cells ability to undergo differentiation into myotubes. When confluent cells were challenged, by low serum, CLP-1 +/- cells showed a complete block in myotube formation. This was indicated by the absence of differentiation marker myogenin by western blot **(Fig. 5A)**. Also, proliferation was assessed using antibody to Proliferating Cell Nuclear Antigen (PCNA). PCNA was expressed in control cells in growth (G) condition. But in the CLP-1 +/- cells PCNA was present in D1 and D3 as well suggesting that despite low serum in the medium CLP-1 +/- cells do not undergo differentiation. Next to confirm the differentiation deficiency morphologically, I performed immunofluorescene analysis on cells in growth and differentiation medium. Cells were stained with phalloidin actin stain and co-stained with either anti-CLP-1 antibody (red) or with DAPI nuclear marker (blue) (**Fig 5B**). Under differentiation conditions C2C12 CLP-1 +/+ control cells fused into multi-nucleated myotubes (**Fig. 5B,** *second panel*). In contrast, when challenged to differentiate in low serum medium C2C12 CLP-1 +/- stable cells were incapable of entering the differentiation program, remaining mono-nucleated and maintaining a non-differentiated phenotype (**Fig. 5B,** *last panel*).

Association of CLP-1 with MyoD in C2C12 cells: MyoD and the components of P-TEFb, were previously detected in a multimeric complex in both proliferating and differentiated C2C12 cells (Simone et al., 2002). I, therefore, examined the possible association of CLP-1 with MyoD in C2C12 cells. I performed immunoprecipitation with anti-MyoD antibody using lysates from proliferating C2C12 myoblasts in growth media (G) and cells induced to differentiate for 24 hours (D1) and 72 hours (D3) **(Fig. 6)**. IP



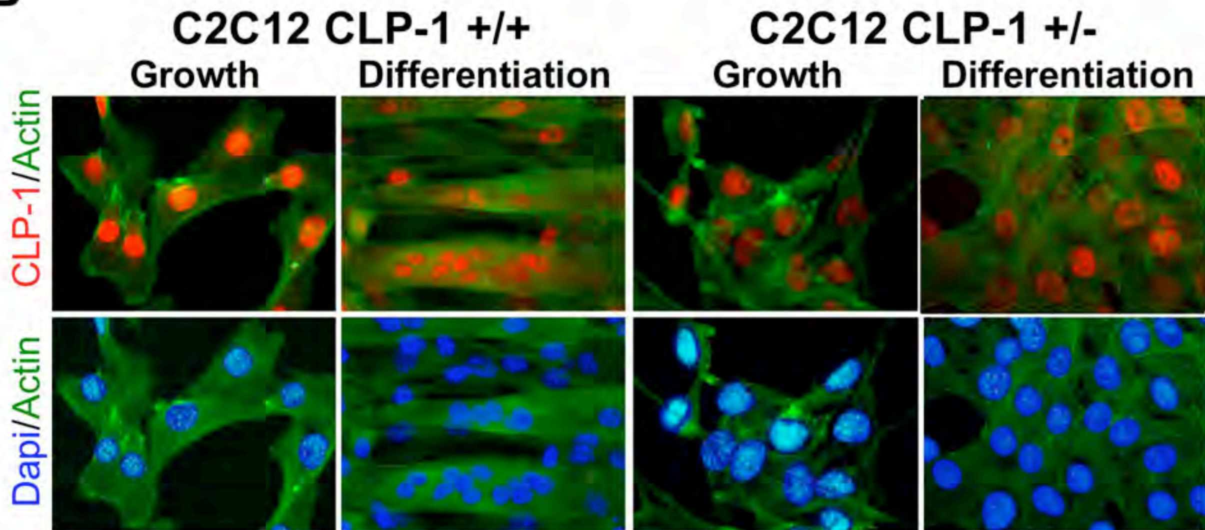


Fig. 5: CLP-1 +/- C2C12 cells are differentiation deficient. *A*, Western blot of control C2C12 CLP-1 +/+ cells and C2C12 CLP-1 +/- cells in growth medium (G), and differentiation medium at 24 hr (D1) and at 72 hr (D3), probed with anti-CLP-1, anti-myogenin, and anti-PCNA antibodies. GAPDH served as a loading control. *B*, Immunofluorescence of C2C12 CLP-1 +/+ and C2C12 CLP-1 +/- cells in growth and differentiation medium with actin-stain (green) and co-stained with anti-CLP-1 antibody (red), or dapi nuclear stain (blue). The analysis was performed on three, independently isolated C2C12 CLP-1 +/+ and +/- cell cultures. Relative CLP-1, myogenin (MyoG), and PCNA levels depicted graphically. GAPDH was used for normalization. Data are mean $\pm$ SE ($P<0.05$) as compared to G.

with antibody alone served as control for non-specific immunoreactivity. The immunoprecipitates were western blotted with anti-CLP-1 antibody. The cells exhibited a robust association between MyoD and CLP-1 in the differentiation stage (D1 & D3) relative to the growth stage (G). There was barely any detectable association of MyoD with CLP-1 in the growth stage (G). Direct western blot of GAPDH served as an input control. The preferential association of CLP-1 with MyoD at D1 suggests, for the first time, that MyoD and CLP-1 are probably synergistically associated in the regulatory aspect of skeletal myogenesis that is restricted to the early differentiation stage.

Association of CLP-1 with HDACs in C2C12 cells: HDACs are implicated in regulation of skeletal muscle by interacting with and regulating MyoD mediated transcription (McKinsey et al., 2001). I raised the question whether CLP-1 regulates myogenesis possibly by recruiting HDACs. I therefore performed co-immunoprecipitation experiments using C2C12 cell lysates as above, with antibodies against HDAC1, 3 and 5 and western blotting for the presence of CLP-1. Results shown in **Fig. 7** indicate that CLP-1 associates with Class I HDACs, 1 and 3 and Class II, HDAC5. Importantly, CLP-1 association with HDACs was highest at the D1 stage of C2C12 cell differentiation. The pattern of association is distinct from HDAC protein levels seen in direct western blotting (**Fig. 7**, *bottom right*). These results are consistent with previous findings in which HEXIM1 associates with HDAC3 in HeLa cells (Fu et al., 2007). This association preferentially at the initiation of differentiation provides new insights into the role of HDACs in myoblast differentiation and into the putative role CLP-1 in control of cell differentiation.

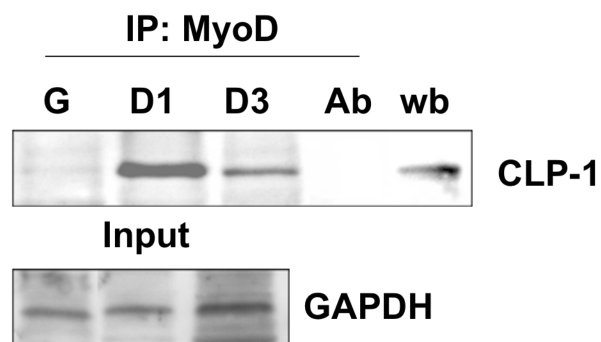
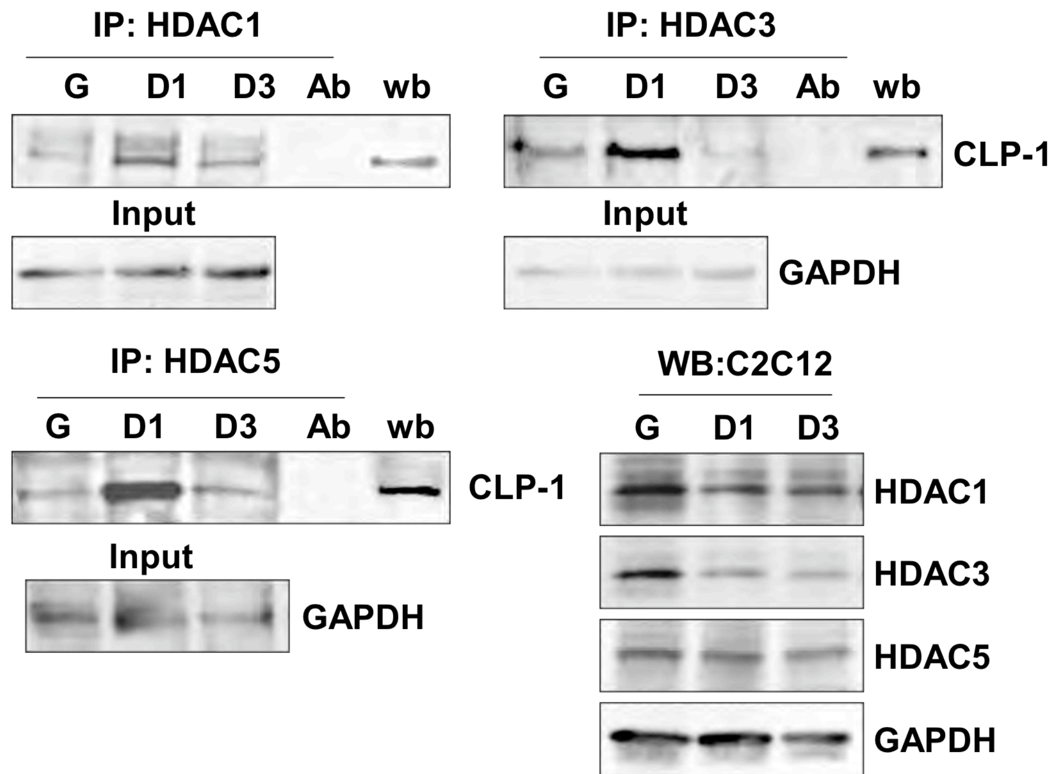


Fig. 6: CLP-1 associates with MyoD at the onset of differentiation. Immunoprecipitation (IP) of C2C12 cell lysates in growth medium (G), and differentiation medium at 24 hr (D1) and at 72 hr (D3) with anti-MyoD antibody and western blotting with anti-CLP-1 antibody. IP with antibody alone (Ab) served as a control. Direct western of CLP-1 (wb) and GAPDH served as input controls. Data shown represent one of three separate experiments.



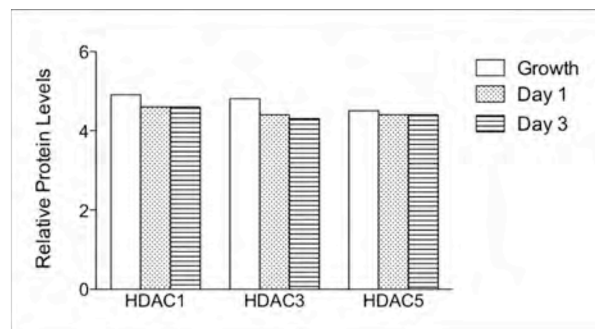


Fig. 7: CLP-1 associates with HDACs at the onset of differentiation. Immunoprecipitations (IP) of C2C12 cell lysates from growth medium (G), and differentiation medium at 24 hr (D1) and 72 hr (D3) with anti-HDAC1, HDAC3, and HDAC5 antibodies and western blotting with anti-CLP-1 antibody. IP with antibody alone (Ab) served as a control. Western blot of CLP-1 (wb) and GAPDH served as an input controls. Data represent one of three separate experiments, for each antibody. *Botom Right,* Direct western blotting of C2C12 cell lysates with anti-HDAC1, 3 and 5 antibodies. GAPDH served as a loading control. Relative HDAC1, 3 and 5 levels depicted graphically. GAPDH was used for normalization. Data are mean $\pm$ SE ($P<0.05$) as compared to G.

Class II HDAC5 is known to translocate from the cytoplasm to the nucleus, while class I HDACs are located in the nucleus only (Gray and Ekstrom, 2001). To examine whether CLP-1 association to HDAC5 is restricted to the nucleus, I performed cell fractionation and co-immunoprecipitation **(Fig. 8)**. By direct western blotting, I observed that CLP-1 was in the cytoplasmic and nuclear fractions, whereas cyclin T1 and MyoD were present in nuclei only and GAPDH was in the cytoplasm only, as expected (*left panel*). Co-immunoprecipitation of the nuclear and cytoplasmic fractions with anti-HDAC5 antibody and western blot with anti-CLP-1 antibody showed that CLP-1 was bound to HDAC5 only in the nuclear fraction (*right panel*).

Association of MyoD with HAT and HDAC: Next, I raised the question whether MyoD association with HDAC1 occurs in growth or differentiation stages. I examined MyoD association with HATs and HDACs using lysates from proliferating cells cultured in growth media (G) and cells differentiated into myotubes for 24 hours (D1) and 72 hours (D3) (**Fig. 9**). I immunoprecipitated with anti-MyoD antibody and western blotted with anti-HDAC1, PCAF, and cdk9 antibodies (*left panel*). IP with antibody alone served as a control for non-specific immunoreactivity. Direct western blot was used to show protein input levels (*right panel*). Results show that MyoD binds to HDAC1 in both growth and differentiation conditions with a preferential increase at D1. Paradoxically, MyoD association with PCAF is also in D1 and it is lower in D3 and G. Previously, it was reported that MyoD and HDAC1 inhibit expression from the myogenin promoter only in growth conditions (Mal and Harter, 2003; Mal et al., 2001). My data indicates that MyoD is able to associate with HDACs preferentially in early

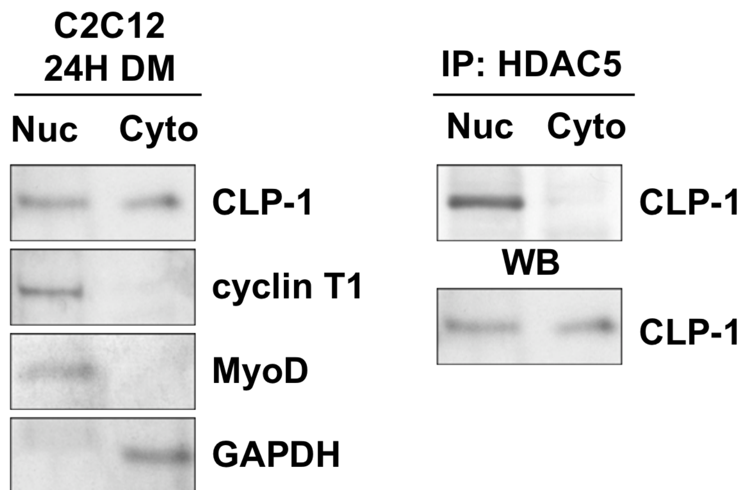


Fig. 8: CLP-1/HDAC5 are nuclear localized. *Left panel*, Cellular fractionation of C2C12 cells in differentiation media for 24 hr followed by direct western blot of nuclear (Nuc) and cytoplasmic (Cyto) fractions with anti-CLP-1, cyclin T1, MyoD and GAPDH antibodies. *Right Panel*, Lysates were subject to immunoprecipitation (IP) with anti-HDAC5 antibody and western blotting with anti-CLP-1 antibody. Direct western (WB) with anti-CLP-1 antibody served as input control.

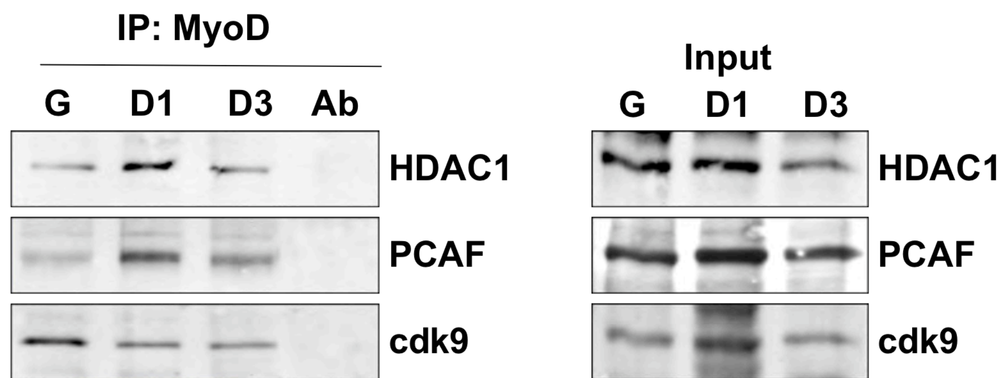
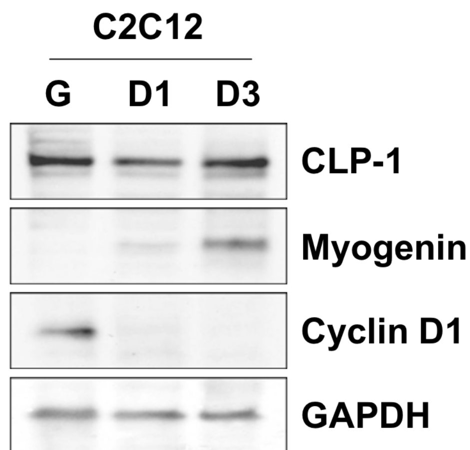


Fig. 9: MyoD associates with PCAF and HDAC1 in myoblasts and myotubes. Immunoprecipitations (IP) using C2C12 cell lysates from growth medium (G), and differentiation medium at 24 hr (D1) and at 72 hr (D3) with anti-MyoD antibody and western blotting with anti-HDAC1, PCAF and cdk9 antibodies. Direct western of HDAC1, PCAF and cdk9 served as input controls.

differentiation conditions and one may envision that MyoD/P-TEFb form a complex with both HDAC and PCAF in differentiation suggesting that both inhibition and activation of distinct gene subprograms involves MyoD, consistent with previous reports (Blais et al., 2005).

Chromatin Immunoprecipitation (ChIP) to localize CLP-1 on MyoD target gene promoter: Since CLP-1 was found to be associated with MyoD and with inhibitory proteins HDACs in early differentiation, I hypothesized that this complex may be involved in down-regulation of cell cycle genes, such as cyclin D1, to allow for expression of differentiation specific genes. I first examined the cyclin D1 protein expression in C2C12 cells by western blotting of total cell lysates from proliferating myoblasts (G), and differentiating myotubes at 24 hours (D1) and 72 hours (D3) **(Fig. 10)**. The results show that cyclin D1 is expressed only in proliferating myoblasts (G), while differentiation marker myogenin was expressed only in D1 and D3. I then performed Chromatin Immunoprecipitation assay (ChIP) to examine whether this multimeric complex was associated to the promoter of MyoD-target cyclin D1 gene. The mouse cyclin D1 promoter contains two E-box sequences upstream to the transcription start site **(Fig. 11A)**. I used C2C12 myoblasts in growth medium (G) and myotubes in differentiation medium for 24 hours (D1) and 72 hours (D3). DNA/protein was cross-linked with paraformaldehyde, nuclear lysates were prepared and DNA was fragmented by sonication. A fraction of the lysate was used for DNA quantification and equal concentrations of DNA were used for IP with antibodies against MyoD, CLP-1, HDAC1, HDAC3, HDAC5, and RNA Pol II. IP with rabbit IgG and anti-actin antibody served as negative controls and PCR of input DNA represents 10% of total chromatin used in each



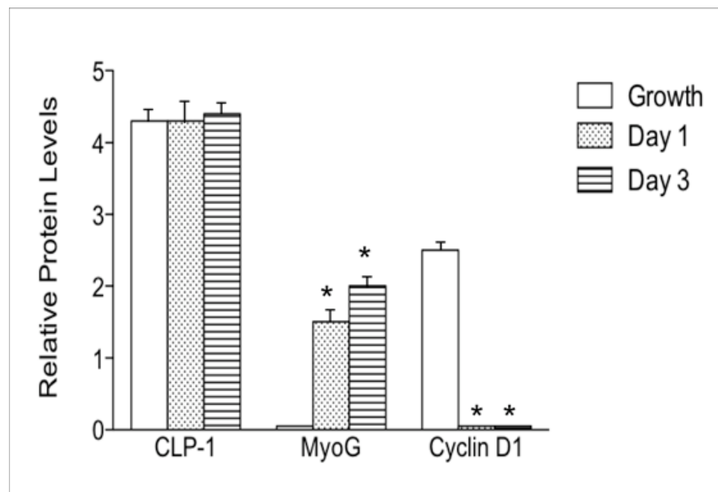


Fig. 10: Western blot analysis for expression of Cyclin D1 protein in C2C12 cell differentiation. Analysis of C2C12 cells from growth medium (G), and differentiation medium at 24 hr (D1) and at 72 hr (D3) by western blotting with anti-CLP-1, myogenin and cyclin D1 antibodies. GAPDH served as a loading control. Relative CLP-1, myogenin (MyoG), and Cyclin D1 levels depicted graphically. GAPDH was used for normalization. Data are mean ± SE ($P<0.05$) for three independent experiments as compared to G.

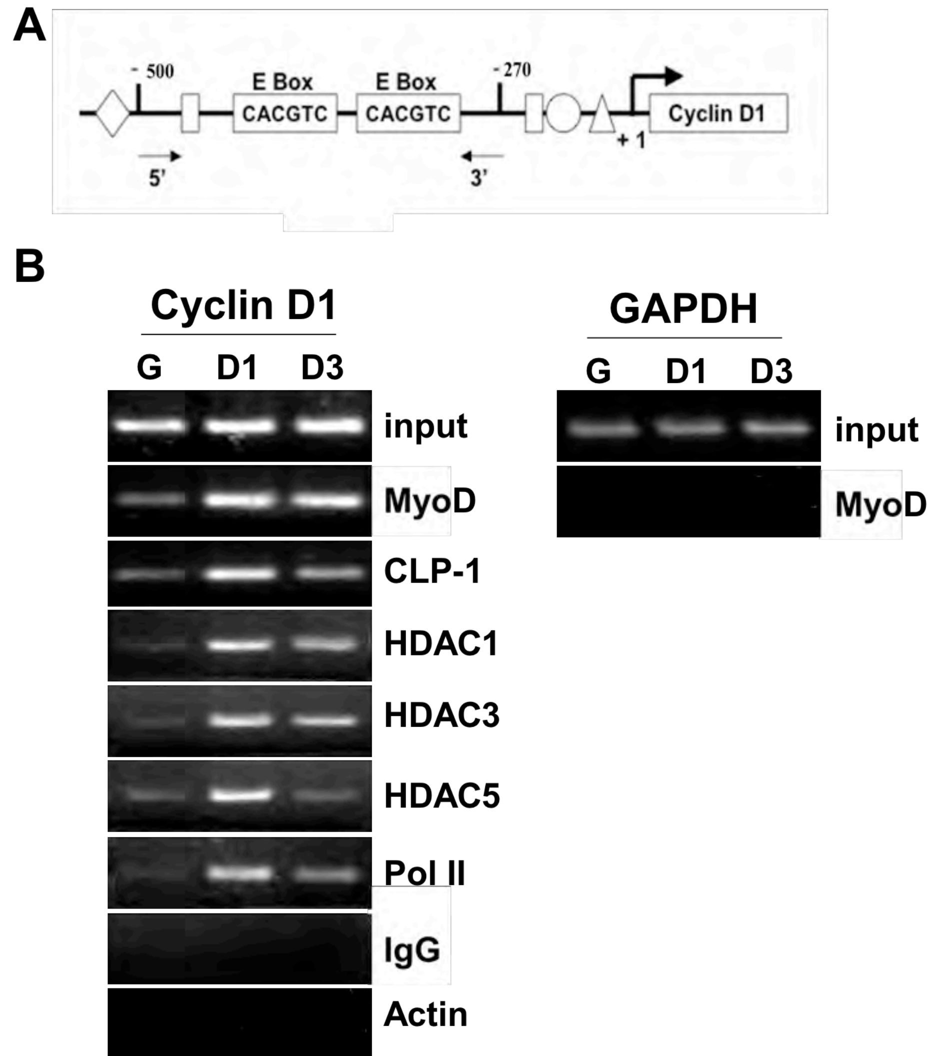


Fig. 11: Chromatin Immunoprecipitation (ChIP) analysis of the Cyclin D1 promoter in C2C12 cell differentiation. *A*, Schematic representation of the genomic region of the mouse cyclin D1 promoter amplified by PCR following ChIP (+1 denotes transcription start sight). *B,* ChIP was performed with anti-MyoD, CLP-1, HDAC1, HDAC3, HDAC5 and RNA Polymerase II (Pol II) antibodies on C2C12 cells in growth medium (G), and differentiation medium at 24 hr (D1) and at 72 hr (D3). A non-specific IgG and anti-actin antibody served as negative controls. Precipitated DNA was amplified by PCR for regions of the cyclin D1 gene corresponding to the 5' upstream promoter region encompassing two E-box sequences. Input DNA represents 10% of total chromatin used in each reaction. Primers specific to GAPDH were used before (input) and after immunoprecipitation as a control to monitor immunoprecipitation specificity. Data shown are one representative of three separate experiments.

reaction. Immunoprecipitated DNA was amplified by PCR using primers flanking two E-box sequences in the cyclin D1 promoter and visualized on an agarose gel by ethidium bromide staining (size 188 bp) **(Fig. 11B)**. Results demonstrate that the CLP-1/MyoD/HDAC containing complex was indeed associated with the cyclin D1 promoter at MyoD-target binding. MyoD and CLP-1 association were maximal in D1 and D3, and HDACs 1 and 3 had little association in G as compared to D1 and D3, while HDAC5 association was highest only in the D1 stage. These results which are consistent with IP experiments above show unambiguously that CLP-1 associates with MyoD and HDACs and the complex binds to promoter DNA of growth-related gene, cyclin D1 in the early stage of differentiation of myoblasts.

Analysis of CLP-1-associated proteins in CLP-1 -/- mice embryos: Finally, I chose to evaluate the function of CLP-1 in skeletal muscle tissue from CLP-1 wild-type, +/-, and -/- embryos at E12. CLP-1 heterozygote male and female mice were *time-mated* and embryos were collected at 12 days post-coitum. DNA was isolated from the embryos for genotype analysis and total protein was isolated from skeletal muscle tissue. Equal amounts of protein was examined by western blotting **(Fig. 12)**. CLP-1 was not expressed in -/- embryos as expected, while CLP-2, a homolog of CLP-1, was expressed. CLP-2 does not compensate for CLP-1, since we reported previously that CLP-1 -/- embryos die at about E17/18. We observed that cdk9 and cyclin T1 levels remained steady while MyoD was down in CLP-1 -/- embryos and differentiation markers myogenin and Myosin Heavy Chain (MHC) were significantly decreased in CLP-1 -/- embryos. These findings suggest that the absence of CLP-1 in the knock-out mice might

lead to markedly reduced myogenesis, which may partly be responsible for the embryonic lethality.

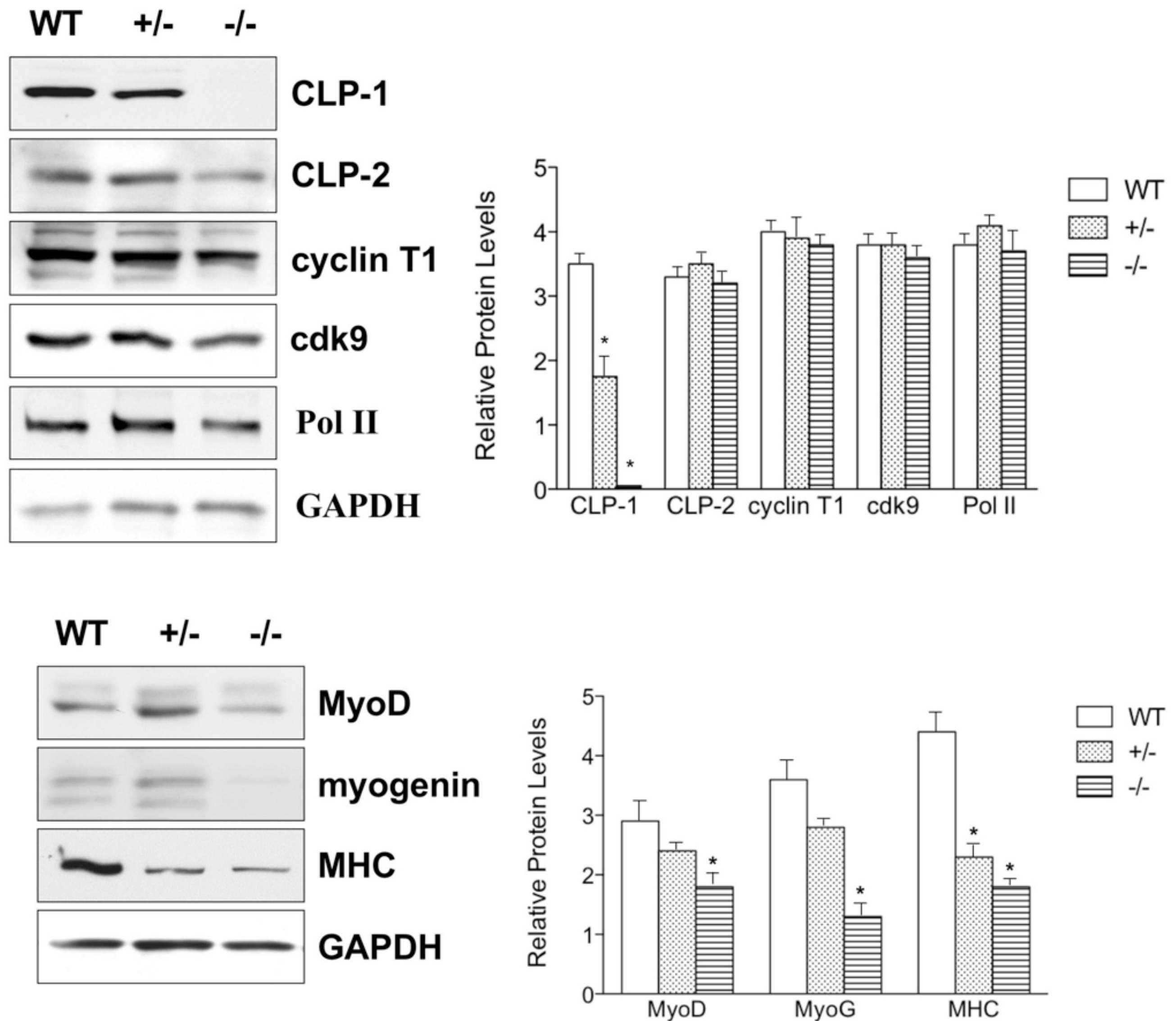


Fig. 12: Analysis of skeletal muscle from embryonic day 12 mice. Western blot of skeletal muscle tissue lysates from mouse embryos, wild-type (WT), heterozygote (+/-), and homozygote (-/-) for the CLP-1 allele using anti-CLP-1, CLP-2, cyclin T1, cdk9, RNA Polymerase II (Pol II), MyoD, myogenin and myosin heavy chain (MHC) antibodies. GAPDH served as a loading control. Data represent one of three separate experiments with one embryo of each genotype per experiment. Relative MyoD, myogenin and MHC levels depicted graphically. GAPDH was used for normalization. Data are mean $\pm$ SE (*P<0.05) as compared to wildtype.

DISCUSSION

Understanding the mechanism(s) of transcriptional control of skeletal muscle cell differentiation is important for future development of therapeutic approaches. Skeletal muscle is a terminally differentiated tissue which is replenished throughout our lives by satellite stem cells (Bischoff and Heintz, 1994; Dhawan and Rando, 2005). Skeletal muscle loss can be caused by pathological conditions such as cancer, AIDs, diabetes and muscular dystrophies and injuries resulting from accidents, disease and trama, as well as prolonged bedrest. In these patients muscle replenishment by satellite cells is insufficient or unavailable and there are limited options for successful treatment.

Mechanistically, muscle cell development and its maintenance is attributed to the critical balance between transcriptional activators and inhibitors and their effect on specific genes. Current data supports the notion that the active recruitment of P-TEFb is crucial for expression of the RNA Pol II transcribed genes (Chao and Price, 2001; Shim et al., 2002). At the transcriptional level, it is evident that the P-TEFb complex plays a role in gene expression, but it is not known how P-TEFb is specifically recruited and what other cellular factors cooperate with P-TEFb. A great deal of information is available on the molecular composition of the P-TEFb complex and its role in transcription elongation, however, there is limited knowledge on the role of P-TEFb in skeletal muscle gene control and certainly the involvement of CLP-1 is totally unknown.

In this study, I have examined the potential function of CLP-1 in regulation of skeletal muscle cell differentiation using C2C12 mouse myoblast cells as an experimental model. These cells are well established and are widely used as a model for analysis of cell differentiation. I observed that CLP-1 is expressed in C2C12 cells in both growth

and differentiation conditions. Its association with P-TEFb, which is reflective of its inhibition of P-TEFb activity, however is preferentially at the early stages of cell differentiation. By knocking down expression of CLP-1 in C2C12 cells, I show that CLP-1 has a direct role in promoting differentiation. The evidence indicates for the first time, that CLP-1 associates in a complex with the skeletal muscle specific transcription factor, MyoD. In addition to MyoD, CLP-1 associates with transcriptional repressors, HDACs. These interactions occur preferentially at the initiation of differentiation providing new insights into the regulatory function of CLP-1 in skeletal muscle differentiation. CLP-1's association with P-TEFb is dynamic and it is maximal during early phase differentiation implying that CLP-1 is functionally connected with the transition of myoblasts to myotubes. A recent report (Nojima et al., 2008) also examined HEXIM1's association with P-TEFb in C2C12 cells and found that HEXIM1 dissociated from P-TEFb 30 minutes after switching to low serum, but the association was restored at 2 hours. However, myogenin is expressed only after 12 hours in low serum medium (Simone et al., 2002), therefore the 24 hour and 72 hour time points are representative of early and established differentiation. It is likely that the increase in association to P-TEFb seen in our system begins at around the 12 hour time point and continues on until about 36 hours (*not shown*).

By reducing CLP-1 expression in C2C12 cells, I observed an arrest in the transition of skeletal myoblast to myotubes, implying that CLP-1, and possibly its association with components of P-TEFb are obligatory to this transition. Since the CLP-1 knock-out mice die at embryonic day 17 to 18, CLP-2 expression does not appropriately substitute CLP-1 function in mice despite high homology between the carboxyl-termini of the two

proteins. The CLP-1 heterozygosity in C2C12 cells was sufficient to arrest differentiation in cell culture whereas CLP-1 +/- mice which survive apparently do not encounter the arrest of differentiation. One may speculate that during embryonic development a compensatory molecule that is not available in the C2C12 cell line substitutes partially for CLP-1, as a result the embryos survive. Also, embryonic growth undergoes defined phases of development while the C2C12 cells are immediately challenged to differentiate and therefore possibly bypass the regulatory subprograms of gene expression. During embryonic development CLP-1 +/- embryos appear slightly smaller than their wild-type litter mates. There may very well be a skeletal muscle deficiency as evidenced by reduced skeletal muscle markers during development, a phenomenon that needs to be examined in the future.

Recent reports have examined the effects of HDACs and HATs associated with the P-TEFb complex. It is known that cdk9 is acetylated *in vitro,* but whether this acetylation enhances or inhibits kinase activity is not settled (Fu et al., 2007; Sabo et al., 2008). Fu et al. (2007) showed that cdk9 was deacetylated by HDAC3, which reduced the association of cdk9 with activator Brd4 and promoted the interaction of P-TEFb with HEXIM1. Sabo et al. (2008) showed that cdk9 acetylation by PCAF reduced kinase function and transcriptional activity of P-TEFb. The cyclin T1 component of P-TEFb can also be acetylated which resulted in a decrease in HEXIM1 bound to P-TEFb, and increased cdk9 kinase activity (Cho et al., 2009). In this study, I observed that CLP-1 associates with HDAC1, 3 and 5 in skeletal muscle and that this association was specifically enhanced in the differentiation stage. HDACs are primarily nuclear, but class II HDACs, which includes HDAC5, can translocate to the cytoplasm. HDAC

translocation is the result of an influx of Ca^{++} in response to extra-cellular signals which triggers G protein coupled receptors and leads to phosphorylation of Class II HDACs by several Ca-dependent kinases (Chang et al., 2005). Upon immunoprecipitation, I found that CLP-1 associated with HDAC5 in the nuclear compartment, consistent with the gene regulatory role of both proteins. CLP-1 association with HDACs in skeletal muscle may act to counter the activity of acetylated cyclin T1 or cdk9. I speculate that inactive P-TEFb bound by CLP-1 is actively recruited to silence specific gene promoters.

To understand the dynamics of MyoD association to HATs and HDACs in C2C12 cell differentiation, I performed co-immunoprecipitations to confirm that MyoD binds to HDACs in differentiated C2C12 cells. Mal et al. (2001) have shown previously that MyoD association with HDAC is highest in growth medium and declines in differentiation. Likewise, a previous report indicated that MyoD associated with PCAF in differentiation medium preferentially as compared to growth medium (Mal and Harter, 2003). My results depict MyoD association with HDAC1 in growth and differentiation conditions and show that association with PCAF is less in growth and increases upon differentiation. My findings suggest that the role of MyoD as a repressor or activator is not bound to the growth or differentiation stage respectively, but rather to its association with interacting proteins (HDAC, HAT, CLP-1, etc.).

In this report, I also show that MyoD and HDACs associate with CLP-1 maximally at 24 hours of C2C12 cell differentiation. This would suggest that perhaps both CLP-1 and HDACs are recruited by MyoD to offer an environment optimal for repression of proliferative genes allowing the transition of myoblasts to myotubes. At this junction, one would expect that MyoD must also associate with HAT proteins in a

separate protein complex to facilitate the promotion of muscle specific gene expression. I noted that MyoD associates with HDAC1 and PCAF in the differentiation stage. I believe that both suppression and activation of MyoD activity are likely to co-exist in C2C12 cells at the onset of differentiation to act on distinct promoters.

Since proliferation and differentiation are mutually exclusive processes in myogenesis, I hypothesize that CLP-1's primary role is in down-regulation of proliferative genes. Cell cycle progression occurs via cyclin proteins, each of which binds and activates a specific cdk partner protein. The D type cyclins activate cdk4 and 6, and cyclin E activates cdk2 (Baldin et al., 1993). Normal progression through the G_1 phase of the cell cycle requires cyclin D and cyclin E. Cessation of proliferation by down-regulation of cell cycle genes, such as cyclin D1, is required for initiation of muscle specific gene expression. By direct western blotting of C2C12 cell extracts, I showed that in differentiated C2C12 cells cyclin D1 protein is not expressed. Using the ChIP assay, I localized CLP-1 and its associated proteins, MyoD and HDACs, on the cyclin D1 promoter in C2C12 cells preferentially in the differentiation stage. HEXIM1 expression has been reported to directly correlate with Estrogen receptor-inducible cyclin D1 protein expression and to bind to the E_2-responsive region on the cyclin D1 promoter (Ogba et al., 2008). The regulation of the cyclin D1 gene is complex; multiple sites within the cyclin D1 promoter contribute to the transcriptional output. A more comprehensive analysis using primers to different sites within the promoter will reveal which sites are regulated by CLP-1 mediated inhibition. My studies nonetheless suggest that CLP-1 regulates skeletal muscle by promoting cell cycle exit, which in turn allows for the up-regulation of skeletal muscle specific gene transcription.

Mouse embryonic development is highlighted by distinct stages of muscle cell specification, determination and differentiation, while in the adult the muscle cells are terminally differentiated. The advantage of using C2C12 cells is that the growth and differentiation stages are distinct. For comparison, I looked at skeletal muscle tissue in CLP-1 null embryos. By western blotting of tissue lysates, I showed that in CLP-1 +/- and -/- embryos, differentiation-specific proteins, myogenin and MHC are down regulated. The decrease in expression of skeletal muscle proteins in CLP-1 null embryos suggests that there is a deficiency in the myogenic lineage within these embryos and this may contribute, in part, to the embryonic lethality.

Upon C2C12 cell differentiation two changes in gene programs occur, cell cycle exit and up regulation of skeletal muscle specific genes. Simone et al. (2006) previously reported that upon cell cycle exit P-TEFb and MyoD act to up-regulate muscle genes myogenin and MHC (*See Model* **Fig. 13**, *right panel*). In my work, I examined the cell cycle exit component. Although CLP-1 expression is ubiquitous, it is likely that CLP-1 is recruited to target proliferative genes through interaction with MyoD (*See Model* **Fig. 13**, *left panel*). Another possibility is that P-TEFb is already on the promoter DNA with Pol II for cyclin D1 gene expression and CLP-1 and HDAC arrive to inhibit P-TEFb. Based on my results I can surmise that MyoD guides CLP-1 to MyoD-target DNA. CLP-1 was reported to bind directly to HDACs (Fu et al., 2007), therefore, its recruitment to MyoD may be mediated either by association with HDACs or with P-TEFb. It is likely that CLP-1 association with HDACs is functionally significant acting to oppose the HATs that has been reported to associate with active P-TEFb and inhibit transcriptional activity of MyoD. These hypotheses are speculative and require experimental validation.

The signaling that triggers specific interaction of these molecules is not known, neither is the sequence of association of various proteins and the signaling events that dictate the target promoter. Future studies to characterize the fundamental regulatory events mediated by CLP-1 would provide further insights into CLP-1's physiological role in skeletal muscle cells. One potential pathway, which is of interest to our laboratory is the Jak2/STAT3 signaling pathway. It was recently reported that upon C2C12 differentiation there is an increase in activated STAT3 (Wang et al., 2008; Yang et al., 2009). Also, it was reported that STAT3 associates with MyoD at about 12 hours of differentiation (Yang et al., 2009). It will be interesting to see if CLP-1 can potentially regulate MyoD associated STAT3, specifically in C2C12 cell differentiation. Also, it will be interesting to look further into the skeletal muscle phenotype in the CLP-1 +/- mice and CLP-1 -/- embryos. Specifically we could look at the muscular make up of the mice, such as the ratio of "slow-twitch" to "fast-twitch" muscle fibers. Also, the CLP-1 +/- mice can be subjected to stress, such as muscle injury or swimming, to see if CLP-1 deficiency has an effect on regenerative capacity of skeletal muscle. Furthermore, it is plausible that CLP-1 +/- cells can be used in muscle replenishment of adult muscle similar to stem cells. This could be studied using a muscle disease model such as muscular dystrophy. Since CLP-1 +/- cells maintain their proliferative capacity maybe they offer a unique opportunity to regenerate muscle in dystrophic mice.

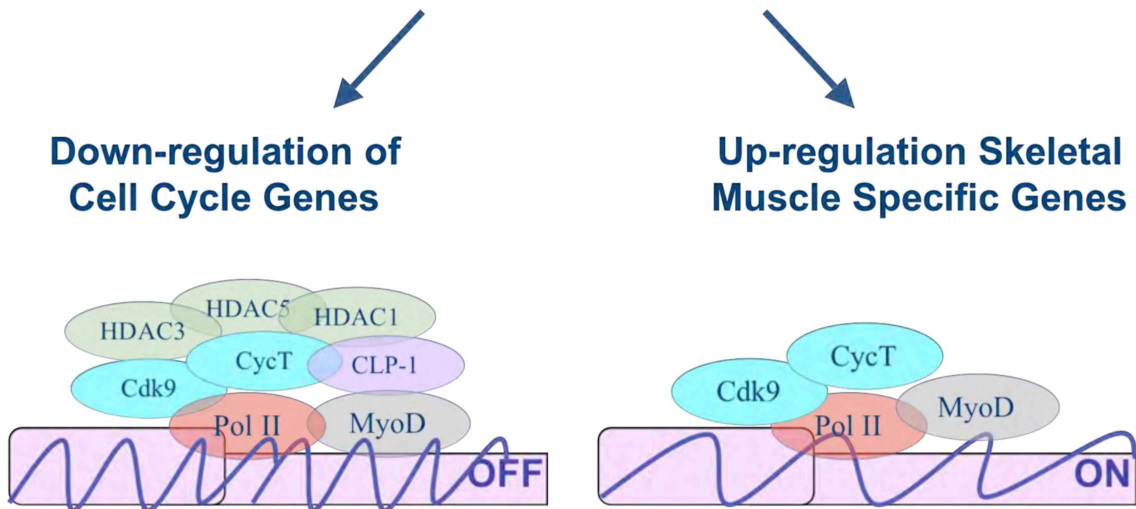


Fig. 13: Putative model for the role of the CLP-1/MyoD/HDAC complex in C2C12 cell differentiation. Upon cell differentiation, skeletal muscle myoblasts must permanently exit the cell cycle by turning *off* expression of proliferative genes potentially through cooperativity of CLP-1 and HDACs with MyoD and P-TEFb (*left panel*). While for up-regulation of skeletal muscle specific gene transcription it is likely that CLP-1/HDACs are released from MyoD/P-TEFb (*right panel*).

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APPENDIX

In addition to my thesis work above, I was involved in the following experiments that were published in the Journal of Cellular and Molecular Medicine (Mathew et al., 2009).

Nished, a novel transcription factor, was isolated in our laboratory from a cDNA library created from the mesodermal germ layer of a chicken embryo (Dhar et al., 1997). The cDNA called Nished contains an insert of 1167 bp with an open reading frame of 137 amino acids, which encodes a protein of about 15 kDa. Our laboratory has shown that Nished mediates transcription of cardiac specific Myosin Light Chain-2v (MLC-2v) gene via binding to two regulatory elements, the positive Intronic Regulatory Element (IRE) and the negative upstream element, Cardiac Specific Sequence (CSS) (Mathew et al., 2004). Nished acts as a positive regulatory protein by its association with histone acetylase protein p300 and binding to the IRE element of MLC-2v gene which was reported to promote cardiac specific MLC-2v gene expression (Mathew et al., 2004).

Our laboratory hypothesized that Nished may also act as a negative regulator. Our laboratory has shown that Nished and the co-repressor mSin3A are part of the same complex on the CSS in non-cardiac tissue (Mathew et al., 2004). mSin3a does not directly interact with DNA binding proteins, rather it functions as a scaffold upon which proteins like HDACs associate to form a co-repressor complex (Ayer, 1999). To investigate the mechanism of Nished's negative regulatory role, I examined the MLC2v gene promoter in skeletal muscle cells by Chromatin Immunoprecipitation (ChIP) assay. I used antibodies to histone H3 Lysine 9 mono methylated a marker of inactive chromatin to show that the IRE was in the inactive conformation in non-cardiac tissue (*See* **Fig. 3B**

in manuscript attached). Also, I stably transfected C2C12 mouse skeletal muscle cells with an expression vector for Nished. Clones were antibiotic-selected, and several independent colonies were picked and screened for Nished overexpression (*See* **Fig 5A** *in manuscript attached*).

To further investigate the mechanism by which Nished inhibits MLC2v gene transcription in non-cardiac tissue, I examined the possibility that HDACs may act in conjunction with Nished at the CSS. To investigate if HDAC's participate in the Nished co-repressor complex, I performed transient luciferase expression assays in C2C12 mouse skeletal muscle cells using MLC2v promoter + luciferase (pMutIRELuc) in which IRE was mutated and CSS was intact, cotransfected with Nished expression vector. Results showed that Nished represses the promoter activity. When HDAC inhibitor Tricostatin A (class I and II) was used the repression was relieved in a dose-dependent manner. Class II HDAC inhibitors Sirtinol and M15 showed no effect suggesting the selectivity for the class I and II HDACs (*See* **Fig. 7A** *in manuscript attached*).

Next, I investigated whether Nished associates with endogenous HDAC's. I performed co-immunoprecipiations using lysates from mouse skeletal muscle tissue lysates and found that Nished interacts with HDAC3 and 5 and not with HDAC 1 or 4. (*See* **Fig. 8A** *in manuscript attached*). To identify the HDAC binding domain in Nished I used in vitro transcription/translation pull-down assays with Nished deletion mutants previously created in our lab and Flag tagged HDAC3 and HDAC5 expression vectors (kindly provided by Dr. Eric Verdin) (*See* **Fig. 8B** *in manuscript attached*). I found that amino acids 37-64 of Nished interact with HDACs.

These experiments provide new insights as to how a cardiac specific gene is kept in the off-state in non-cardiac tissue. I examined two regulatory elements IRE and CSS that were previously characterized in out laboratory and showed that in non-cardiac tissue the IRE is inaccessible because it is in the closed conformation. Also, I showed that Nished is in a complex with transcriptional repressor proteins HDACs.

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Repression of the cardiac myosin light chain-2 gene in skeletal muscle requires site-specific association of antithetic regulator, Nished, and HDACs

Sumy Mathew, Josephine Galatioto, Eduardo Mascareno, M.A.Q. Siddiqui *

Center for Cardiovascular and Muscle Research and Department of Anatomy and Cell Biology, State University of New York Downstate Medical Center, Brooklyn, NY, USA

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Abstract

The transcriptional activation mechanisms that regulate tissue-specific expression of cardiac muscle genes have been extensively investigated, but little is known of the regulatory events involved in repression of cardiac-specific genes in non-cardiac cells. We have previously reported that Nished, a ubiquitous transcription factor, interacts with a positive sequence element, the Intron Regulatory Element (IRE) as well as a negatively acting element, the Cardiac-Specific Sequence (CSS), in myosin light chain-2 (MLC2v) gene to promote activation and repression of the gene in cardiac and skeletal muscle cells respectively. Here, we show that the negative regulation of cardiac MLC2v gene in skeletal muscle cells is mediated *via* the interaction of Nished with histone deacetylase (HDAC) co-repressor. Treatment of cells with the HDAC inhibitor, Trichostatin A (TSA), alleviates the repressor activity of Nished in a dose-dependent manner. Co-transfection studies in primary muscle cells in culture and in Nished expressing stable skeletal muscle cell line demonstrate that Nished down-regulates the cardiac MLC2 gene expression when its association is restricted to CSS alone. Chromatin immunoprecipitation data suggest that the CSS-mediated repression of cardiac MLC2v gene in skeletal muscle cells excludes the participation of the positive element IRE despite the presence of an identical Nished binding site. Taken together, it appears that the negative control of MLC2v transcription is based on a dual mode of regulations, one that affords inaccessibility of IRE to Nished and second that promotes the formation of the transcription repression complex at the inhibitory CSS site to silence the cardiac gene in skeletal muscle cell.

Keywords: Nished • myosin light chain-2v • transcription repression • histone deacetylases

Introduction

Repression of gene transcription is obligatory for establishment of cell-specific gene expression and cellular differentiation. Eukaryotic gene transcription is regulated in part by the enzymatic activity of histone acetylases (HATs) and deacetylases (HDACs) (see ref. [1 and 2] for review). Analysis of genetically manipulated mice has documented the functions of these chromatin-modifying enzymes as central to control of gene expression in an animal context. HDACs promote chromatin compaction and repress transcription by limiting the access of transcription factors to DNA. Hypoacetylated histones, therefore, are generally found in transcriptonally silent genes. Several recent studies have implicated HDACs in control of muscle development and differentiation through their association with myogenic transcription factors MEF-2 and MyoD to their cognate binding sites [3–5]. The class II HDACs (HDACs 4,5,7) associate with MEF-2 and inhibit the MEF-2-dependent activation of target genes. The class I HDACs 1,2,3, on the other hand, do not interact with MEF-2, but associate with MyoD and inhibit the MyoD-mediated gene transcription that involves recruitment of co-repressors [6]. During skeletal myogenesis, MyoD-dependent expression of muscle gene requires the association of MyoD with members of the MEF-2 family. Thus, the events that target the chromatin remodelling enzymes for interactions with myogenic transcription factors are important and orchestrate the positive and negative regulation of muscle genes during myogenic cell development and differentiation.

*Correspondence to: M.A.Q. SIDDIQUI,
Box 5, Department of Anatomy & Cell Biology,
State University of New York Downstate Medical Center,
450 Clarkson Avenue, Brooklyn, NY 11203, USA.
Tel.: 718-270-1014
Fax: 718-270-3732
E-mail: maq.siddiqui@downstate.edu

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The cardiac muscle cell-restricted expression of myosin light-chain 2 (MLC2v) gene offers an attractive experimental paradigm for uncovering the role of specific transcription factors and their co-regulators in modulation of gene activity in response to different muscle cell type signalling [7–9]. The expression of chicken cardiac MLC2v is regulated positively in cardiac muscle and negatively in skeletal muscle cells, even though the basal promoter architecture of both cardiac and skeletal muscle MLC2 genes is almost identical. In order to understand the underlying mechanism(s), we have identified and characterized several cis-elements in MLC2v gene and their cognate DNA binding proteins [10–15]. The activation of chicken MLC2v gene transcription is orchestrated by the combinational interaction of MEF-2 [7], SRF [11] and Nished [15] with the proximal promoter elements in concert with the co-activators NFAT-c4 and p300 [15], whereas its inhibition in skeletal muscle cell requires an intact upstream element (CSS) [10] and a downstream modulator element [14]. We have recently reported that a ubiquitous transcription factor, Nished, recognizes the palindrome sequence present in both the negative CSS and the positive IRE elements to facilitate the negative and positive transcription activity of MLC2v gene, respectively [15].

In this report, we provide evidence that Nished promotes repression of the cardiac MLC2v in skeletal muscle cells *via* its association with histone deacetylases co-repressor complex at the CSS site. The HDAC inhibitor, TSA, releases the Nished-mediated repression of MLC2v gene transcription in a dose-dependent manner. Chromatin immunoprecipitation data suggest that IRE is inaccessible to Nished in skeletal muscle cells affording thereby the preferential binding of Nished to CSS and the concurrent repression of MLC2v gene transcription. Taken together, our data define the mechanism in which Nished plays a key role *via* its interaction with HDAC. In addition, the inaccessibility of the activator IRE site influences the formation of a functional complex promoting the repression of the cardiac MLC2v gene in skeletal muscle cell.

Materials and methods

Construction of mutant CSS and IRE reporter plasmids

A 2.1 kb Sma I/Stu I blunt-ended fragment of MLC-2v gene derived from pILC5.2 [16] was cloned into the Sma I site of the promoterless vector, pGL2Basic, that carries the coding region for firefly (Photinus pyralis) luciferase (Promega, Madison, WI, USA) to generate the pMLC2.1Luc reporter plasmid. GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) was used to introduce mutations within CSS and IRE sequences of pMLC2.1Luc as per the manufacturer's instructions. Mutagenic oligonucleotides for CSS and IRE were synthesized (see below). The underlined sequence denotes the mutation within the core motif. CSSMutB: 5′-CGAGGAGGTAGTACTACCCTGAAGCAAAAG-3′ MutIRE: 5′-GCAGAGAGCAAGGGTACCCCGGGGGTCTGATGGC-3′. CSS oligonucleotides corresponding to −355 to –323 bp as shown below were used as probes for GMSA. CSS 5: 5′-GACGAGGAGGTACTTCTACCCTGAAGCAAAAGG-′3 and CSS 3: 5′-CCTTTTGCTTCAGGGTAGAAGTACCTCCTCGTC-3′.

Cloning of Nished cDNA sequence in mammalian expression vector pcDNA6 V5/HisB

The coding region of Nished was amplified by PCR using the primer pair Nished-V5 5′Hind III: 5′-CCCAAGCTTGCCACCATGTGCAGGAATTCCCGCCA-3′ and Nished-V5 3′Not 1: 5′-ATAAGAATGCGGCCGCCCCCGGGAGGTGACAGAAGTGA-3.' The amplified PCR DNA fragment was cloned into pcDNA6-V5-HisB vector DNA and transformed into DH5a competent cells. Plasmid DNA isolated from the clones was sequenced to ascertain that no errors were introduced into Nished cDNA during PCR and that it was in frame with the C-terminal V5 epitope.

Generation of N-terminal and C-terminal mutants of Nished

The primer pair used to generate the N-terminal mutants, (pNΔ1) are NΔ1 5′ Hind III: 5′-ACCAAGCTTGCCGCCACCATGAACAAGGGAGGA-3′ where the underlined sequence corresponds to 177–188 bp of Nished cDNA and NΔ1 3′ Not I: 5-′ ATAAGAATGCGGCCGCCCCCGGGAGGTGACAGAAGTGA-3′ where the underlined sequence corresponds to 468–488 bp of Nished cDNA. The primer pair for pNΔ2 corresponding to 345–356 bp and 468–488bp of Nished cDNA are NΔ2 5′ Hind III: 5′ACCAAGCTTGCCGCCACCATGATGCAGCAGCCA-3′ and NΔ2 3′ Not I: 5′-ATAAGAATGCGGCCGCCCCCGGGAGGTGACAGAAGTGA-3′. To generate the C-terminal mutants, pCΔ1 primer pairs corresponding to 78–97 bp and 252–269 bp are shown below: CΔ1 5′ Hind III: 5′-CCCAAGCTTGCCACCATGTGCAGGAATTCCCGCCA-3′ and CΔ1 3′ Not I: 5′-ATAAGAATGCGGCCGCCTGCCCAGACAAAGCCGAA-3′. The primer for pCΔ2 corresponds to 78–97 bp and 405–422 bp are CΔ2 5′ Hind III: 5′-CCCAAGCTTGCCACCATGTGCAGGAATTCCCGCCA-3′ and Cdel2 3′ Not I: 5′-ATAAGAATGCGGCCGCCGCACGCAAACCAGAACCC-3′. N-and C-terminal mutants were cloned into the mammalian expression vector, pcDNA6-V5/HisB. ^{35}S-methionine was used to synthesize radiolabelled proteins and the gel was subjected to fluorography using EN3HANCE (NEN). After electrophoresis, the gel was fixed in 10% glacial acetic acid, 30% methanol for 1 hr after which it was impregnated with ENHANCE, washed, dried and exposed to X-ray film.

Cell culture

Hearts of the 11-day-old White Leghorn chicken embryos (Charles River Laboratories, CT, USA) were collected for primary cardiac skeletal muscle cell cultures as described previously [10, 15]. C2C12 mouse myoblast cells were grown in DMEM containing 20% heat-inactivated foetal bovine serum. Primary cells were transfected using FuGENE 6 transfection reagent according to the manufacturer's conditions (Roche Applied Science) with 1 μg of luciferase vectors mixed with 100 ng of pcDNA6 vector, Nished-V5 or its N-and C-terminal mutants. C2C12 cells were transfected as above with 1 μg Nished-V5 or pcDNA6 vector. After Blasticidin S selection (10 μg/ml), individual clones were selected based on the existence of Nished and/or Blasticidin S resistant gene (BGH) transcripts.

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Gel mobility shift assay (GMSA)

Tissues from either fresh or frozen embryonic hearts and skeletal soleus muscles were minced finely and the dissociated cells were lysed in lysis buffer as described previously [10, 15]. Nuclei were lysed in the nuclei lysis buffer (20 mM HEPES, pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100) with 1 mM DTT, 1 mM PMSF, 2.5 mM Na-vanadate, 10 mM NaF and protease inhibitor cocktail (Sigma, P8340; Sigma, St Louis, MO, USA) and extracts were used for binding reactions [7, 10, 15]. Protein -DNA complexes were separated by electrophoresis on 6–8% non-denaturing polyacrylamide gels as previously described [7, 13]. When Nished antibodies were used, 5 ml of 1:2.5 diluted antibodies in PBS were pre-incubated on ice with nuclear extracts for 20–30 min. followed by incubation with radiolabelled probe. Similar conditions were used for pre-immune serum incubations. NIH Image (version 1.57) software was used to determine the relative density of the DNA-protein complexes.

Immunoprecipitation and Western blot analysis

For co-immunoprecipitations, 10 μg of antibody against HDAC 3 (ab47237) and 5 (ab1439) (Abcam) was incubated overnight with 2.5 mg of pre-cleared cell or tissue lysate. Western blotting was carried out as described earlier with anti-Nished antibody [15].

Microaffinity purification

Microaffinity purification of CSS or IRE-binding proteins was performed using chemically synthesized CSS or IRE oligonucleotides containing 5′ biotin (Bt) on a flexible linker (Invitrogen). Forty pmol of duplex Bt-IRE was incubated with 500 μg of cardiac and skeletal muscle nuclear extracts from embryos or with ^{35}S methione labelled *in vitro* translated proteins in the presence of 5 μg of poly (dI/dC) in a 1000-μl volume of a buffer containing 4.5% (v/v) glycerol, 5 mM $MgCl_2$, 10 mM KCl, 0.42 mM EDTA, 0.8 mM DTT, 4 mM HEPES (pH 7.5) for 1 hr at 4°C. Proteins bound to Bt-CSS or -IRE were captured by addition of 50 μl of a 50% (v/v) slurry of streptavidin-agarose beads (Thermo Scientific, Pierce, Rockford, IL, USA) and washed twice in binding buffer. Bt-CSS or IRE-binding proteins were then eluted by SDS-PAGE loading buffer for Western immunoblot analysis.

ChIP assays

The ChIP assay was perfromed following manufacturer's protocol (ChIP Assay Kit Upstate 17-295). The frozen tissue (−100–500 mg) was cut into small pieces with a razor blade and cross-link with 1% formaldehyde in 1× PBS plus protease inhibitor Sigma (P8340) for 15 min. at room temperature. Then, the formaldehyde was quenched with glycine at room temperature for 5 min. Tissue was homogenized on ice in 1× PBS plus protease inhibitors. The cell pellet was washed twice with cold 1× PBS with protease inhibitors and lysed in SDS-lysis buffer (50 mM Tris-HCl, ph = 8.1, 1% SDS, 10 mM EDTA) with protease inhibitors. Then, the extracts were sonicated with 30 sec. pulses (30 times) and resuspended in Chip Dilution Buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 0.01% SDS, 1% Triton X-100, 2 mM EDTA). The immunoprecipitation was performed by pre-clearing with Salmon Sperm DNA/Protein A Agarose-50% slurry for 30 min. Then, 5 ug of antibodies H3K4M3, and H3K9M1 (Abcam) were incubated with 10–15 mg DNA/sonicated extract overnight with rotation at 4°C followed by addition of 60 ml protein A, per IP and incubated 1 hr at 4°C. The immunoprecipitates were washed as recommended by the manufacturer (Upstate ChIP assay kit) for 5 min., 1 wash low salt immune complex wash buffer, 1 wash high salt immune complex wash buffer, 1 wash LiCl immune complex wash buffer, 2 washes 1× TE buffer. The elution was obtained with 1% SDS, 0.1 M $NaHCO_3$. Rotate 15 min. 250 ml 2 times. Add 20 ml of 5 M NaCl and reverse cross-linked at 65°C overnight. Add 10 ml of 0.5 M EDTA, 20 ml 1 M Tris-HCL, pH 6.5, 1 ml 20 mg/ml proteinase K, 45°C 1 hr. The DNA was recovered using the Qiagen QIAquick kit followed by PCR. The primer pairs used for the PCR reactions are; IRE-F: 5′-CCTGTG-GCACATGCGTTCTCATT-3′, IRE-R: 5′-TGCCCCCAAATGACCTGTGG-3′, GAPDH-F: 5′-AGAGAGCTCGATGGGGATG-3′, GAPDH-R:5′-CCGTTGACTC-CGACTTTCAC-3′, and ApoB-F: 5-′AAAACCAACCCAACAACTGG-3′, ApoB-R: 5′-CCACCTCAGAGGGAGAATGA-3′.

Statistical analysis

Data are expressed as mean ± S.E. Differences between experimental groups were evaluated for statistical significance using either one sample t-test with Bonferroni correction or one-way ANOVA test. Tamhane's test were performed *post hoc* to test for significant differences; *P*-values <0.05 were considered statistically significant.

Results

Role of CSS and IRE in tissue-specific expression of MLC2v gene

We have previously identified a negative regulatory cis-element (CSS) in the MLC2v promoter that contains a palindrome sequence 5′GAAGCTTC3′ [10]. We observed subsequently that an identical sequence motif resides in the downstream activator element IRE located in the first intron of the MLC2v gene [15]. To examine the possibility of functional interaction between CSS and IRE in transcription regulation of the cardiac MLC2v gene, transient transfection was done with gene constructs that contain mutations in either IRE or in both IRE and CSS sequences. Specifically, the GAAGCTTC palindrome in IRE was mutated in the 2.1 kb SmaI-StuI genomic fragment that extends from −1293 to +815 bp and encompasses the promoter and the first intron of the gene. The DNA fragment was linked to the luciferase reporter (pMutIRLuc) (see Materials and Methods). The same domain was also mutated in CSS as above to generate a double mutant pMutCSS+pMutIRELuc (Fig. 1A). The activity of these constructs was examined in transient transfection assay. In the wild-type construct, the IRE positive activity apparently overrides the inhibitory activity of CSS. Mutation in IRE in pMutIRELuc resulted in a significant loss (70%) of the promoter function. However, when the same mutation was introduced in CSS as well as in IRE in the double mutant pMutIRE+MutCSSLuc, there was a loss of inhibition (Fig. 1B), suggesting that CSS and IRE interact with common DNA binding protein(s) that recognizes the conserved GAAG/CTTC motif present in both regulatory elements. Indeed, we have previously identified a transcription factor, Nished,

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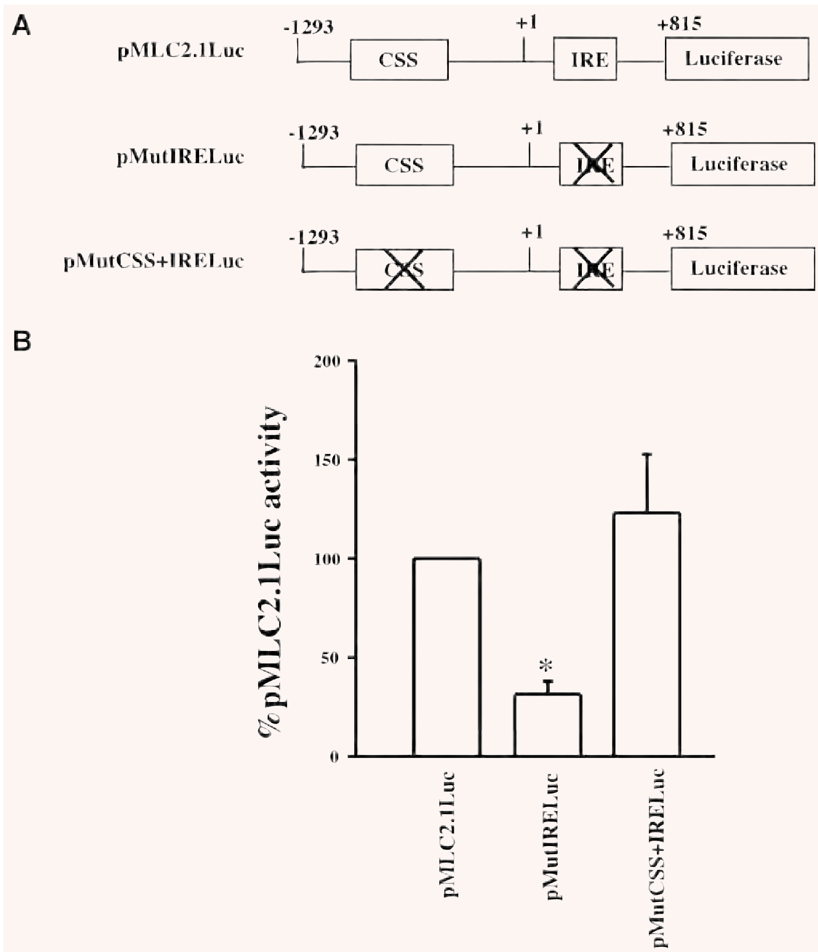

Fig. 1 (**A**) Schematic representation of the chicken cardiac MLC2 promoter constructs with either the wild-type (pMLC2.1Luc), IRE mutant, (pMutIRELuc) or mutations in both CSS and IRE, (pMutCSSIRELuc). (**B**) Transient transfection and MLC2 promoter activity. Chicken primary skeletal muscle cells in culture were transfected with plasmids pMLC2.1Luc, pMutIRELuc or pMutCSSIRELuc and activity of luciferase was measured as described in 'Material and Methods'. Data are shown as percentage of wild-type promoter activity. ($n = 4$ in triplicate; □ one sample t-test with Bonferroni correction, $P < 0.0167$).

that binds with sequence specificity to the palindrome in CSS [14] as well as in IRE where the IRE/Nished complex mediates the activation function of MLC2v gene promoter in cardiac cells [15].

In order to test whether the CSS/Nished complex is formed in skeletal muscle cells, we performed the gel shift assay with CSS DNA as probe using the skeletal muscle nuclear extract (Fig. 2). We observed that one major (CSSBPA) and two minor (CSSBP1 & 2) complexes are formed and that preincubation of the extracts with anti-Nished antibody disrupts the major complex, CSSBPA, and one of the minor complexes, CSSBP1. Thus, Nished appears to be the common CSS and IRE DNA binding protein that is likely to be involved in both activation and repression mechanism of transcription of cardiac MLC2v gene in the distinct cellular environments, *i.e.* cardiac and skeletal muscle.

Nished represses cardiac MLC2v gene in skeletal muscle cells

In previous studies, Arnold and co-workers [16] have noted the existence of a DNase-I hypersensitive region (HR1) in the first intron of the cardiac MLC2v gene. Since IRE appeared to be the only functional sequence element in that region [15], we speculated that IRE is in an open conformation in cardiac cells, and perhaps not in skeletal muscle cells where the gene is repressed. To test this possibility, we performed the ChIP assay (see Materials and Methods) to read the histone code in the vicinity of IRE in cardiac and skeletal muscle cells. Euchromatin is characterized by the presence of histone modifications such as acetylated H3K9 and dimethylated H3K4. The heterochromatin is enriched by the presence of mono-, di- and trimethylated H3K9. We used anti-K4 methyl antibody to immunoprecipitate chromatin from both heart and skeletal muscle tissues. Anti-K4 methyl antibody was raised against the methylated lysine residue 4 and serves as an indicator of transcriptionally active DNA. PCR was performed with DNA primers flanking the IRE sequence. Results in Fig. 3 confirmed that the IRE containing DNA is in open conformation and hence accessible in cardiac cells, but under identical conditions it was not accessible in skeletal muscle cells. The use of monomethylated H3K9 antibody revealed the closed conformation in skeletal muscle. The expression of GAPDH gene was measured as a control reaction for evaluating the transcriptionally active selection of

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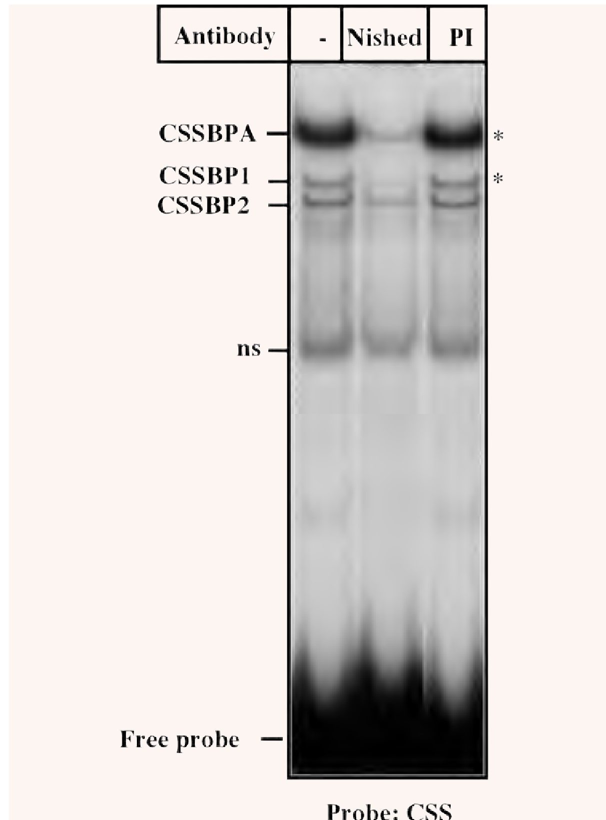

Fig. 2 Gel mobility shift assay. Nuclear extracts of Chicken skeletal muscle cells pre-incubated with anti-Nished antibody (Nished) or pre-immune serum (PI) were incubated with radiolabelled CSS oligonucleotide and subjected to gel mobility shift assay as described in 'Materials and Methods'. □ denotes the DNA-protein complexes disrupted by anti-Nished antibody.

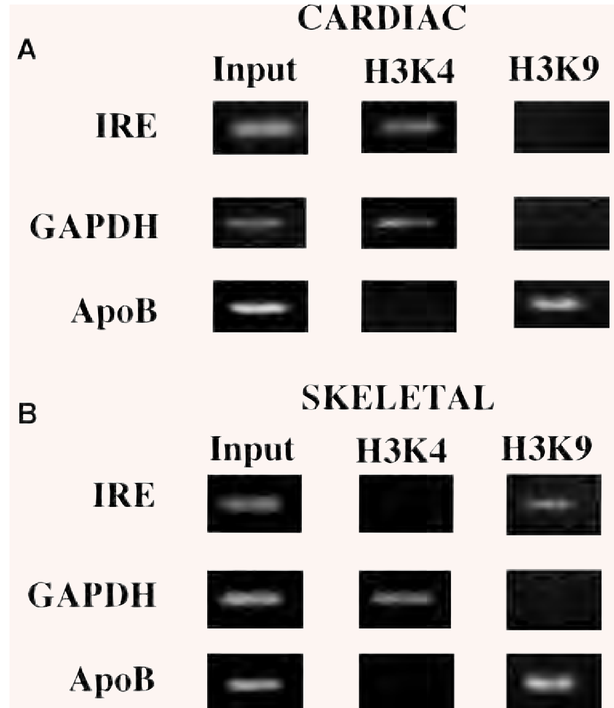

Fig. 3 Chip assay with extracts from Chicken cardiac and skeletal muscle tissues. (**A**) Upper panel shows the ChIP assay performed in cardiac tissue for detection of active chromatin antiH3K4 (trimethylated) and inactive chromatin anti-H3K9 (monomethylated) antibodies. We used primer flanking the IRE region in the cMLC2 gene, and as positive control the GAPDH primer, and as negative control primers that recognize the ApoB gene. (**B**) Lower panel shows a representative PCR using the same antibodies and target primers described in A but using skeletal muscle tissue.

Anti-K4 methyl antibody. Conversely, inactive chromatin activity was determined by evaluating the close chromatin status of the ApoB gene.

We then examined the functional activity of Nished/IRE complex in transient cotransfection assay using the wild-type promoter, pMLC2.1Luc, and mutant IRE- containing plasmid, pMutIRELuc, as reporters along with the Nished expression vector pNished-V5 in primary cardiac cells (see Materials and Methods). We reasoned that in pMutIRE plasmid the presence of non-functional IRE will simulate the transcriptional state of the gene in skeletal muscle cells where IRE is not accessible to DNA binding protein(s). We observed (Fig. 4) that the ectopic expression of Nished does not repress the activity of MLC2v promoter with wild-type IRE, supporting our argument (see Fig. 1) that IRE/Nished interaction overrides the repressor activity of the CSS/Nished complex. Repression of promoter activity occurs when IRE is mutated. Apparently, the absence of wild-type IRE and loss of IRE/Nished interaction would drive Nished to CSS to cause inhibition of transcription.

To further investigate the role of Nished as repressor, we used the mouse skeletal muscle cell line C2C12 to produce Nished expressing stable transfectants, N1, (see 'Materials and Methods'). Immunoprecipitation and Western blotting of cell extracts with anti-Nished antibody showed an enhanced level of Nished expression in N1 cells relative to the parent cells C2C12. GHAPDH levels were detected as loading control (Fig. 5A). Upon transient transfection of mutant MLC2 promoter (pMutIRELuc) in N1 cells, there was repression of the reporter plasmid compared to the level of expression in the parent cell line C2C12 (Fig. 5B). Taken together, these results suggest that Nished effectively down-regulates the MLC2v promoter activity when its binding is restricted to CSS alone, an environment mimicking that of the skeletal muscle cell.

DNA binding domain in Nished

In an attempt to localize the DNA binding domain in Nished, we created two N-terminal, NΔ1 (34–137) and NΔ2 (90–137) and two

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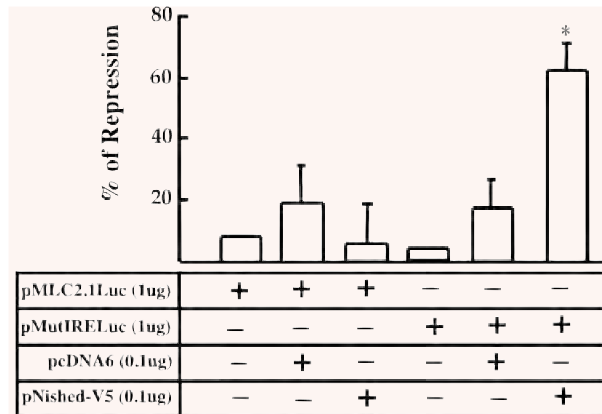

Fig. 4 Primary chicken skeletal cells were co-transfected with the DNA constructs containing wild-type IRE, (pMLC2.1Luc) or IRE mutant (pMutIRELuc) along with Nished expression vector, pNished-V5, or the control mammalian expression vector (pcDNA6V5/HisB). Data are plotted as percentage increase or decrease of promoter activity relative to that of pMLC2.1LucDNA. Experiments were performed in triplicate ($n = 7$). ☐ signifies differences with one sample t-test with Bonferroni correction, $P < 0.008$.

C-terminal CΔ1 (1–64) and CΔ2 (1–115) mutants (Fig. 6A) (see Materials & Methods). The wild-type Nished and the mutants in lanes NΔ1, CΔ1, NΔ2 and CΔ2 were tested by micro-affinity isolation assay (see Materials & Methods) where the *in vitro* translated and S^{35}-labelled products were subjected to binding to the biotinylated oligonucleotide containing CSS. Results in Fig. 6B show that mutants CΔ1 (1–64) and NΔ2 (90–137) that lack the segment do not bind CSS suggesting that the DNA binding activity resides within this region. The weak signals might be due to the proteolysis activity during transcription/translation assay. The results were nonetheless reproducible. The input of the reaction mixtures used in the assay, where the oligonucleotide was omitted, is shown in Fig. 6C. Specificity of the binding reaction was demonstrated by competition (Fig. 6B) with 10-fold (10×) excess of non-labelled *in vitro* translated wild-type protein pNishedV5 product. Then, each mutant was evaluated for their repression activity on the pMutIRELuc, and we observed that NΔ1 lacking the first 34 aa although binds to the CSS motifs, yet fails to repress the promoter activity suggesting that the trans-repression domain might be located within the first 34 aa region of the protein (Fig. 6D).

Nished associates with HDACs co-repressor proteins

To evaluate the mechanism by which Nished inhibits MLC2v gene transcription in non-cardiac cells, we examined the involvement of HDAC activity in MLC-2v gene repression. Upon exposure to Trichostatin A (TSA), a Class I-II HDAC inhibitor, the promoter

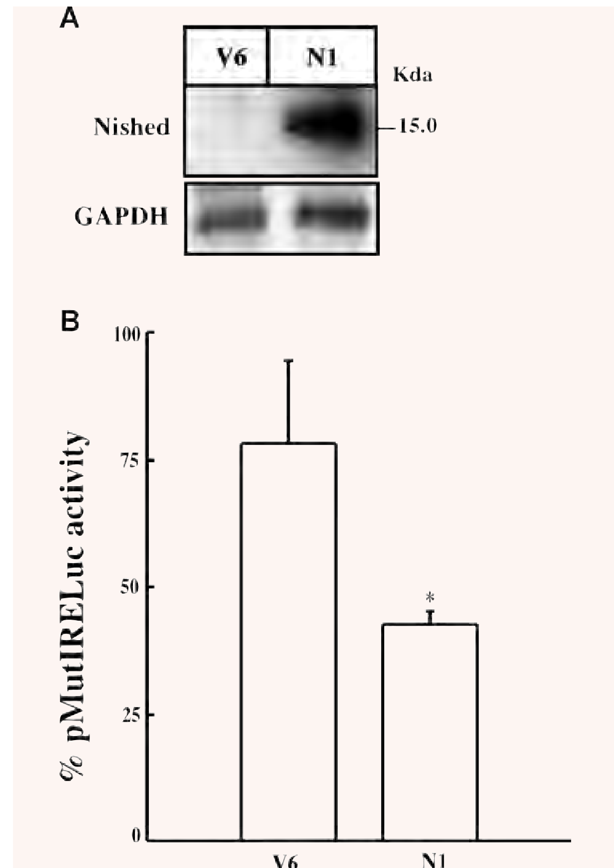

Fig. 5 Western blot of total cell lysates from C2C12 cells stably transfected with pcDNA6 vector (V6) or with plasmid expressing Nished (N1). (**A**) cell lysates were immunoprecipitated and blotted with anti-Nished antibody or pre-immune serum. As loading control we used equal amount of proteins from each cell line, and by Western blot determined the GAPDH levels. (**B**) Transient transfection of plasmid pMutIRELuc in C2C12 cells stably transfected with pNished (N1) or vector alone (V5). Data are expressed as percentage activity of pMutIRELuc transfected in wild-type C2C12 cells. ($n = 4$ in triplicate; ☐, one sample t-test; $P < 0.05$).

activity of the pMutIRELuc reporter in skeletal muscle cells was activated in a dose-dependent manner (5, 10, 25 nM), to nearly 2.5 fold (Fig. 7A), whereas the pMutIRELuc was not activated in primary cardiac cells exposed to TSA (Fig. 7B). The differential response of MLC-2v gene to TSA in cardiac and skeletal muscle cells indicates the specific requirement of HDAC activity for active repression in skeletal muscle cells. The selective requirement of classes I and II HDACs was demonstrated by lack of release of inhibition by the HDAC class III inhibitors, sirtinol [17] and M15 [18]. To identify the HDAC, which interacts with Nished, tissue extracts of heart and skeletal muscle were immunoprecipitated with anti-Nished antibody followed by Western blotting with

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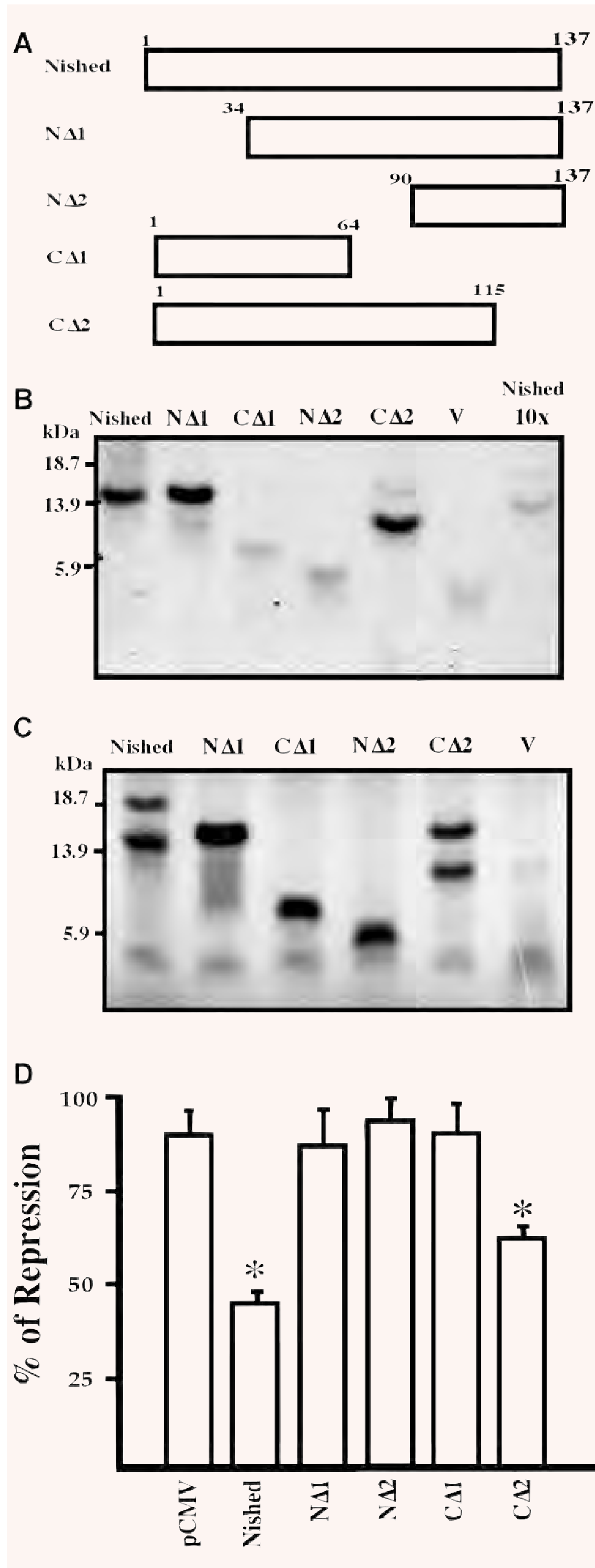

Fig. 6 Schematic representation of Nished and its C-and N-terminal deletion mutants. (**A**) The deletions were made and cloned in the mammalian expression vector, pcDNA6V5/HisB, as described in 'Material and Methods'. Numbers denote the amino acids in the constructs. (**B**) microaffinity isolation of S^{35} methionine labelled Nished and its N and C-terminal deletion proteins generated by coupled transcription-translation reaction with biotinylated CSS oligonucleotide as described in 'Material and Methods'. (**C**) One tenth of reaction mixture volume of S^{35} methionine labelled Nished and its N-and C-terminal deletions proteins were electrophoresed on an 18% SDS gel. (**D**) Functional analysis of Nished deletion mutants. Primary skeletal muscle cells were co-transfected with the IRE mutant (pMutIRELuc and Nished or the N and C terminal deletions mutants). The luciferase activity was normalized against the internal control, Renilla luciferase activity. ($n = 7$ in triplicate, □ one sample t-test with Bonferroni correction, $P < 0.05$). Nished full length protein; NΔ1 (34–137 a.a); NΔ2 (90137 a.a.); CΔ1 (1–64 a.a); CΔ2 (1–115 a.a); V, Vector alone (negative control); Nished 10X, competition with lysate contouring vector alone.

antibodies against HDAC3, HDAC5 and Nished. We observed interaction of Nished with HDAC 3 and HDAC 5 in extracts from skeletal muscle only (Fig. 8A), indicating that Nished's association with HDAC3 and 5 might be important in repression of cardiac MLC2v gene in skeletal muscle. In an attempt to identify the putative HDAC3/5 binding domain(s) in Nished, we tested the deletion mutants of Nished for Nished/HDAC3/5 binding assay as above. Figure 8B shows that NΔ2 does not bind with HDAC3 and 5, whereas NΔ1 binds with HDAC 3 but not with HDAC5, suggesting that the optimal binding activity, at least for HDAC3, resides in CΔ2, and involves amino acids 37–64 in Nished.

Discussion

Our laboratory has characterized the MLC2v gene extensively and identified several proximal and distal regulatory elements and their cognate DNA binding proteins. More recently, we have demonstrated the activation role of IRE bound Nished in conjunction with co-activators in hypertrophy agonist-induced up-regulation of the MLC2v gene [15]. The function of IRE and the role of Nished as an activator becomes further evident by the finding that the αBE-4 element in B-crystallin gene, which shares sequence homology with IRE, is needed for the maximal expression of the B-crystallin gene in myocardial cells [19, 20]. Nished or an analogous DNA binding protein is likely to be involved in this context in activation of this gene in cardiomyocytes. The question that remains unresolved, however, is the mechanism by which Nished causes the inhibition of expression of cardiac-specific MLC2v gene in skeletal muscle.

The regulatory association of muscle-specific proteins with activators and repressors of cardiac genes have been amply documented in a large number of studies, yet the precise mechanism

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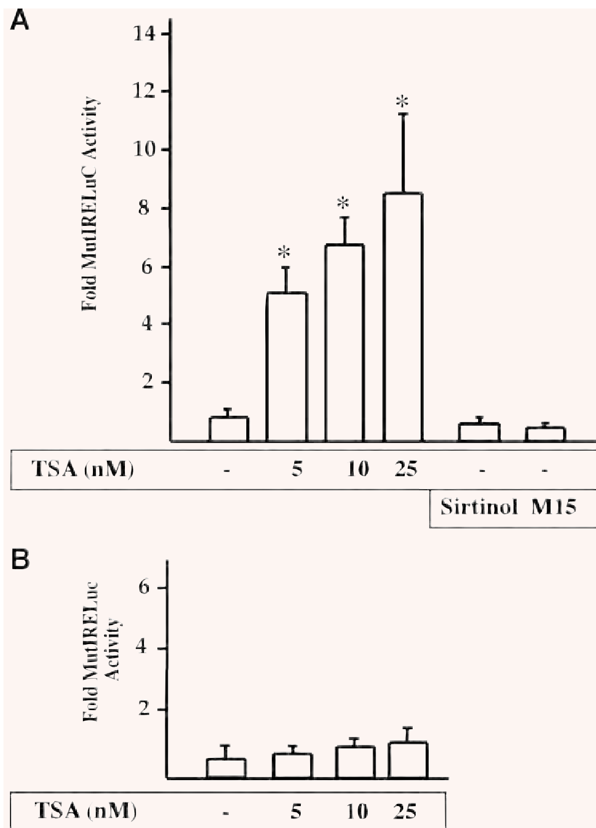

Fig. 7 Tricostatin A releases CSS mediates repression of MLC2v promoter in C2C12 cells. (**A**) C2C12 cells were transfected with MLC2v promoter containing mutated IRE (pMutIRELuc). After 24 hrs, cells were treated with Tricostation A (TSA), an inhibitor of Class I and II HDACs, and 25 μM each of Sirtinol and M15, inhibitors of Class III HDACs. The HDACs inhibitors were dissolved in DMSO. Luciferase assays were performed 18 hrs after treatment. Nished repression of MLC2v promoter was relieved by TSA in a dose-dependent manner, whereas Sirtionol and M15 had no effect. (**B**) Similar transient transfection experiment was performed in chicken primary cardiac cells, however, TSA treatment failed to modify the MLC2v promoter activity ($n = 4$ in triplicate, □ one sample t-test, $P < 0.05$).

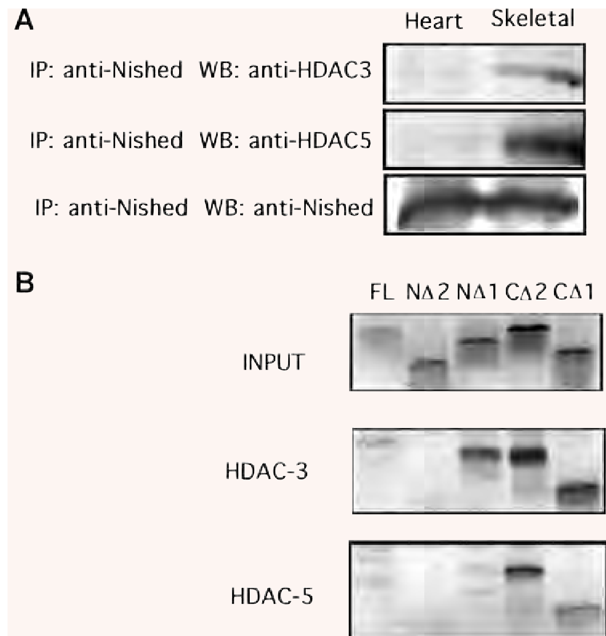

Fig. 8 (**A**) Total cell lystate of mouse cardiac and skeletal muscle tissue was co-immunoprecipicated with anti-Nished antibodies and immunoblotted with anti-HDAC3, anti-HDAC5 and anti-Nished antibody (loading control). (**B**) *In vitro* transcription/translation pull-down assay was done using pcNishedV5, CΔ1, CΔ2, NΔ1, NΔ2, pcHDAC3′ flag, pcHDAC5′ flag as described in 'Material and Methods'. Nished proteins were labelled with S^{35} methionine and combined with HDAC3 or HDAC5. Proteins were pulled down using M2 anti-flag agarose, and run on a 18% polyacrylamide gel. Panel 1 (INPUT) shows translated Nished proteins. Panel 2 shows HDAC3 binding to Nished mutants, no binding is seen with NΔ2; panel 3 shows HDAC5 binding to Nished mutant proteins (no binding is seen with NΔ2).

involved in silencing the cardiac genes in skeletal muscle remains unsolved. Post-translational modifications of chromatin such as acetylation, deacetylation and methylation have been shown to be involved in regulation of gene transcription. Deacetylation of the histones results in chromatin compaction blocking gene transcription. Our data presented in this study suggest that the repression of MLC2v in skeletal muscle involves two steps; one where the IRE sequence becomes inaccessible to the putative activator(s) of transcription, and second where CSS/Nished tissue-specific association with the deacetylases HDAC3/5 mediates suppression of the MLC2v gene expression in skeletal muscle, perhaps *via* inhibition of the MEF-2 protein. Indeed, MEF-2 interaction with element B is essential for the transcriptional activity of the MLC2v promoter [21], and this Element B-protein interaction is disrupted in presence of CSS binding protein (data not shown). The repression of MLC2v transcription in skeletal muscle cells, however, is underscored by the selectively of the CSS site and the lack of access of the IRE site in physiological conditions. These findings are consistent with the reported role of other co-repressors such as SMRT, SiN3, NuRD and their functional association with class I and II HDACs [22]. The association of HDACs with these distinct corepressors constitutes a family of transcription repression complexes that interfere with the activation role of transcription factors such as MEF-2 [23, 24].

Our results here suggest that the antithetical role of Nished, *i.e.* to potentiate the promoter activity of MLC2v gene in cardiac cells and to inhibit the same gene in skeletal muscle is afforded by the ability of Nished to interact with activators [15] or repressors (this report). Moreover, the association of DNase hypersensitivity and histone modifications in specific cell types suggests that developmentally established chromatin changes dictate the spatial and temporal expression of genes. In higher eukaryotes, histone

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modification of the type that involves methylation of histone H3 at lysines 4 and 9 (K4 and K9) leads to complementary functions; methyl K4 is enriched in transcriptionally active regions and methyl K9 in silent regions [25, 26]. Our data show that the histone H3 K4 residue is methylated around the IRE sequence in cardiac cells, suggesting that IRE is accessible to the transcriptional machinery in the cardiac tissue and inaccessible in non-cardiac tissues. IRE must, therefore, reside in a developmentally established instructive chromatin conformational environment that directs the transcription of MLC2v gene in cardiac cells. This is consistent with the methylation profile of H3 K9 and K4 residues across the chicken βglobin locus that similarly dictates the erythroid development stage-specific §-globin gene expression [27]. Likewise, chromatin modification at the myogenin loci is marked by the interaction of Pbx/Meis complex with MyoD, which, in association with other factors, regulates the temporal expression of myogenin during muscle differentiation through a feed-forward mechanism [28].

We have previously reported that IRE–Nished interaction serves as the target of hypertrophy-induced signalling in expression of MLC2v gene [15]. Nished interacts with transcription factor, NFATc4, and recruits p300 at the IRE site. The DNA–protein interaction is stimulated by the hypertrophic agonists angiotensin II. In this context, the dual role of Nished is significant given the identical Nished binding sequence present in the two disparate elements (CSS and IRE) in the MLC2v promoter. The association of Nished with HDAC and with p300 thus identifies the antithetical role of Nished in mediating transcription regulation. Our data identify Nished as a regulator of both positive and negative transcription in the target muscle cells of distinct lineages. That Nished is expressed in both adult cardiac and skeletal muscle cells, as well as in non-muscle cells, makes it likely that it has a role(s) in processes other than that in myogenesis.

Acknowledgement

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CLP-1 Associates with MyoD and HDAC to Regulate Skeletal Muscle Differentiation

Josephine Galatioto, Eduardo Mascareno and M.A.Q. Siddiqui*

Department of Cell Biology, Center for Cardiovascular and Muscle Research, State University of New York Downstate Medical Center, Brooklyn, New York 11203

*Author for correspondence (e-mail: maq.siddiqui@downstate.edu)

Summary

Emerging evidence suggests that eukaryotic gene transcription is regulated primarily at the elongation stage by association/dissociation of the inhibitory protein, CLP-1/HEXIM-1, from the Positive Transcription Elongation Factor b (P-TEFb) complex. It was reported recently that P-TEFb interacts with skeletal muscle-specific regulatory factor, MyoD, suggesting a linkage between CLP-1 mediated control of transcription and skeletal myogenesis. To examine this, we produced CLP-1 knock-down skeletal muscle C2C12 cells by homologous recombination and demonstrate that the C2C12 CLP-1 +/- cells failed to differentiate when challenged by low serum in the medium. We also show here that CLP-1 interacts with both MyoD and Histone Deacetylases (HDACs) maximally at the early stage of differentiation of C2C12 cells. This led us to hypothesize that the association might be critical to inhibition of MyoD-target proliferative genes. Chromatin immunoprecipitation analysis revealed that the CLP-1/MyoD/HDAC complex binds to the promoter of the cyclin D1 gene, which is down regulated in differentiated muscle cells. These findings suggest a novel transcriptional paradigm where CLP-1, in conjunction with MyoD and HDAC, may act to inhibit growth

related gene expression, a requirement for myoblasts to exit the cell cycle and transit to myotubes.

Key words: P-TEFb, HEXIM1, CLP-1, MyoD, Skeletal muscle

Introduction

Skeletal muscle development involves coordinated expression of transcription factors which control specification of mesodermal progenitors to the muscle fate and differentiation of committed myoblasts into myotubes (Stockdale, 1992). The development of skeletal muscle is directed by four myogenic regulatory factors (MRFs), MyoD, myf-5, MRF4 and myogenin (Sabourin and Rudnicki, 2000; Yun and Wold, 1996). MyoD and myf5 are expressed in proliferating myoblasts and influence lineage restriction, whereas myogenin, a target of MyoD, is induced upon differentiation (Rudnicki and Jaenisch, 1995). The MRFs hetero-dimerize with ubiquitous E-proteins, bind to conserved E-box sequences (CANNTG) in the promoter of muscle specific genes and control transcription (French et al., 1991). The transcriptional activity of MyoD is influenced by its interaction with an array of coactivators and corepressors (McKinsey et al., 2001). The repressor protein histone deacetylase 1 (HDAC1) was reported to interact with MyoD and deacetylate it and consequently suppress MyoD's transcriptional activity (Mal et al., 2001). Activator proteins, histone acetylases (HATs), on the other hand, are known to stimulate MyoD dependent transcription by engaging histone acetylases p300 and PCAF which, in turn, promote acetylation of MyoD itself and increase its affinity for target-gene promoters (Puri et al., 1997; Sartorelli et al., 1999). It is well known that the

Positive Transcription Elongation Factor b (P-TEFb) complex, which consists of cdk9 and cyclin T1 (or the minor forms T2a and b), mediates the transcription of RNA Polymerase II (Pol II) genes (Fu et al., 1999; Peng et al., 1998). P-TEFb phosphorylates the Carboxyl-terminal domain (CTD) of the largest subunit of Pol II at Serine 2 of a 52-tandem heptapeptide, which is required for transcription to change from the abortive to productive phase of transcriptional elongation (Price, 2000; Zhou et al., 2000).

We and others have demonstrated that the mouse Cardiac Lineage Protein-1 (CLP-1) (Huang et al., 2002; Huang et al., 2004), and its human homolog HEXIM1 (Schulte et al., 2005), function as transcriptional repressors. In HeLa cells, P-TEFb exists in equilibrium between active and inactive forms by way of association and dissociation of HEXIM1 from the P-TEFb complex (Nguyen et al., 2001; Yang et al., 2001). Our laboratory knocked-out the CLP-1 gene in mice, which resulted in lethality in late fetal stages (E17-E18) due to massive growth of the embryonic hearts (Huang et al., 2002; Huang et al., 2004). Subsequently, studies in our laboratory demonstrated that introduction of CLP-1 heterozygosity +/- in the background of cardiac specific cyclin T1 overexpression enhanced RNA Pol II phosphorylation at Serine 2 (Espinoza-Derout et al., 2009).

Skeletal muscle differentiation, characterized by silencing of the proliferative genes and up-regulation of muscle specific genes, is prominently influenced by MyoD which is known to target more than 300 genes controlling several subprograms of skeletal muscle gene expression (Bergstrom et al., 2002). The reported link between MyoD and P-TEFb prompted us to examine if CLP-1 is involved in control of P-TEFb activity during the transition of myoblasts of myotubes. In this study, we report that CLP-1 associates with MyoD and HDAC proteins in the early phase of differentiation of C2C12 myoblasts and

conclude that CLP-1/MyoD/HDAC complex is critical in control of P-TEFb activity and in regulation of skeletal muscle cell differentiation.

Results

Association of CLP-1 with P-TEFb and MyoD in C2C12 cell differentiation

To investigate the role of CLP-1 in skeletal muscle cell differentiation, we first examined its expression in C2C12 skeletal muscle cells, which represent a highly suitable model for analysis of myogenic differentiation. In high serum (10% FBS) growth medium, C2C12 myoblasts proliferate until they reach confluency. Differentiation into multi-nucleated myotubes is triggered by switching to low serum (2% horse serum) differentiation medium. As shown in Fig. 1A, CLP-1 was present in C2C12 cells in both growth (G) and differentiation medium at 24 hours (D1) and 72 hours (D3). Myogenin, a known marker for differentiation, is expressed at D1 and D3, but not in G. MyoD, is expressed in both myoblasts and myotubes, as expected.

Next, CLP-1 association to the P-TEFb complex was analyzed by immunoprecipitation (IP) with anti-cdk9 antibody followed by western blotting with anti-CLP-1 antibody (Fig. 1B). There was a prominent level of association of CLP-1 with P-TEFb in early differentiation (D1) whereas that observed in both proliferative cells (G) and the terminally differentiated cells (D3) was negligible. Thus it appeared that there is a clear shift in P-TEFb equilibrium to the CLP-1 bound state in C2C12 cell differentiation, which suggests that CLP-1 mediated regulation of P-TEFb activity might have a role in the transition of C2C12 cells from growth to differentiation.

Since the kinase activity of P-TEFb is influenced by the association of CLP-1, one may envision that association of CLP-1 to the P-TEFb/MyoD complex will regulate MyoD mediated transcriptional activity. We co-immunoprecipitated with MyoD antibody using lysates from proliferating C2C12 myoblasts (G) and from cells induced to differentiate for 24 hours (D1) and 72 hours (D3). We observed that there was association between MyoD and CLP-1 in differentiation stage D1 while in D3 and in G it was barely visible (Fig. 1C).

HDACs are implicated in the regulation of skeletal myogenesis by their interaction with MyoD and in regulating MyoD mediated gene transcription (Mal et al., 2001). HDAC1 binding to MyoD is implicated in blocking the function of MyoD in initiating the myogenic program. To examine whether MyoD association with HDAC1 occurs in growth and/or differentiation conditions, co-immunoprecipitations were performed on proliferating C2C12 cells (G) and differentiating C2C12 cells at D1 and D3 (Fig. 1D). The results show that MyoD binds to HDAC1, as well as PCAF in both growth and differentiation conditions, albeit at a relatively lower level in G. MyoD's association with HATs and HDACs suggests that both activation and suppression mechanisms co-exist perhaps in directing distinct promoters in myoblasts toward differentiation.

Association of CLP-1 with HDACs in C2C12 cell differentiation

We then examined CLP-1's interaction with HDACs in C2C12 cells by immunoprecipitation with antibodies against HDAC1, HDAC3 and HDAC5 and western blotting for the presence of CLP-1. As seen in Fig. 2A, B, CLP-1 associates with Class I HDACs 1 and 3 and Class II, HDAC5 preferentially at the D1 stage of differentiation.

HEXIM1 was previously reported to bind directly to HDAC3 in HeLa cells (Fu et al., 2007). Also, upon immunoprecipitation with HDAC5 and western blotting with anti-cdk9 antibody, we observed that association of cdk9 is also highest in D1 (Fig. 2B). The pattern of CLP-1 association with HDACs was distinct from HDAC protein expression as seen by direct western blotting (Fig. 2C). It is known that Class II HDACs translocate from the nucleus to the cytoplasm, while Class I are restricted to the nucleus. To ascertain whether CLP-1 association to HDAC5 is nuclear or cytoplasmic, we fractionated C2C12 cells and immunoprecipitated with anti-HDAC5 antibody. When western blotted with CLP-1 antibody, CLP-1 was seen in both the nuclear and cytosolic fractions, whereas cyclin T and MyoD were present in nuclei only, as expected (Fig. 2D). Co-immunoprecipitation analysis showed that the CLP-1/HDAC5 complex was localized only in the nuclear fraction (Fig. 2D).

CLP-1 associates with MyoD and HDAC on the cyclin D1 promoter

In order to gain mechanistic insights as to how CLP-1 regulates skeletal muscle differentiation, we hypothesized that CLP-1 associates with MyoD and HDAC and is involved in down-regulation of cell-cycle genes, for example cyclin D1, to allow expression of differentiation-specific genes. We examined association of this inhibitory complex to the cyclin D1 promoter because cyclin D1 is a cell cycle regulatory protein whose proteins level were reported to change in response to HEXIM1 (Ogba et al., 2008). Cyclin D1 protein in C2C12 cells is expressed only under growth conditions (G) when examined by western blotting (Fig. 3A). Chromatin immunoprecipitation (ChIP) assay revealed that the inhibitory complex was associated with the promoter of the MyoD-

target cyclin D1 gene. DNA was amplified using primers flanking two putative E-box sequences within the cyclin D1 promoter (insert size 188 bp). CLP-1/MyoD/HDAC containing complexes were associated with the cyclin D1 promoter at MyoD-target binding sites preferentially in differentiating muscle cells (D1, D3) (Fig. 3B). MyoD association was maximal in D1 and D3, and HDAC1, 3 and 5 had little association in G as compared to D1 and D3. These results are consistent with our IP experimental data above.

Knock-down of CLP-1 inhibits C2C12 cell differentiation

To further investigate the putative function of CLP-1 in early myogenic differentiation, we chose to knock-down CLP-1 in C2C12 cells by homologous recombination. C2C12 cells were first transfected with a CLP-1 replacement gene-targeting vector (Huang et al., 2004) and then selected for neomycin resistance. Drug-resistant colonies were isolated and examined for expression of the recombinant allele by PCR (Fig. 4A). The colonies were also assayed for CLP-1 protein level by western blot (Fig. 4B). CLP-1 protein level was lower in heterozygote (+/-) C2C12 cells compared to wild-type (+/+) C2C12 cells, as expected. When C2C12 CLP-1 +/- cells were challenged to differentiate by switching to low serum medium, the cells were unable to differentiate as indicated by the absence of myogenin protein in D1 and D3 (Fig. 4C). The CLP-1 +/- cells continued to proliferate as indicated by the presence of Proliferating cell nuclear antigen (PCNA) in differentiation conditions (Fig. 4C). To confirm the differentiation deficiency, we performed Immunofluorescence analysis on cells in growth and differentiation medium (Fig. 4D). Under differentiation conditions, wild-type C2C12 cells fused into multi-

nucleated myotubes. In contrast, when challenged to differentiate in low serum medium, C2C12 CLP-1 +/- cells remained mono-nucleated, maintaining a non-differentiated phenotype.

Analysis of CLP-1-associated proteins in CLP-1 -/- mice embryos

Since the data above on association of CLP-1 with MyoD and HDAC was obtained in in vitro cultured C2C12 cells, we addressed the question if such functional association exists in vivo in the animal. We used embryonic day 12 embryos and adult wild type skeletal muscle for direct western blotting. In adult muscle CLP-1 and cdk9 were very low compared to embryo at day 12 (Fig. 5A, input). Likewise, CLP-1 association with cdk9 and MyoD was not observed in adult (Fig. 5A). Embryos at day 12 contain myoblasts as well as myotubes while in the adult there are only myotubes in which CLP-1 cooperativity with cdk9 and MyoD is no longer necessary. Next, we examined CLP-1 wild-type, +/-, and -/- day 12 stage embryos by western blot for expression of the components of P-TEFb and MRFs. CLP-1 was not expressed in -/- embryos as expected, while CLP-2, a homolog of CLP-1, was expressed (Fig. 5B). Cdk9, cyclin T1 and Pol II levels remained steady (Fig. 5B). Skeletal muscle marker MyoD was down in CLP-1 +/- and -/- embryos and the differentiation marker myogenin and myosin heavy chain (MHC) were also decreased markedly (Fig. 5C). These finding suggest that the absence of CLP-1 in the knock-out mice might lead to reduced myogenesis, which may partly be responsible for embryonic lethality. It was also clear that CLP-2 does not compensate for the loss of CLP-1 since CLP-1 -/- mice die at E17/18 despite CLP-2 expression.

Discussion

Mechanistically, eukaryotic cell development and its maintenance are attributed to a critical balance between transcriptional activators and inhibitors and their effect on specific genes. At the transcriptional level, it is evident that the P-TEFb complex plays a role in expression of tissue and developmental stage specific genes. Current data support the notion that the active recruitment of P-TEFb is crucial for the expression of these genes, but it is not known how P-TEFb is specifically recruited and what cellular factors cooperate with P-TEFb. A great deal of information is available on the molecular composition of the P-TEFb complex and its role in transcription elongation. However, there is limited knowledge on the role of P-TEFb in skeletal muscle gene control, and certainly the involvement of CLP-1 is totally unknown.

In this study, we have examined the role of CLP-1 in skeletal muscle cell differentiation using C2C12 mouse myoblast cells. We noted that CLP-1 association with P-TEFb is dynamic and it is maximal during the early phase of differentiation, implying that CLP-1 is functionally connected with the transition phase of myoblasts to myotubes. A recent report also examined HEXIM1 association with P-TEFb in C2C12 cells and found that HEXIM1 dissociated from P-TEFb 30 minutes after switching to low medium. But the association was shown to be restored at 2 hours (Nojima et al., 2008). However, myogenin is expressed only after 12 hours in low serum medium (25). We therefore used 24 hours as early phase and 72 hours as fully established phase of C2C12 cell differentiation.

By reducing CLP-1 expression in C2C12 cells, we see an arrest in the transition of skeletal myoblast to myotubes, implying that CLP-1, and possibly its association with

components of P-TEFb are obligatory to this transition. Since the CLP-1 knock-out mice die at embryonic day 17 to 18, CLP-2 expression does not appropriately substitute CLP-1 function in mice despite high homology between the carboxyl-termini of the two proteins. While the CLP-1 heterozygosity in C2C12 cells was sufficient to arrest differentiation in cell culture, the CLP-1 +/- mice survive. One may speculate that during embryonic development CLP-2 or some compensatory molecule that is not available in the cell line substitutes for CLP-1. Furthermore embryonic growth undergoes defined phases of development while the C2C12 cell are immediately challenged to differentiate and therefore possibly bypass the regulatory subprograms of gene expression. During early embryonic stages CLP-1 +/- embryos appear smaller than their wild type litter mates (data not shown), and after birth they appear phenotypically similar. There may very well be a skeletal muscle deficiency as evidenced by reduced skeletal muscle markers during development, a phenomenon that needs to be examined in the future.

In this report, we also show that MyoD and HDACs associate with CLP-1 maximally at 24 hours. This would suggest that perhaps both CLP-1 and HDACs are recruited by MyoD to offer an environment optimal for repression of proliferative genes allowing the transition of myoblasts to myotubes. At this junction, one would expect that MyoD must also associate with HAT proteins in a separate protein complex to facilitate the promotion of muscle specific gene expression. In support of this notion Mal and Harter (2003) have shown that once myoblasts undergo differentiation, MyoD actively engages in recruitment of HAT proteins at skeletal muscle promoters. In our present studies, we noted that MyoD associates with HDAC1 and PCAF in the differentiation stage. MyoD's role as a repressor or activator can be dictated by its association with interacting proteins

(HDAC, HAT). Recent reports have examined the effect of HDACs and HATs association with the P-TEFb complex and it was shown that cdk9 is acetylated, but whether this acetylation enhances or inhibits the kinase activity is not settled (Fu et al., 2007; Sabo et al., 2008). Fu et al., (2007) showed that cdk9 was deacetylated by HDAC3, which reduced the association of cdk9 with activator Brd4 and promoted the interaction of P-TEFb with HEXIM1. Sabo et al., (2008) showed that cdk9 acetylation by PCAF reduced kinase function and transcriptional activity of P-TEFb. The cyclin T1 component of P-TEFb can also be acetylated which resulted in a decrease in HEXIM1 bound P-TEFb, and increase in cdk9 kinase activity (Cho et al., 2009; Sabo et al., 2008). In our study, we observed that CLP-1 associates with HDAC1, 3 and 5 in skeletal muscle and this association is specifically enhanced in the early differentiation phase. CLP-1 associated with HDAC in skeletal muscle may act to counter the activity of acetylated cyclin T1 or cdk9. We speculate that inactive P-TEFb bound by CLP-1 is actively recruited to silence specific gene promoters.

In summary, we presented evidence that CLP-1 interacts with MyoD and hypothesize that CLP-1 associated with MyoD is required for down-regulation of proliferative genes and to steer the myoblast to myotube transition. HEXIM1 expression has been reported to correlate with Estrogen receptor-inducible cyclin D1 protein expression and binding to the E_2-responsive region on the cyclin D1 promoter (Ogba et al., 2008). Using ChIP assay, we localized CLP-1 and its associated proteins, MyoD and HDACs, on the cyclin D1 promoter in C2C12 cells, which occurs preferentially in the differentiation stage. Based on our results, we speculate that MyoD guides CLP-1 to MyoD-target DNA, which is in complex with HAT and HDAC proteins. We believe that both suppression

and activation of MyoD activity are likely to co-exist in C2C12 cells at the onset of differentiation to act on distinct promoters. The signaling that triggers specific interaction of these molecules is not known. Future studies to characterize the fundamental regulatory events mediated by CLP-1 would provide further insights into CLP-1's physiological role in skeletal muscle cells. Our data collectively highlight a distinct function of CLP-1 in cell-cycle exit, suggesting that CLP-1's regulation of P-TEFb activity is likely to play a pivotal role in targeting skeletal muscle cells toward differentiation.

Materials and Methods

Antibodies

Polyclonal rabbit anti-CLP-1 antibody was generated to the peptide HRQQERAPLSKFGD (Proteintech Group Inc.). Cdk9 (C-20, D-7), cyclin T1 (H-245), GAPDH (FL-335), HDAC1 (H-11), HDAC3 (B-12), HDAC5 (H-714), Pol II (N-20), myogenin (F5D), MyoD (5.8A, C-20), HEXIM2 (M-90), PCAF (E-8), PCNA (PC10) and Actin (C-2) antibodies were from Santa Cruz Biotechnology. Anti-Myosin Heavy Chain (MF20) antibody was from Developmental Studies Hybridoma Bank. Anti-cyclin D1 antibody was from Abcam Inc. HDAC antibody sampler kit from Cell Signaling Technology. ChIP Grade antibodies anti-HEXIM1 and anti-MyoD (C-20x) from Abcam Inc. and Santa Cruz Biotechnology respectively.

Cell culture

C2C12 mouse myoblasts obtained from ATCC were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin/amphotericin b (growth medium). To induce differentiation, growth medium was substituted with differentiation medium (DMEM supplemented with 2% horse serum and penicillin/streptomycin/amphotericin b). Cells were incubated at 37°C with 5% CO_2.

Stable transfections

CLP-1 allele replacement vector DNA (pKO-HR-CLP-1neo) (Huang et al., 2004) was transfected into C2C12 cells at 10% confluency using Fugene 6 (Roche) transfection reagent. Neomycin (G418) antibiotic was used to isolate resistant clones. C2C12 cells heterozygous for the targeted CLP-1 allele were determined by PCR. DNA was isolated and genotype determined by PCR with previously described primers (Huang et al., 2004).

Coimmunoprecipitation

Cells were lysed in Buffer A (Michels et al., 2004) supplemented with 1 mM dithiothreitol, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor cocktail (Sigma) and RNasin (Promega). Lysate was subjected to "freeze" on dry ice, and "thaw" at 37°C, followed by centrifugation at 16,000 X *g*. Equal concentration of lysates were incubated with antibody overnight at 4°C with rotation. For protein capture, protein A/G plus agarose beads (Santa Cruz Biotech) were added at 4°C for 2 h with rotation. After extensive washing with Buffer A, bound proteins were eluted by boiling in 1X SDS loading buffer.

Cell Fractionation

Cells were lysed in Cell Fractionation Buffer A (10 mM HEPES, pH 7.4, 15 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA) supplemented with 1 mM dithiothreitol, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail (Sigma), and RNasin (Promega) with homogenization using a dounce homogenizer and monitored microscopically with trypan blue. Samples were centrifuged (1,500 X *g*) at 4°C for 5 min and supernatant was collected as cytoplasmic extract. Pellet was incubated with Cell Fractionation Buffer B (10 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.5% NP-40) supplemented with 1 mM dithiothreitol, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, protease inhibitor cocktail (Sigma) and RNasin (Promega). Samples were subjected to "freeze", on dry ice, and "thaw", at 37°C, with vortexing. After centrifugation (16,000 X *g*) at 4°C for 10 min, the supernatant was collected as nuclear extract.

Whole-Cell Lysis

Cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100) supplemented with 1 mM dithiothreitol, 1 mM PMSF, 10 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail (Sigma).

Western blot analysis

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane in electroblotting buffer (20 mM Tris, 150 mM Glycine, 20% methanol) for 1 hr. The membranes were blocked in TBS-T with 5% nonfat dry milk. Membrane was probed

with primary antibody in TBS-T overnight followed by incubation with an HRP-conjugated secondary antibody for detection with enhanced chemiluminescence reagent.

Immunofluorescence analysis

C2C12 cells were seeded in 6 well tissue culture dishes containing sterile glass coverslips. Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100. After blocking in 5% FBS in PBS, cells were incubated with primary antibody at 4°C overnight, followed by washes in PBS and incubation with secondary antibody (1:200) conjugated to either Alexa-488 or Alexa-594 (Invitrogen) for 1 hr. For actin staining, cells were incubated for 20 min with 2 units of Alexa-594 phalloidin (Invitrogen). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Imaging was captured at 40X magnification using Zeiss Axiokop microscope, Axio CamMRc camera and Axio vision software.

Chromatin Immunoprecipitation Assay

Proteins were cross-linked to DNA in culture medium using 1% formaldehyde (10 minutes, room temperature). Cells were lysed (10 mM Tris, pH 8.1, 1.5 mM $MgCl_2$, 10 mM KCl) and nuclei were sedimented by centrifugation (1,500 X *g*) for 5 min at 4°C. Pellet was resuspended in Nuclear Lysis Buffer (50 mM Tris-HCl, 10 mM EDTA, 1% SDS). Chromatin was sonicated five times, for 30 s each, generating DNA fragments from 250 to 1,000 bp. DNA concentration was quantified and 10 mg of DNA was used per immunoprecipitation. Lysates were diluted with ChIP dilution buffer (20 mM Tris,

pH 8.1, 150 mM NaCl, 2 mM EDTA, 0.01 % SDS, 1 % Triton X-100) and antibodies were added. After overnight rotation at 4°C, the immune complexes were collected by the addition of Protein G agarose beads/salmon sperm DNA (Millipore). After extensive washes, immune complexes were eluted (1% SDS, 0.1 M $NaHCO_3$) and cross-linking was reversed by the addition of 190 mM NaCl overnight at 65°C. DNA was purified using a PCR purification kit (Qiagen) and amplified by PCR. Sequence of PCR primers:

Cyclin D1 5'-TATCCTGGAAGGGCGACTAA-3',

5'-AATTCCAGCAACAGCTCAAGA-3'

GAPDH primer control

5'-CGGTGCGTGCCCAGTTG-3', 5'-GCGACGCAAAAGAAGATG-3'.

DNA was amplified for 18 cycles (annealing 60°C). Product was visualized on 2% agarose gel by ethidium bromide stain and 8-bit digital camera.

Animals

CLP-1 knock-out mice have been described previously (Huang *et al., 2*004). All experiments were performed in accordance with the Guidelines of the National Institute of Health. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee. For protein expression studies, male and female CLP-1 +/- mice were "time-mated".

Statistical Analysis

For quantitative western blot analysis films were scanned and the band signal intensities determined using ImageJ software. The densitometry values were expressed as a fold

level relative to the control, and standardized to corresponding total GAPDH densitometry values obtained from the same sample. Statistical analysis were performed using a paired student's *t*-test. *P* values of < 0.05 were considered significant.

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Figure Legends

Fig. 1. Association of CLP-1 to cdk9 and MyoD is dynamic in C2C12 cell differentiation. (A) Whole cell lysates were collected from growth medium (G) and differentiation medium at 24 hours (D1) and at 72 hours (D3). Equal amounts of protein were subjected to SDS-PAGE followed by immunoblotting. Myogenin served as a marker of differentiation. GAPDH served as a loading control. Data shown represent one of three separate experiments. (B) Immunoprecipitation (IP) of C2C12 cell lysates from growth medium (G) and differentiation medium at 24 hours (D1) and at 72 hours (D3) with anti-cdk9 antibody and western blotting with anti-CLP-1 antibody. IP with antibody alone served as a control for immunoreactivity (Ab). Western blot with anti-cdk9 antibody served as a control of total immunoprecipitated protein. Data shown represent one of three separate experiments. (C) Immunoprecipitation (IP) of C2C12 cells in growth medium (G) and differentiation medium at 24 hours (D1) and at 72 hours (D3) with anti-MyoD antibody and western blotting with anti-CLP-1 antibody. IP with antibody alone served as a control for immunoreactivity (Ab). Western blot with anti MyoD antibody served as a control of total immunoprecipitated protein. Data represent one of three separate experiments. (D) Immunoprecipitation (IP) using C2C12 cell lysates from growth medium (G) and differentiation medium at 24 hours (D1) and at 72 hours (D3) with anti-MyoD antibody and western blotting with anti-HDAC1 and PCAF antibodies. IP with antibody alone served as a control for immunoreactivity (Ab). Direct western of HDAC1 and PCAF served as input controls.

Fig. 2. CLP-1 associates with HDACs at the onset of C2C12 cell differentiation. (A) Immunoprecipitation (IP) of C2C12 cell lysates from growth medium (G) and differentiation medium at 24 hours (D1) and at 72 hours (D3) with anti-HDAC1 and HDAC3 antibodies and western blot with anti-CLP-1 antibody. IP with antibody alone served as a control for immunoreactivity (Ab). Western blot with anti-CLP-1 and anti-GAPDH antibodies served as an input controls. Data represent one of three separate experiments, for each antibody. (B) IP with anti-HDAC5 antibody and western blot with anti-CLP-1 and cdk9 antibodies. IP with antibody alone served as a control for immunoreactivity (Ab). Western blot with anti-CLP-1 and anti-GAPDH antibodies served as an input controls. Data represent one of three separate experiments. (C) Western blot of total cell lysates with anti-HDAC1, 3 and 5 antibodies. Western blot with GAPDH served as input control. (D) Left panel, nuclear (Nuc) and cytoplasmic (Cyto) fractions of C2C12 cells in differentiation medium at 24 hours were subjected to direct western blotting with anti-CLP-1, cyclin T1, MyoD and GAPDH antibodies. Right panel, lysates were subjected to immunoprecipitation (IP) with anti-HDAC5 antibody and western blotting with anti-CLP-1 antibody. Direct western of CLP-1 served as an input control.

Fig. 3. Chromatin immunoprecipitation (ChIP) analysis of the cyclin D1 promoter in C2C12 cell differentiation. (A) Analysis of C2C12 cells in growth medium (G), and differentiation medium at 24 hours (D1) and 72 hours (D3) by western blotting with anti-CLP-1, myogenin, and cyclin D1 antibodies. GAPDH served as a loading control. (B) ChIP was performed with anti-MyoD, CLP-1, HDAC1, 3 and 5 antibodies on C2C12

cells in growth medium (G) and differentiation medium at 24 hours (D1) and at 72 hours (D3). A non-specific IgG and anti-actin antibody served as negative controls. Precipitated DNA was amplified by PCR for regions of the cyclin D1 gene corresponding to the 5' upstream promoter region encompassing two putative E-box sequences. Input DNA represents 10% of total chromatin used in each reaction. Primers specific to GAPDH were used before (input) and after immunoprecipitation as a control to monitor immunoprecipitation specificity. Data represent one of three separate experiments.

Fig. 4. C2C12 CLP-1 +/- cells are differentiation deficient. (A) PCR using DNA from C2C12 CLP-1 +/+ and +/- cells. Primers generate a 457 base pairs product for the CLP-1 gene and a 383 base pairs product for the mutated allele. (M) denotes DNA size ladder. Data shown is one representative clone. (B) Western blot of C2C12 CLP-1 +/+ cells and C2C12 CLP-1 +/- cells in growth medium probed with anti-CLP-1 antibody. GAPDH served as a loading control. Western blot shown is one representative clone. The analysis was performed on three independently isolated C2C12 CLP-1 +/+ and +/- cell cultures and means are depicted graphically, GAPDH was used for normalization (*$P<0.05$). (C) Western blot of lysates from C2C12 CLP-1 +/+ and +/- cell cultures in growth medium (G) and differentiation medium at 24 hours (D1) and at 72 hours (D3), probed with anti-CLP-1, myogenin and PCNA antibodies. GAPDH served as a loading control. (D) Immunofluorescence of C2C12 CLP-1 +/+ and C2C12 CLP-1 +/- cells in growth and differentiation medium using actin stain and co-stained with anti-CLP-1 (red) or dapi nuclear stain (blue). This analysis was performed on three independently isolated C2C12 CLP-1 +/+ and +/- cell cultures.

Fig. 5. Analysis of expression of P-TEFb and MRF's in mouse skeletal muscle. (A) Immunoprecipitation (IP) of skeletal muscle lysates from wild type embryonic day 12 stage embryo and adult, with anti-cdk9 and MyoD antibodies and western blotting with anti-CLP-1 antibody. IP with antibody alone served as a control for immunoreactivity. Western blot of total protein served as an input control. GAPDH served as a loading control. (B) Western blotting of skeletal muscle tissue lysates from mouse embryos (day 12) wild-type (WT), heterozygote (+/-), and homozygote (-/-) for the CLP-1 allele with anti-CLP-1, CLP-2, cdk9, cyclin T1 and RNA polymerase II (Pol II) antibodies. GAPDH served as a loading control. (C) Western blotting of skeletal muscle tissue lysates with anti-MyoD, myogenin, and myosin heavy chain (MHC) antibodies. GAPDH served as a loading control. Data represent one of three separate experiments with one embryo per experiment. Relative MyoD, myogenin, and MHC levels depicted graphically, GAPDH was used for normalization (* $P<0.05$).

Figure 1

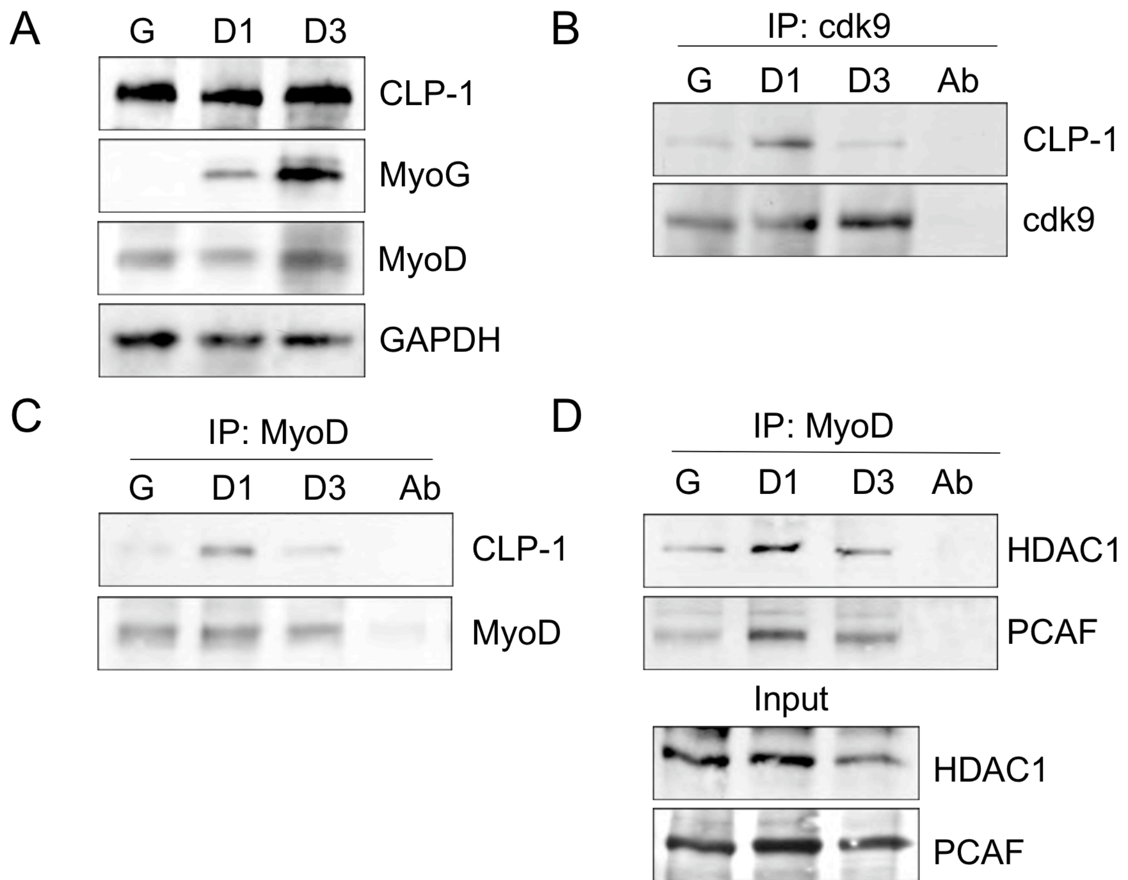
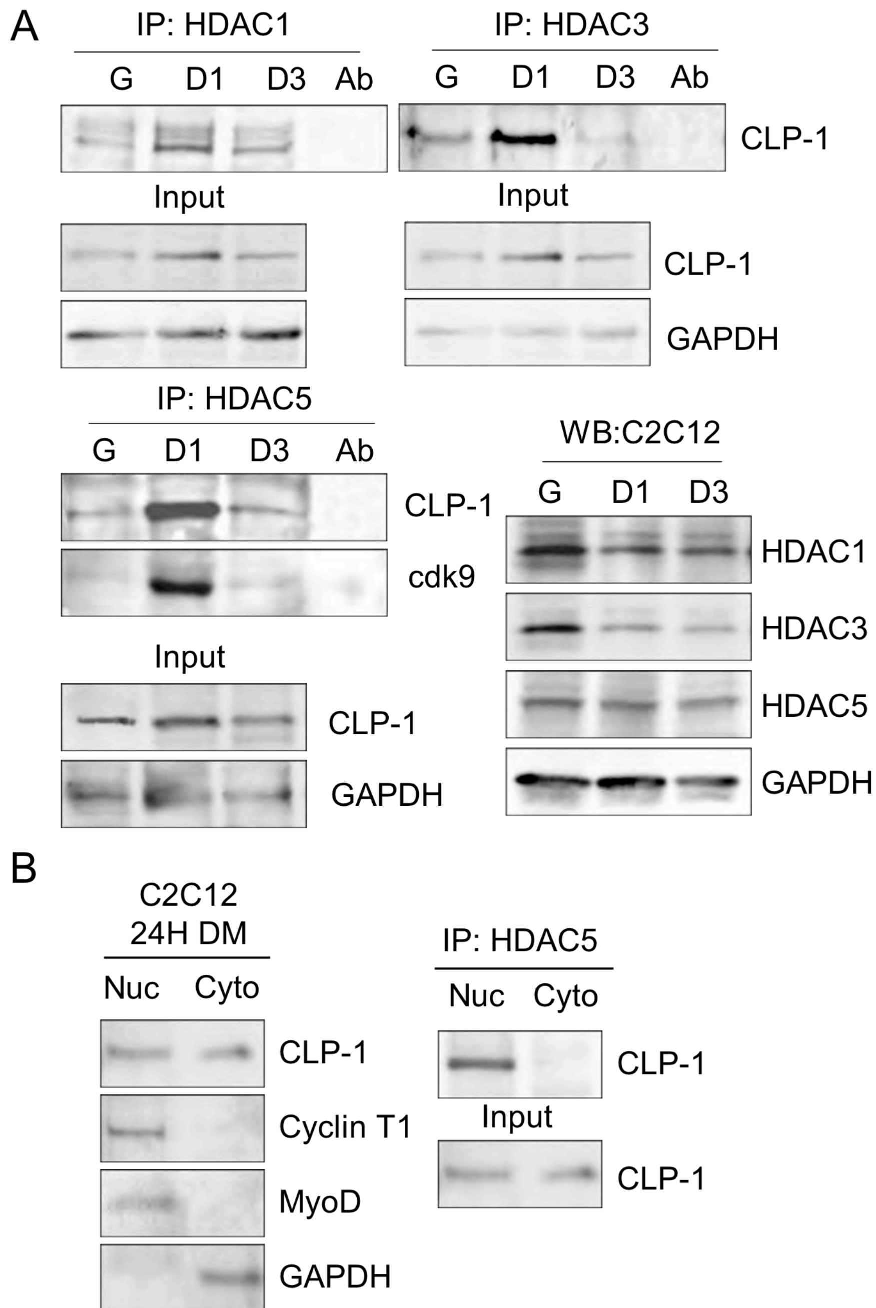


Figure 2



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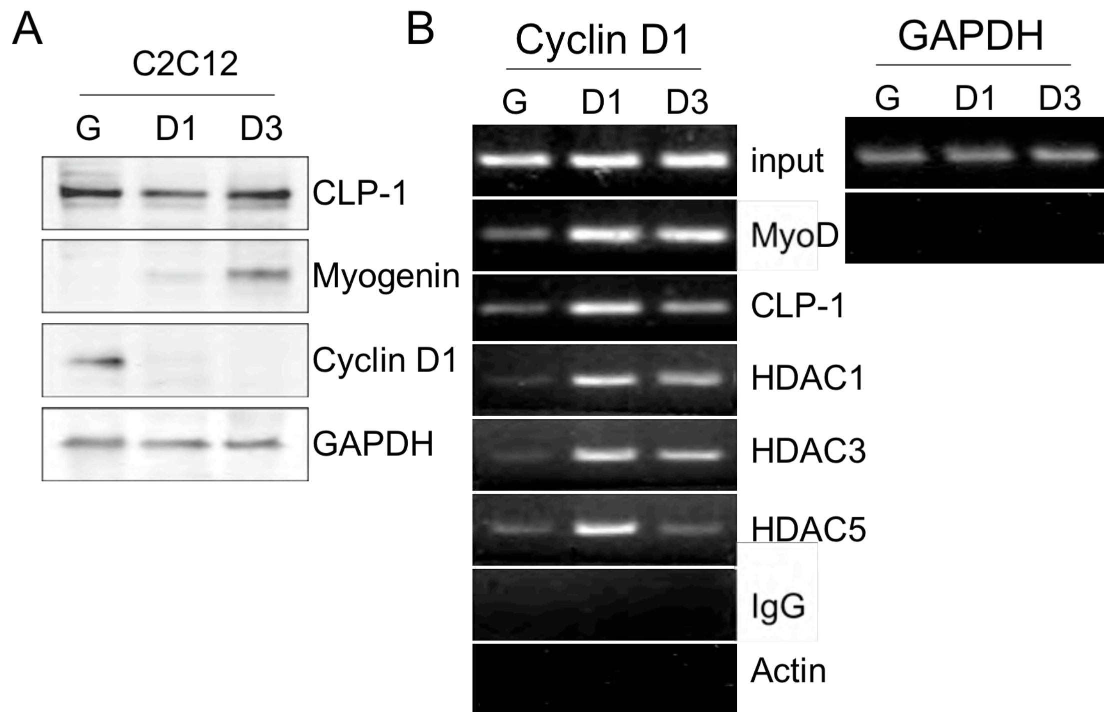

Figure 4

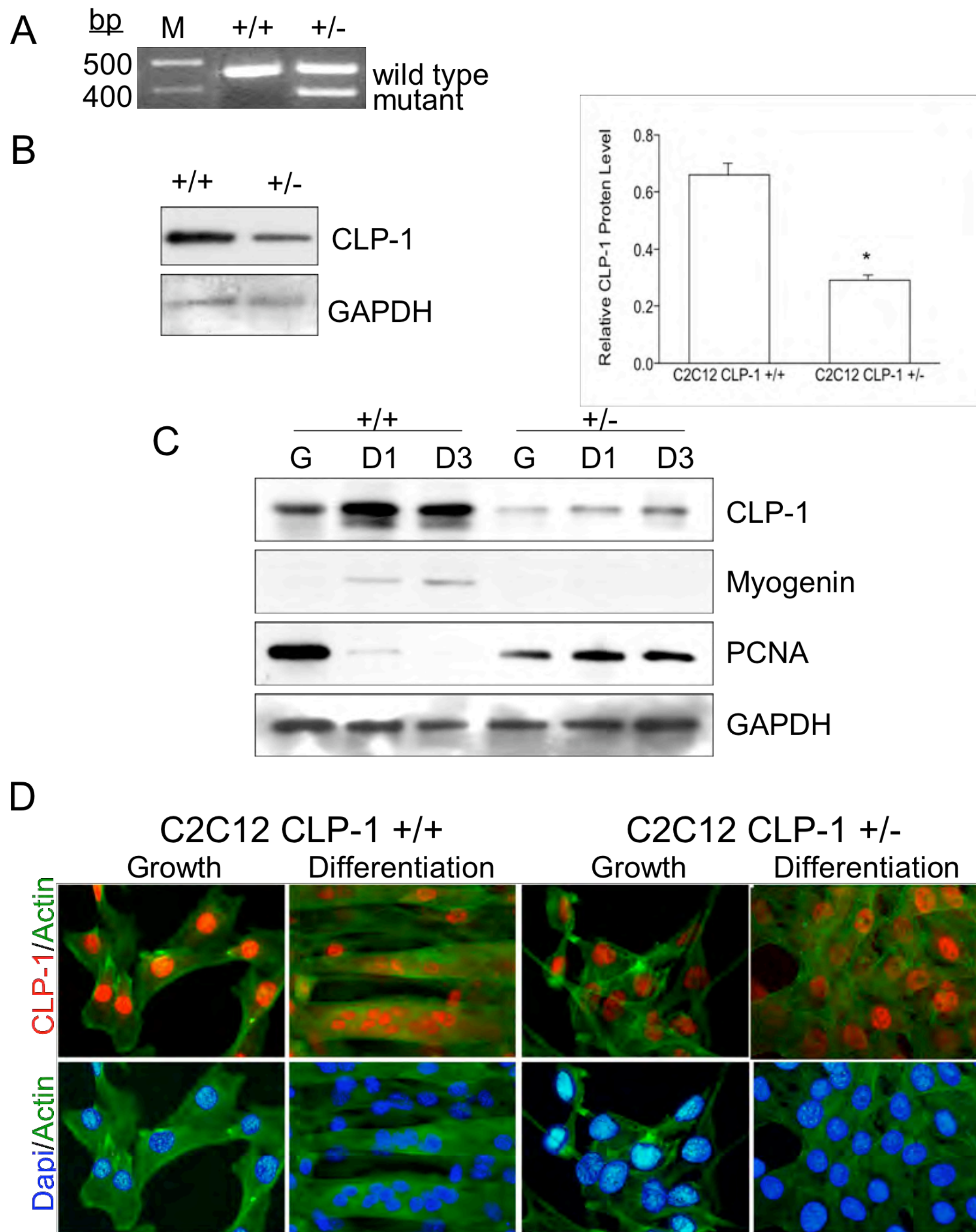

Figure 5