Effect of p300 HAT Activity on Myogenic Differentiation

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Abstract

Skeletal muscle specification and differentiation programs are regulated by the myogenic regulatory factors which include Myf5, MyoD, myogenin and Mrf4. Upstream of the MRFs, the transcription co-activators and other intracellular and extracellular signals play crucial roles in regulating skeletal myogenesis. Histone acetyltransferase activity of p300 is required for Myf5 and MyoD expression. Furthermore, the MyoD core enhancer region is indispensable for MyoD expression. However, the mechanism by which p300 activates *MyoD* gene expression is to be determined. The histone acetyltransferase activity of p300 can be inhibited by small molecule inhibitors such as curcumin. Thus, using the inhibitor approach on stem cells is useful to investigate the role of p300 in activating *MyoD* expression during myogenesis. We here show that curcumin was able to inhibit stem cell determination and differentiation into skeletal myocytes. We also show that p300 is present, and histone acetylation is high at the core enhancer region. Therefore, we provide evidence that p300 is directly involved in *MyoD* gene expression during skeletal myogenesis.

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Ac: Acetylation

α –MEM: Minimum Essential Medium-α

BD: Bromodomain

bHLH: basic Helix-Loop-Helix

BMP4: Bone Morphogenetic Protein 4

cAMP: Adenosine 3',5'-cyclic-monophosphate

CBP: CREB Binding Protein

CER: Core Enhancer Region

CH: Cysteine and Histidine-rich regions

ChIP: Chromatin Immunoprecipitation

CREB: cAMP Response Element Binding protein

Dach: Dachshund

DMEM: Dulbecco's Modified Eagle Medium

DML: Dorsal Medial Lip

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic Acid

DRR: Distal Regulatory Region

DTT: Dithiothreitol

ES: Embryonic Stem cells

Eya: Eyes absent

EB: Embryoid Body

EC: Embryonal Carcinoma

EDTA: Ethylenediaminetetraacetic Acid

EEE: Early Epaxial Enhancer

FGF: Fibroblast Growth Factor

FBS: Fetal Bovine Serum

FCS: Fetal Calf Serum

FAT: Factor Acetyltransferase

GCN5: Spt-Ada-Gcn5-Acetyltransferase

Gli: Glioma-associated oncogene homolog

HAT: Histone Acetyl Transferase

HDAC: Histone deacetylase

H3K9Ac: Histone3 Acetylation on Lysine 27

H3K4me: Histone3 Methylation on Lysine 4

HS: Horse Serum

HI-FBS: Heat Inactivated - Fetal Bovine Serum

ICM: Inner Cell Mass

IP: Immunoprecipitation

IgG: Immunoglobulin G

IF: Immunofluorescence

Kb: Kilo basepair

K1X: Binding site of CREB

LXR: Liver X Receptor

MADS: MCM1, Agamous, Deficienns and human serum response factor

MRF: Myogenic Regulatory Factor

Myf5: Myogenic Factor 5

MyoD: Myogenic Differentiation Antigen

Mrf4: Muscle Regulatory Factor 4

MEF2: Myocyte Enhancer Factor 2

Me: Methylation

MHC: Myosin Heavy Chain

mRNA: Messenger RNA

Msx1: Methionine sulfoximine 1

Pax3: Paired Box 3

Pax7: Paired Box 7

PBS: Phosphate Buffered Saline

PPAR: Peroxisome Proliferator Activated Receptor

PXR: Pregnane X Receptor

PCAF: p300/CBP-Associated Factor

PMSF: Phenylmethylsulfonyl fluoride

RA: Retinoic Acid

RAR: Retinoic Acid Receptor

RXR: Retinoid X Receptor

RT-PCR: Reverse Transcription Polymerase Chain Reaction

Shh: Sonic Hedge Hog

Six: Sine oculis

SID: Steroid receptor co-activator-1 Interaction Domain

SDS: Sodium Dodecyl Sulfate

THR: Thyroid Hormone Receptor

TE: Tris-EDTA buffer

VDR: Vitamin D Receptor

VLL: Ventral Lateral Lip

Wnt: Wingless/Integrated

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1. Introduction

Skeletal muscles are highly specified tissues that are made up of differentiated myocytes (Miller, 1991). Myogenic regulatory factors have been extensively studied to understand the mechanism and regulation of skeletal myogenesis. Moreover, intracellular and extracellular signaling is very crucial during muscle development. However, the interaction mechanism between the MRFs, acetyltransferases, signaling proteins and/or muscle genes has yet to be determined. Our study aims to understand the skeletal myogenesis at the molecular level. We are focusing to elucidate the direct involvement of p300, a co-activator that is crucial for skeletal myogenesis, in *MyoD* regulation.

Skeletal myogenesis

Skeletal myogenesis is a complex process that requires commitment of mesodermal progenitors to the skeletal muscle lineage and transcriptional activation of numerous muscle genes. The main source of progenitors for all body muscles such as epaxial, hypaxial and deep back muscles is the somites, which are formed from the paraxial mesoderm in the mouse embryo. The formation of somites takes place as pairs of epithelial spheres of paraxial mesoderm on either side of the neural tube/notochord during axis formation (Christ & Ordahl, 1995; Nowicki & Burke, 2000). After several hours of epithelialization, the ventral region of the somite, referred to as the sclerotome, will form the cartilage and bone of the vertebral column and the ribs, whereas the dorsal part of the somites makes the dermomyotome, which is a sheet of columnar cells that produces all the body musculature progenitor lineages (Pownall, Gustafsson, & Emerson, 2002). The newly formed dermomyotome consists of two types of cells, medial

dermomyotome cells and lateral dermomyotome cells which are located at the dorsal medial lip (DML) and ventral lateral lip (VLL) respectively. Each type gives rise to different groups and response to distinct developmental signals. The DML gives rise to epaxial muscle progenitors, the first to be produced in newly formed somites of mouse embryos, which form the myotomal deep back muscles. However, VLL gives rise to hypaxial muscle progenitors that eventually form limb muscles, intercostal muscles and abdominal wall muscles (Buffinger & Stockdale, 1995; Christ & Ordahl, 1995; Cinnamon, Kahane, & Kalcheim, 1999; Denetclaw, Berdougo, Venters, & Ordahl, 2001; Denetclaw et al., 2001).

Myogenic regulatory factors

Skeletal myogenesis has been extensively studied as it is essential for the survival of the organism (Devlin & Emerson, 1978; Konieczny & Emerson, 1984; Konigsberg, 1963; Yaffe, 1968). The formation of skeletal muscles during vertebrate embryogenesis requires the expression of myogenic regulatory factors (MRFs) including Myf5, MyoD, myogenin and Mrf4 in which they initiate myoblast identity and terminal differentiation (Hasty et al., 1993; Rudnicki et al., 1993; Nabeshima et al., 1993).

Investigations of the expression, function and regulation of MRFs in mouse embryos have revealed that the MRF genes are the key regulators of the determination and the terminal differentiation of skeletal muscle lineage. Skeletal myogenesis is a multistep process which involves specification of the mesodermal precursors into a muscle lineage, followed by formation of myoblasts and multinucleated muscle fibers, and finally activation of muscle-specific genes (Pownall et al., 2002).

During skeletal myogenesis, transcription factors bind to sequence-specific DNA motifs located at the regulatory regions of muscle genes. This binding causes chromatin modifications that lead to loosening of the DNA in the nucleosomes, which allows the recruitment and activation of the transcriptional machinery in order for the genes to be expressed (Sartorelli & Caretti, 2005). Cell specification, proliferation and differentiation into skeletal muscles are complex pathways orchestrated by the action of different transcription factors and co-regulators. Pivotal in the biology of myogenesis are the myogenic regulatory factors (Buckingham, 1994; Weintraub, 1993). They belong to a basic-helix-loop-helix (bHLH) family of transcription factors that dimerize with other HLH proteins and bind DNA to regulate gene expression (Braun, Rudnicki, Arnold, & Jaenisch, 1992; Braun & Arnold, 1995). This family consists of four distinct master transcription regulators: Myf5, MyoD, myogenin and Mrf4 (Gianakopoulos et al., 2010; Puri et al., 1997; Tapscott, 2005). The MRFs act by dimerizing E proteins to bind the ubiquitously expressed bHLH sequence E-box (CANNTG), located at muscle gene enhancers and/or promoters where they regulate muscle-specific gene expression (Berkes & Tapscott, 2005; Blackwell & Weintraub, 1990; Sartorelli & Caretti, 2005). Previous studies have illustrated the crucial function of MRFs (Gianakopoulos et al., 2010; Puri et al., 1997; Rudnicki et al., 1993; Tapscott, Lassar, & Weintraub, 1992). However, the direct interaction between MRFs and chromatin modifying factors, transcription regulators and other signaling proteins has yet to be characterized. MRFs are exclusively expressed in the skeletal muscles and enforce skeletal muscle formation when culturing non-myogenic cell types (Buckingham, 1992). Hence, each of these myogenic regulatory factors has been postulated to play a major role in muscle cell specification and terminal

differentiation (Gianakopoulos et al., 2010; Polesskaya & Harel-Bellan, 2001; Puri et al., 1997; Rudnicki et al., 1993; Tapscott et al., 1992; Tapscott, 2005; Hasty et al., 1993; Nabeshima et al., 1993) (Figure 1).

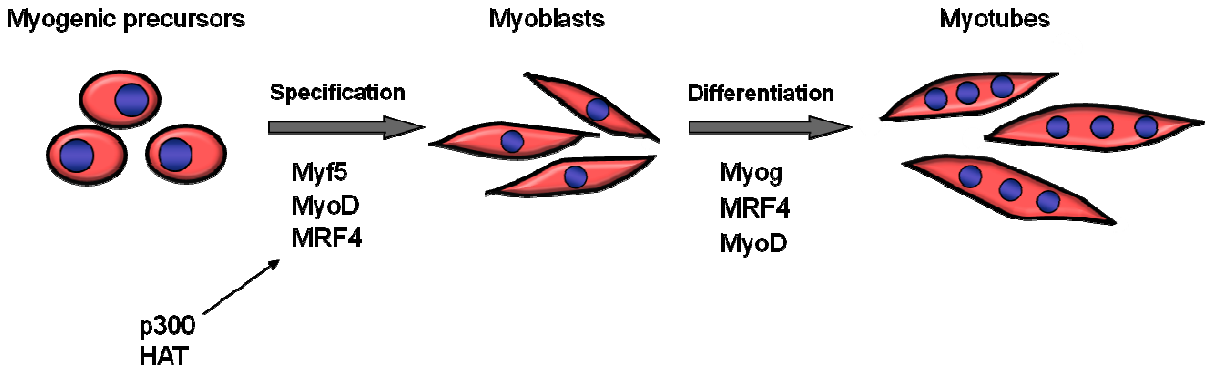


Figure 1: **Involvement of MRFs in skeletal myogenesis**. Myf5 and MyoD expression is the key step that results in commitment of myogenic precursors into muscle lineages. Mrf4 is involved in the specification, and along with MyoD and myogenin, is involved in terminal differentiation.

In conjunction with MRFs, the myocyte enhancer factor 2 (MEF2) MADS (MCM1, Agamous, Deficienns and human serum response factor)-box transcription factors including MEF2a, MEF2b, MEF2c and MEF2d are essential for myogenesis (Black & Olson, 1998; Buckingham et al., 2003; Naya & Olson, 1999; Tapscott, 2005). MEF2s need to collaborate with members of the myogenic bHLH proteins during myogenic development in culture to activate myogenic genes expression. This

collaboration is mediated by direct protein-protein interactions between MEF2 and the heterodimers formed between MRFs and E protein (Molkentin, Black, Martin, & Olson, 1995), implying that MEF2 family alone is not sufficient to induce myogenesis.

Each member of the MEF2 family is expressed at a different time point during myogenesis. MEF2d, for example, is expressed in proliferating myoblasts just before they undergo differentiation, MEF2a is expressed when cells begin to enter differentiation status and MEF2c is expressed later during differentiation (Black & Olson, 1998; Black, Molkentin, & Olson, 1998; Breitbart et al., 1993; Nguyen, Bodmer, Abmayr, McDermott, & Spoerel, 1994). Unlike MRF genes, MEF2 genes also function in cardiac and smooth muscles development (Black & Olson, 1998; Black et al., 1998; Edmondson, Lyons, Martin, & Olson, 1994; Leifer et al., 1993; Lyons, Micales, Schwarz, Martin, & Olson, 1995; Buckingham, 1992; Lin, Schwarz, Bucana, & Olson, 1997).

MRFs functions

As mentioned above, the roles of MRFs are indispensable for skeletal muscle specification and differentiation. It has been demonstrated that *Myf5* and *MyoD* induce early specification of epaxial muscle lineage since deletion of both genes (*Myf5* and *MyoD*) results in inhibition of myoblast formation and hence, absence of skeletal muscle appearance. However, a single mutation in either *Myf5* or *MyoD* gene did not show any defect in muscle development (Rudnicki et al., 1993), suggesting that *Myf5* and *MyoD* have overlapping functions in muscle cell specification. This finding also suggests that *Myf5* and/or *MyoD* expression are crucial for the commitment of multipotential somite cells to the myogenic lineage. Furthermore, *Myf5* and *MyoD* are expressed in the

myoblasts and they are able to convert fibroblasts into myoblasts (Braun, Buschhausen-Denker, Bober, Tannich, & Arnold, 1989; Montarras, Pinset, Chelly, Kahn, & Gros, 1989; Wright, Sassoon, & Lin, 1989), which again indicates that the establishment and maintenance of muscle lineage is governed by Myf5 and/or MyoD (Pownall et al., 2002). *Myf5* and *MyoD* genes have the ability of remodeling chromatin and opening gene loci, which leads to further muscle differentiation (Gerber, Klesert, Bergstrom, & Tapscott, 1997).

Myogenin acts genetically downstream of Myf5 and MyoD to turn on the muscle differentiation program through activation of muscle differentiation genes (Hasty et al., 1993; Nabeshima et al., 1993; Rawls et al., 1995). Myf5 and MyoD are essential for *myogenin* gene transcription (de la Serna et al., 2005; Rawls et al., 1995). Studies have shown that null mutations in *myogenin* result in very poor development of skeletal muscles, although myoblasts are present, indicating that *myogenin* has an important function in myoblast terminal differentiation (Hasty et al., 1993). Previous studies have shown that Mrf4 is essential during early somitogenesis, but is not essential for myocyte formation (Braun & Arnold, 1995; Patapoutian et al., 1995). However, later studies of *MyoD/Mrf4* double mutants resulted in a lethal deficiency of differentiated skeletal muscle (Rawls et al., 1998), a phenotype that was similar to that of *myogenin* mutants (Hasty et al., 1993), suggesting that *MyoD* and *Mrf4* have a partial function redundancy during the activation of muscle differentiation program (Rawls et al., 1998). Although myoblasts from either *myogenin* or *MyoD/Mrf4* double mutant mice are unable to form myofibers *in vivo*, myoblasts from these mutants are capable of differentiating into muscles when cultured *in vitro* (Nabeshima et al., 1993; Rawls et al., 1995; Valdez,

Richardson, Klein, & Olson, 2000). Myoblasts from *myogenin/Mrf4/MyoD* triple mutants are unable to differentiate into muscles *in vitro*. Nevertheless, *myogenin-/-/Mrf4-/-/MyoD+/-* myoblasts are able to form differentiated myocytes *in vivo*, suggesting that *MyoD* is able to partially rescue myogenesis in the absence of both myogenin and Mrf4. Since MRFs act in a cascade fashion, it has been demonstrated that they can also regulate their own and each other's expression (Braun et al., 1989; Brennan, Edmondson, & Olson, 1990; Naidu, Ludolph, To, Hinterberger, & Konieczny, 1995; Thayer et al., 1989). Overall, each MRF mutant results in a distinct phenotype, suggesting that each myogenic bHLH gene has a unique function during myogenesis. Therefore, the spatial and temporal expression of each bHLH transcription factor is crucial during embryogenesis (Tapscott, 2005).

Genetic analysis of *Myf5* and *MyoD* myogenic functions

During embryogenesis, muscle formation is regulated by the MRFs among which Myf5 is the first to be expressed and is the earliest marker of myoblast specification in the dorsal dermomyotome (Kablar et al., 1998; Tapscott, 2005; Rudnicki et al., 1993). In addition to playing a role in myogenic specification, MyoD is considered to be the master switch for the skeletal differentiation program (Gu et al., 1993; Halevy et al., 1995; Zhang, Zhao, Wei, & Paterson, 1999; Lassar, Paterson, & Weintraub, 1986).

In mice, Myf5 is expressed in the mature somites and its transcript is accumulated in the myotome, the first skeletal muscle to appear (Ott, Bober, Lyons, Arnold, & Buckingham, 1991). Moreover, in epaxial, hypaxial and head muscles, *Myf5* expression is controlled by a set of lineage-specific transcription enhancer elements, implying that

different mechanisms control cell determination at different stages of myogenesis in the embryo (Carvajal, Keith, & Rigby, 2008; Ott et al., 1991). *In vivo*, inactivation of *MyoD* causes up regulation of *Myf5* gene expression and they do not exhibit defects in skeletal myogenesis (Rudnicki, Braun, Hinuma, & Jaenisch, 1992). This indicates that there is a compensatory mechanism that contributes to their functional redundancy, suggesting that *Myf5* can functionally substitute for *MyoD*, at least for a short time, during myogenesis (Rudnicki et al., 1993). However, mice embryos lacking *Myf5* suffer from a severe rib defect which leads to abnormalities in the respiratory function and perinatal death (Braun et al., 1992). This suggests that *Myf5* expression during the myotomal development is required for directing the signals to the neighbor sclerotomal cells to form the ribs (Grass, Arnold, & Braun, 1996). Mice lacking *MyoD*, *myogenin* and *Mrf4* seem to have normal myoblasts but fail to form differentiated muscle fibers. However, *myogenin/Mrf4* and *MyoD/Mrf4* mutant mice are able to express *Myf5*. These results suggest that *myogenin* and/or *MyoD* are essential for maintaining *Myf5* expression and maintaining the myogenic differentiation program (Valdez et al., 2000). In P19 cells, Myf5 transcript is detected during myogenic specification, which indicates the commitment of cells to skeletal muscle lineage (Francetic T. et al., 2012). Moreover, *Myf5* mutant studies showed that *Myf5* has a function in the control of progenitor cell proliferation (Montarras, Lindon, Pinset, & Domeyne, 2000), indicating the upstream regulatory function of *Myf5* over *MyoD* in epaxial and hypaxial myotome progenitors. (Pownall et al., 2002). Altogether, the early function and expression of Myf5 before the other MRFs indicate that Myf5 works at the top of the myogenic cascade to start myogenesis (Buchberger, Nomokonova, & Arnold, 2003).

MyoD expression is activated ~2 days after *Myf5* expression in the epaxial progenitors (Tajbakhsh & Buckingham, 2000). Although it has been demonstrated that *MyoD* has an essential regulatory function in progenitor cell specification, later studies showed that MyoD is also required for muscle differentiation since MyoD-deficient myoblasts fail to undergo differentiation (Delfini, Hirsinger, Pourquie, & Duprez, 2000; Tapscott, 2005). *MyoD* mutant mice are viable and fertile. However, cell proliferation and regeneration are abnormal, indicating that *MyoD* has an essential function in adult muscles (Cornelison, Olwin, Rudnicki, & Wold, 2000; Megeney, Kablar, Garrett, Anderson, & Rudnicki, 1996; Montarras et al., 2000). It has also been demonstrated that *MyoD* and *Myf5* activation is controlled in muscle progenitor lineages through both interactive and independent function of developmental signaling ligands and their signal transduction effectors, most likely via direct regulation of *Myf5* and *MyoD* transcription enhancers (Pownall et al., 2002). In embryonic stem (ES) cells, exogenous *MyoD* expression promotes chromosomal *MyoD* activation but does not initiate a complete skeletal muscle differentiation program (Dekel, Magal, Pearson-White, Emerson, & Shani, 1992). Moreover, exogenous *MyoD* expression allows ES cells to differentiate into muscles during aggregation and non-proliferation stage (Kato & Gurdon, 1993), implying that *MyoD* myogenic function requires particular cell signaling environment to activate a complete muscle differentiation program. *MyoD* is therefore able to activate auto-regulation and expression of some early muscle differentiation genes but not the later regulatory program of muscle differentiation (Pownall et al., 2002). As mentioned before, *MyoD* has myogenic regulatory function downstream of *Myf5* in epaxial and hypaxial myotome progenitor and is expressed 2.5 days after *Myf5* expression in wild-type mice

(Tajbakhsh & Buckingham, 2000). However, this is not the case with *Myf5* mutants, in which *MyoD* expression is delayed by one day. Thus, *Myf5* regulates the timely activation of *MyoD*, but the compensatory mechanism takes place for the delayed *MyoD* expression in the absence of *Myf5* in these myotomal lineages (Pownall et al., 2002). Since MyoD has also been found to be acetylated in proliferated myoblasts (Polesskaya et al., 2000), other mechanisms must therefore be involved in MyoD activation during myogenesis.

Enhancer elements of *MyoD*

Given the crucial role of *MyoD* in skeletal muscle specification and differentiation programs (Delfini et al., 2000; Rudnicki et al., 1993; Tapscott, 2005), a mechanistic understanding of this gene and how its expression is activated will provide powerful information on how *MyoD* is controlled in the transcriptional context.

It has been demonstrated that a highly conserved core enhancer region (CER, ~20Kb 5' of human *MyoD*) is indispensable for *MyoD* activation in the somites and limb buds (Faerman, Goldhamer, Puzis, Emerson, & Shani, 1995; Goldhamer et al., 1995; Kablar et al., 1998; J. C. Chen, Love, & Goldhamer, 2001; J. C. Chen & Goldhamer, 2004). Recent studies showed a dramatic accumulation of H3.1 around the *MyoD* CER, implying the involvement of *MyoD* during myogenic differentiation (J. H. Yang et al., 2011).

In mature muscles, the distal regulatory region (DRR, ~5Kb upstream of *MyoD*) is important for *MyoD* expression in which DRR sequence is unrelated to CER (Asakura, Lyons, & Tapscott, 1995; J. C. Chen et al., 2001; Goldhamer et al., 1995; Kablar et al.,

1998; Tapscott et al., 1992). Moreover, the activity of DRR is exclusive to differentiated skeletal muscles *in vivo* (Kablar et al., 1997), and its activity is completely MRF dependent (Kablar et al., 1999). Previous studies demonstrated that CER loses its activity once adult muscles develop, whereas DRR stays active and results in a similar expression pattern of endogenous *MyoD*. Also, deletion of DRR results in MyoD mRNA reduction. These findings suggest that in adult muscles, DRR is necessary to sustain the normal *MyoD* expression. It has previously been shown that DRR and *Myf5* mutant mice embryos express *MyoD* at the appropriate time in the limb and branchial arches (J. C. Chen, Ramachandran, & Goldhamer, 2002), indicating that the DRR is not crucial for *MyoD* expression and the CER and other enhancers might compensate for the absence of DRR (Pownall et al., 2002).

In addition, Tapscott and colleagues have identified a proximal regulatory region (PRR, ~ -275 bp to +1) which is, along with DRR, sufficient to activate the transcription of muscle genes when cultured *in vitro* (Tapscott et al., 1992). Like DRR, PRR is not sufficient for early expression of *MyoD* in the limb muscles, but it contains essential regulatory elements to maintain endogenous *MyoD* expression (Asakura et al., 1995). Therefore, each one of these enhancers/promoters has a specific regulatory function during myogenesis. Further studies are required to individually study the role of each enhancer/promoter in MRFs expression during myogenesis.

Extracellular developmental signaling in epaxial/hypaxial muscles

Gene expression studies demonstrated that some developmental signaling ligands from surrounding tissues are known for their roles in muscle development. These signaling proteins include Sonic hedgehog (Shh), Wingless/Integrated (Wnt) family, Bone Mophogenetic Proteins (BMPs), Notch, Fibroblast Growth Factors (FGF), and Retinoic Acid (RA), in which all positively or negatively control *Myf5* and *MyoD* activation in muscle epaxial progenitors (Pownall et al., 2002). Shh is produced by notochord and floor plate cells (Fan & Tessier-Lavigne, 1994), BMPs are secreted from the lateral plate and mesodermal cells (A. G. Borycki et al., 1999; Dietrich, Schubert, Healy, Sharpe, & Lumsden, 1998; Munsterberg, Kitajewski, Bumcrot, McMahon, & Lassar, 1995; Pourquie et al., 1996; Tajbakhsh et al., 1998), and Wnt is expressed in the neural tube and dorsal ectoderm (Parr, Shea, Vassileva, & McMahon, 1993). These signals act upstream of Myf5, MyoD, Mrf4 and myogenin. Also, these signals play essential roles in *Myf5* activation during epaxial progenitor specification (A. G. Borycki, Mendham, & Emerson, 1998; Munsterberg et al., 1995).

Previous studies have shown that Shh signaling targets *Myf5* but not *MyoD* through Gli transcription factor in epaxial progenitors (A. G. Borycki et al., 1999; Gustafsson et al., 2002). It has also been demonstrated that Wnt1 regulates Shh signaling to coordinate *Myf5* activation in epaxial progenitors during somites formation by stabilizing β-catenin (A. Borycki, Brown, & Emerson, 2000; Wodarz & Nusse, 1998), while Wnt7a preferentially activates *MyoD* (Tajbakhsh et al., 1998) through Shh, where which *Myf5* has to be present in order for the Shh to activate MyoD (A. G. Borycki et al.,

1999; McDermott et al., 2005). Hence, Wnt and Shh are essential for the activation of *Myf5* and *MyoD* in epaxial muscles (Tajbakhsh et al., 1998).

Furthermore, the Six-Eya-Dach family of transcription factors (Six-1 to Six-6) has been found to be essential for the proliferation and differentiation of muscle cells (Kawakami, Sato, Ozaki, & Ikeda, 2000; Kumar, 2009; Laclef et al., 2003; Laclef, Souil, Demignon, & Maire, 2003; Ozaki et al., 2004), where Six-1 is crucial for skeletal muscle development (Li et al., 2003). Previous studies showed that *Six-1* mutant mice die at birth due to primary myogenesis and respiratory failures (Laclef et al., 2003; Laclef, Souil et al., 2003; Li et al., 2003). However, mice lacking only *Six-4* develop normally, and double *Six-1/Six-4* mutant mice studies result in more apparent defect in myogenesis than in *Six-1* mutant mice (Grifone et al., 2005). These findings suggest that Six-4 has an important function during muscle development when cooperating with Six-1. In addition, these results suggest that Six-1 has a crucial function during early stages of muscle development. Six-1 and Six-4 play a key role in myogenesis including regulation of MRFs expression. For instance, *Six-1* has been demonstrated to be essential for *MyoD* and *myogenin* activation in the limb buds (Laclef et al., 2003). Furthermore, the Six family also plays a significant role in regulating the hypaxial promoter of Pax (Paired box protein)-3 (Franz, Kothary, Surani, Halata, & Grim, 1993; Grifone et al., 2005; Grifone et al., 2007).

Pax3 is a transcription factor with homeo and paired domain motifs. It is a member of the developmentally transcriptional regulators family and plays a crucial role in skeletal muscle formation (Sato, Rocancourt, Marques, Thorsteinsdottir, & Buckingham, 2010). It has been shown that *Pax3* mutant mice result in skeletal muscle

impairment (Daston, Lamar, Olivier, & Goulding, 1996; Franz et al., 1993; Grifone et al., 2007). Pax3 is required for progenitor migration to the limb buds (Daston et al., 1996), since *Pax3* null mice result in severe muscle loss (Alvares et al., 2003; Dietrich et al., 1999; Epstein, Lam, Jepeal, Maas, & Shapiro, 1995; Grifone et al., 2005; Tajbakhsh, Rocancourt, Cossu, & Buckingham, 1997). Moreover, Pax3 is expressed in the somite before becoming restricted to the dermomyotome and muscle cells (Goulding, Lumsden, & Paquette, 1994; Williams & Ordahl, 1994) and its induction is also essential for Myf5, MyoD and myogenin expression (Maroto et al., 1997). However, a dominant negative Pax3 in P19 cells leads to loss of MyoD and myogenin expression and therefore loss of myogenesis (Ridgeway & Skerjanc, 2001).

Pax3 and *Myf5* single and double mutations have been analyzed to examine their ability to undergo myogenesis. It has been shown that *Myf5* mutant embryos result in a defect in myogenesis. It has also been shown that Pax3 directly regulates Myf5 expression via the limb bud enhancer of *Myf5* (Bajard et al., 2006). Since *MyoD* is not expressed in the trunk and limb muscles of *Pax3/Myf5* double mutant mice, it has been demonstrated that *MyoD* acts genetically downstream from these two genes (*Myf5* and *Pax3*) for the initiation of skeletal myogenesis (Tajbakhsh et al., 1997). Therefore, in cooperation with Six family of proteins and its cofactor Eya, Pax3 and Pax7 regulate MRFs expression (Ridgeway & Skerjanc, 2001).

RA is required for proper somite formation during development (Maden, Gale, Kostetskii, & Zile, 1996; Maden, Graham, Zile, & Gale, 2000; Niederreither, Subbarayan, Dolle, & Chambon, 1999). RA is a derivative of vitamin A (Chambon, 1996), and exists as two isomers, all-trans and 9-cis RA (Ricaud, Vernus, & Bonnieu,

2005). Moreover, RA functions through two families of nuclear receptors, RAR and RXR, in which both consist of α, β, and γ subunits (Chambon, 1996).

The RAR and RXR are nuclear receptors which are required for proper development (Chiba, Clifford, Metzger, & Chambon, 1997). RAR binds and is activated by both all-trans and 9-cis RA isomers (Ricaud et al., 2005). Previous studies showed that animals lacking RAR-α or RAR-γ display postpartum lethality (Lohnes et al., 1993). The RXRs heterodimerize with different nuclear receptors such as thyroid hormone receptor (THR) and vitamin D receptor (VDR) (Maden, Sonneveld, van der Saag, & Gale, 1998; Mic, Molotkov, Benbrook, & Duester, 2003; Szanto et al., 2004). RXR-α mutant mice die in the uterus due to hypoplastic myocardium (Kastner et al., 1994; Kastner, Mark, & Chambon, 1995). However, *RXR- γ -/-* mutant mice are viable and do not display defects in muscles (Dolle, 2009), which suggests that the loss of RXR- γ is compensated for by RXR- α (Tanaka & De Luca, 2009). Thereby, these findings indicate that RAR and RXR signals are essential during muscle development.

Role of p300 HAT activity in MRFs regulation

The formation and maintenance of skeletal muscle requires the proper orchestration of myogenic regulatory factors, transcription co-regulators and signal transduction pathways. As the extracellular signals are critical for proper myogenic regulation, nuclear factors are also crucial for gene expression in response to several physiological processes, such as proliferation, apoptosis and differentiation (Brownell & Allis, 1996; Montminy, 1997). Co-regulators are required to cooperate with MRFs as co-regulators do not directly bind to DNA, but that are recruited to enhancer/promoter

regions via interaction with sequence-specific DNA binding proteins. These co-regulators could be transcription factors (Novitch, Mulligan, Jacks, & Lassar, 1996; Sellers et al., 1998), histone deacetylases (HDAC) (Lu, McKinsey, Zhang, & Olson, 2000; McKinsey, Zhang, Lu, & Olson, 2000; Steinbac, Wolffe, & Rupp, 2000), and histone acetyltransferases (Eckner, Yao, Oldread, & Livingston, 1996; Missero et al., 1995; Puri et al., 1997; Yuan, Condorelli, Caruso, Felsani, & Giordano, 1996).

MyoD and myogenic bHLH have been shown to interact with the co-activator p300/CBP in which *MyoD* and *Myf5* determine the myogenic identity of mesodermal cells, whereas *myogenin*, *Mrf4* and MyoD contribute to the terminal differentiation (Black et al., 1998; Tapscott, 2005; Buchberger et al., 2003; Delfini et al., 2000; J. F. Roth et al., 2003). p300 and CBP were first characterized as partners for adenoviral E1A protein and cAMP response element binding protein (CREB) respectively (Chrivia et al., 1993; Eckner et al., 1994). They are global transcriptional co-activators that are ubiquitously expressed and capable of interacting with different transcription factors to regulate a wide variety of cellular processes, such as proliferation and differentiation (Eckner et al., 1996; J. F. Roth et al., 2003; J. C. Chen et al., 2001; Goodman & Smolik, 2000; Shiama, 1997). p300 functions to regulate transcription and open chromatins, thereby facilitates diverse signaling. In physiology, p300 regulates transcription factors that are responsible to control differentiation within a particular cell line (Shiama, 1997). *In vivo* studies provided direct evidence that p300 is crucial for cell cycle regulation and cell differentiation (Yao et al., 1998). Histone acetyltransferase activity has been shown to have important functions in transcription (Puri, Sartorelli et al., 1997; S. Y. Roth, Denu, & Allis, 2001). Previous *in vitro* studies have illustrated the contribution of p300

and CBP acetyltransferases in the specification and terminal differentiation of skeletal muscle by regulating MRF genes. Mutations in the HAT active domain of p300/CBP have also been shown to eliminate their transactivation capability (J. F. Roth et al., 2003; S. Y. Roth et al., 2001).

p300 and CBP function to regulate transcription activity and influence chromatin structure as they possess an intrinsic histone acetyltransferase (HAT) domain that is essential for myogenesis (J. C. Chen et al., 2001; Ogryzko, Schiltz, Russanova, Howard, & Nakatani, 1996; J. F. Roth et al., 2003). Histone modifications have been implicated in orchestrating gene expression, particularly histone acetylation (ac) and methylation (me). Generally, histone acetylation associates with gene activation. p300 regulates chromatin structure through histone acetylation, which makes the chromatin more accessible for transcriptional targeting (Ramos et al., 2010). However, histone methylation relates to both gene activation and gene silencing, with the histones H3K4me3 and H3K9me2 activating and silencing genes, respectively. The molecular mechanism by which histone acetylation governs transcription remains to be fully appreciated. *In vivo*, histone acetyltransferases are often able to acetylate many lysine (K) residues (Jin et al., 2011). Histone acetyltransferase regulates gene expression by catalyzing targeted acetylation of the lysine residues on histone and non-histone proteins (Sterner & Berger, 2000; X. J. Yang, 2004). p300 acetylates MyoD, H3 and H4 to promote transcription initiation (Dilworth, Seaver, Fishburn, Htet, & Tapscott, 2004; Jin et al., 2011; Puri, Sartorelli et al., 1997; Sartorelli et al., 1999). Besides histone acetylation, p300 acts as a scaffold protein for the transcriptional initiation assembly, and as a bridge between the sequence specific factors and the basal transcriptional machinery. p300 can also acetylate

transcription factors and non-histone proteins, which often leads to an increase in the transcriptional activity (J. C. Chen et al., 2001; Imhof et al., 1997; Chan & La Thangue, 2001) (Figure 2)

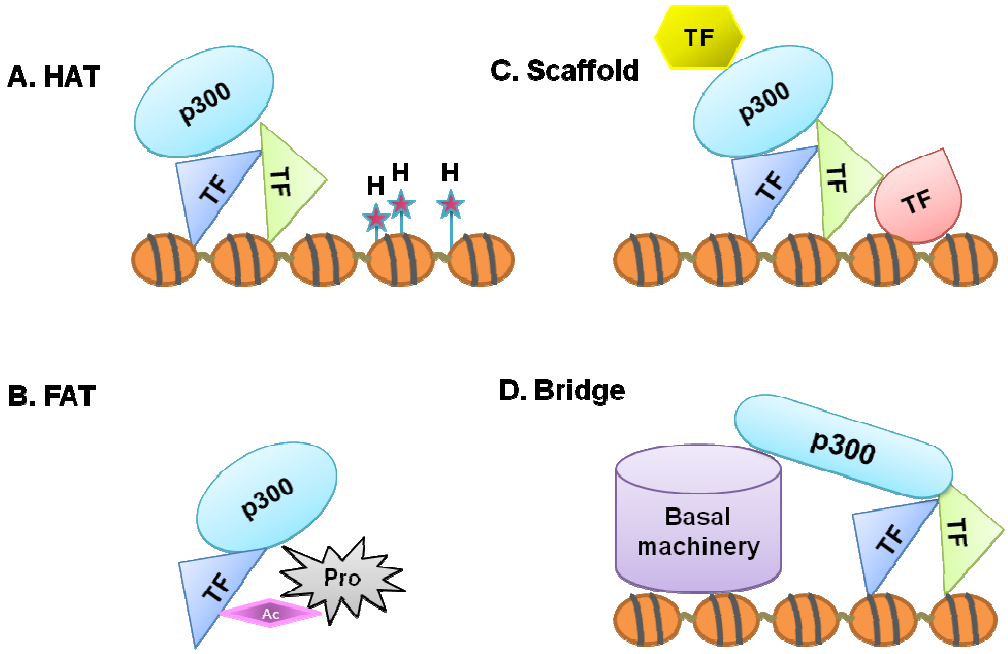


Figure 2: **Transcriptional activation mechanisms of p300.** (A) p300 acetylates histones to facilitate transcriptional activity (HAT). (B) It can also acetylate non-histone proteins (FAT). (C) p300 provides a scaffold for the transcriptional initiation assembly. (D) p300 also acts as a bridge between the sequence specific factors and the basal transcription machinery.

Previous studies have illustrated that acetyltransferases, particularly p300, are present at enhancers and promoters (Hatzis & Talianidis, 2002; Wang, Carroll, & Brown, 2005). Moreover, microarray and ChIP sequencing assays demonstrated that p300

binding sites possess similar characteristics of enhancers. However, it has also been found that many other predicted enhancers were lacking p300 binding sites (Heintzman et al., 2007).

Histone acetyltransferases

Co-activators, as their name implies, are able to activate transcription and interact with the basal transcriptional machinery, as well as act as a scaffold for the assembly of transcriptional complexes and induce chromatin remodeling (Bastien & Rochette-Egly, 2004; Rosenfeld, Lunyak, & Glass, 2006). The co-activators p300 and CBP have different functions, but are also highly related with overlapped involvement. They are indispensable during myogenesis (Ramos et al., 2010), and they interact with transcription factors through conserved domains (CH1, CH3, K1X and SID) (Figure3).

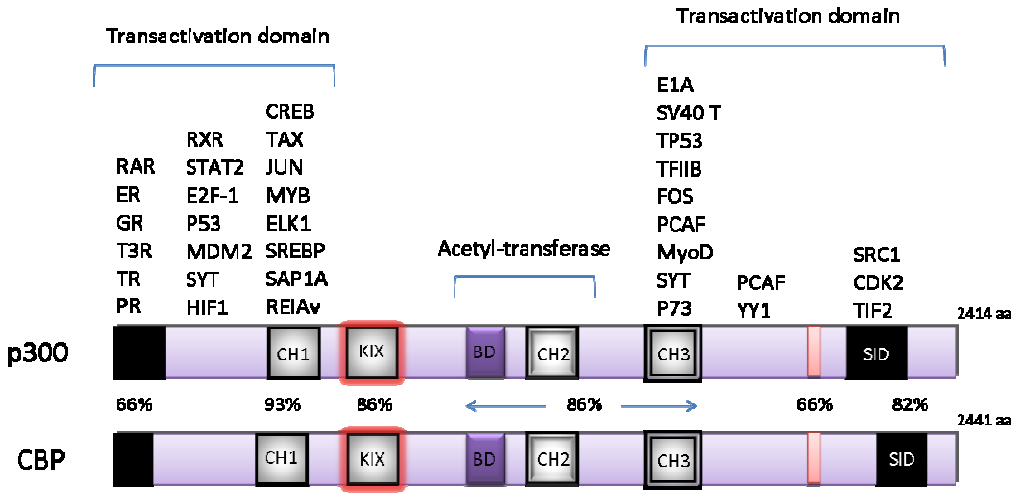


Figure 3: Schematic representation of p300/CBP homologous regions and functional domains. Selected proteins that bind to specific sites of p300/CBP are shown. CH1-3, cysteine and histidine-rich regions 1-3; KIX, binding site of CREB; BD, bromodomain; SID, steroid receptor co-activator-1 interaction domain. The percentage of amino acid identity between the two proteins is indicated.

In vivo, the full complement of p300 acetyltransferase activity is required for normal epaxial muscle formation. Moreover, p300 knockout studies result in compromised *Myf5* expression, and hence, skeletal muscle impairment in mice embryos. *In vitro*, ES cells lacking p300 acetyltransferase activity result with a severe defect in skeletal muscle formation. On the other hand, equivalent mutations in CBP did not exhibit clear muscle impairment and the CBP mutant cells are still able to form myotubes. Furthermore, in p300 acetyltransferase mutant cells, Myf5 and MyoD fail to be expressed. These results suggest that p300 and its acetyltransferase activity are required for myogenesis both *in vivo* and *in vitro*. Moreover, these results validate the difference between the acetyltransferase activity of p300 and CBP, as well as provide evidence for the essential role of p300 in skeletal myogenesis (J. F. Roth et al., 2003). It has also been demonstrated that p300 HAT activity is involved in specific histone acetylation, such as H3K27, to regulate *Myf5* early enhancer during stem cell differentiation (Francetic T. et al., 2012). Since p300 also acetylates non-histone proteins, previous studies showed that the transcription factor MyoD can be acetylated *in vitro* by p300 (Polesskaya et al., 2000). Histone acetylation at *MyoD* enhancers and promoters has been established (J. H. Yang et al., 2011). However, whether the histone acetylation mediated by p300 at *MyoD* enhancers is direct or indirect has yet to be investigated.

Interestingly, other histone acetyltransferases like Spt-Ada-Gcn5 (GCN5) and p300/CBP associated factor (PCAF) have been characterized to be required for myogenic differentiation in culture (Kuninger, Wright, & Rotwein, 2006; Dyda, Klein, & Hickman, 2000; Puri, Sartorelli et al., 1997; X. J. Yang, Ogryzko, Nishikawa, Howard, & Nakatani,

1996). PCAF has been shown to function in a parallel pathway to p300 but displays a different pattern of substrate specificity *in vitro*. Like p300, PCAF also acetylates MyoD and H3 (Sartorelli et al., 1999). PCAF also activates muscle-specific transcription and preferentially acetylates histone H3 on lysine 14 (Schiltz et al., 1999; Trievel, Li, & Marmorstein, 2000). Previous studies showed that PCAF inactivation leads to inhibition of muscle differentiation, indicating that PCAF is also essential for the myogenic differentiation program. Moreover, inhibiting PCAF or p300/CBP completely abolished the differentiation program, suggesting that p300 and PCAF cannot compensate for each other (Kuninger et al., 2006).

Stem cell differentiation

Embryonic stem (ES) cells are pluripotent cells of early embryos that are characterized by their self-renewal ability, which occurs by an auto-organizing group of ectopic expressed transcription factors that inhibit their differentiation and enhance their proliferation. Mouse ES cells were originally derived from the inner cell mass (ICM) of mouse blastocysts (Evans & Kaufman, 1981; Martin, 1981). Previous studies in the last decades have highlighted the role of transcription factors in ES cells maintenance (Boyer et al., 2005; Chambers et al., 2003). It has been concluded that the activity of these transcription factors depends on the pluripotent cell developmental stage. This indicates that these transcription factors function with the cooperation of other processes (Sieweke & Graf, 1998), and depends on the accessibility of their target genes. This accessibility takes place by the modifications of the DNA, histones and/or chromatin structures of these target genes (Jaenisch & Bird, 2003).

Mouse ES cells are suitable to study differentiation of a variety of different cell types *in vitro* as they are capable to differentiate into skeletal, cardiac, neuronal, epithelial and endothelial lineages (Evans & Kaufman, 1981). The differentiation system of ES cells allows one to analyze the early processes involved in the commitment to particular lineages and the developmental steps during the differentiation into specific cell types. Specific cell lines and cultivation allow the embryoid body (EB) of ES cells to differentiate into different cell types. There are several parameters that contribute to the developmental efficiency of ES cells *in vitro* including the number of cells differentiating in the EBs, the quality of the media, the quality of ES cell lines used and the time points of EBs plating or handling (Wobus, Guan, Yang, & Boheler, 2002).

Similarly, embryonal carcinoma (EC) cell lines are derived from tumors called teratocarcinomas. These cells have been extensively used in culture studies due to the similar normal differentiation behavior to embryonic inner cell mass. The P19 is a type of embryonal carcinoma cell line that is derived from teratocarcinomas and that can develop in some mouse strains by transferring early embryos of the female uterus into the ectopic sites (Stevens, 1970). A 7.5-day-old mouse embryo from mating a C3H/He female with males carrying an X-chromosome, bearing some divergent alleles, was injected into an acceptor C3H/He mouse testis ((McBurney & Rogers, 1982; Nielsen & Chapman, 1977). P19 cell line is an experimentally tractable culture system to study early embryonic mechanisms (McBurney, Jones-Villeneuve, Edwards, & Anderson, 1982). They grow continuously in fetal bovine serum (FBS)-and fetal calf serum (FCS)-supplemented media, which have unknown factors that regulate the process of differentiation (McBurney, 1993; Wilton & Skerjanc, 1999). Moreover, they divide rapidly and have the

ability to differentiate even after many transfers in culture. In addition, they can be efficiently induced to differentiate by the simple altering of culture conditions (Bain, Ray, Yao, & Gottlieb, 1994; McBurney, 1993). These characteristics make P19 cells an experimentally outstanding system to study differentiation and cell genetics (McBurney, 1993). P19 cells can be maintained in culture in an undifferentiated form (Bain et al., 1994). They can also be induced to differentiate into different cell types if the aggregates are exposed to non lethal doses of drugs such as Dimethylsulfoxide (DMSO) and Retinoic Acid (RA). DMSO and RA induce P19 cells to differentiate into a wide variety of multinucleated skeletal and/or mononucleated cardiac muscles (McBurney et al., 1982; van der Heyden & Defize, 2003; Wilton & Skerjanc, 1999).

C2C12 myoblasts are another type of stem cells that are subclones of mouse myogenic cell lines, and have the unique quality in that they are already committed to skeletal muscle lineage. They differentiate extensively and rapidly in culture (Blau, Chiu, & Webster, 1983; Shimokawa, Kato, Ezaki, & Hashimoto, 1998; Yaffe & Saxel, 1977). In addition, they proliferate when they are in high serum concentration but lower in confluences. Nevertheless, they fuse when they are in a low concentration of serum but in high confluences, forming multinucleated myotubes within a few days of serum withdrawal. Density and cellular interaction between neighboring cells are therefore critical factors in the effectiveness of cell maintenance and differentiation (Blau et al., 1983; Silberstein, Inestrosa, & Hall, 1982).

Small molecule inducers and skeletal myogenesis

The cell types formed in DMSO- and RA-treated cultures are skeletal, cardiac muscles and/or neuron. Treating cells with DMSO, a small molecule inducer for muscles, leads to induction of P19 cell aggregates to develop into many of the mesodermal and endodermal cell type characteristics, most commonly skeletal and cardiac muscles (Edwards, Harris, & McBurney, 1983; McBurney et al., 1982). The mechanism of DMSO induced differentiation is still not clear. Previous studies suggest that the effect of DMSO is achieved via cooperative kinetics or through affecting some pathways that are composed of extracellular contents and mediated by other cell to cell contact or some soluble factors (Campione-Piccardo, Sun, Craig, & McBurney, 1985; Smith, Reuhl, Craig, & McBurney, 1987).

Besides DMSO, retinoic acid (RA) can also induce differentiation of P19 cells into skeletal muscles (Pratt, Crippen, & Menard, 2000). RA is a natural derivative and active metabolite of vitamin A. Furthermore, RA plays important roles in different embryonic developmental processes. It is also involved in the activation of many genes that play a role in the specification and development of skeletal muscles (Blomhoff, R et al, 2006; Le May et al., 2011; Niederreither & Dolle, 2008). RA is also required for chromatin changes and activation of transcription (Bhattacharyya et al., 1997; Dey, Minucci, & Ozato, 1994). The RA signal is mediated by the nuclear receptors RAR and RXR with each consisting of alpha, beta and gamma isoforms. These isoforms belong to the nuclear receptor superfamily and bind to response elements of RA target genes to activate cell differentiation (Chambon, 1996; Leid, Kastner, & Chambon, 1992; Rohwedel, Guan, & Wobus, 1999). P19 cells response differently to different RA doses.

They differentiate into skeletal muscles with a low concentration of RA around 10^{-9}-10^{-7} M (Edwards et al., 1983), but they differentiate into glial and neuron tissues with a high concentration of RA (>5X10^{-7} M) (Jones-Villeneuve, McBurney, Rogers, & Kalnins, 1982).

Additionally, a synthetic compound, bexarotene has been found to activate the RXR. This RXR selective compound is capable of transactivating the RXR-RXR homodimer (Lehmann et al., 1992), but is unable to activate RARs (Nau & Blaner, 1999). We have previously shown that bexarotene promotes skeletal myogenesis in both P19 and ES cells via a RAR-independent mechanism (Le May et al., 2011). As we established the bexarotene mechanism in our lab, we used bexarotene along with DMSO and RA in order to induce skeletal muscle differentiation in P19 cells.

Inhibitors of p300 HAT activity

Inhibiting the HAT activity of p300 is a useful system to investigate the role of p300 during myogenesis. One of the well studied small molecule p300 HAT inhibitor is curcumin. Curcumin is a natural product isolated from the root of plant curcuma longa, a member of the ginger family. It is a yellow pigment that has been used as spices as well as in herbal remedies, particularly in China, India, Middle East and Indonesia. In ayurvedic medicine, it is a polyphenolic compound that has been used in India. Curcumin has been proved to have some biological effects as an antioxidant, anti-cancer and anti-inflammatory (Y. Chen et al., 2007; Tayyem, Heath, Al-Delaimy, & Rock, 2006). Previous *in vitro* studies have addressed the inhibitory effect of curcumin on p300 HAT activity but not PCAF. Furthermore, their data suggests that curcumin is a potent p300-

specific inhibitor of HAT activity in the transcriptional context (Y. Chen et al., 2007; Balasubramanyam et al., 2004).

Another p300 HAT inhibitor is C646. It is a small and competitive inhibitor with a Ki of 400 nM. It was found that C646 was relatively potent and highly linear selective against p300 as compared with six other HATs. Hence, compound C646 seemed to be efficient for application requiring selective p300 inhibition. *In vitro*, inhibition of p300 HAT activity by C646 results in blocking the acetylation of histone H3 and H4 in mouse fibroblast cell lines. Furthermore, it inhibits the growth of melanoma and lung cancer cell lines (Bowers et al., 2010). The effect of C646 on stem cell differentiation has not been extensively studied, which is why we are interested in investigating the inhibitory effect of C646 on the differentiation of P19 cells into skeletal muscles.

Hypothesis and significance

Regulatory regions of MyoD have been previously characterized (H. Y. Chen et al., 2004; J. C. Chen et al., 2001; J. C. Chen et al., 2002; J. H. Yang et al., 2011). Furthermore, the roles of transcription factors and co-activators on MyoD expression have also been established (Puri et al., 1997; J. F. Roth et al., 2003; Sartorelli, Huang, Hamamori, & Kedes, 1997). The transcriptional co-activator p300 plays a significant role in MRFs regulation during skeletal myogenesis. However, the molecular mechanism by which p300 regulates *MyoD* gene expression has yet to be investigated. Histone acetylation is an important function of p300 in which the acetylation is a common feature of gene activation. Therefore, we hypothesize that p300 is directly involved in the regulation of *MyoD* expression through histone acetylation at the core enhancer region. Our research aims to gain novel, in depth mechanistic insight into the role of p300 HAT activity in skeletal myogenesis.

II. Materials and Methods

Cell Culture and differentiation

P19 cells (Purchased from the American Type Culture Collection-ATCC) were grown in Minimum Essential Medium α (α-MEM) (Gibco-Invitrogen) supplemented with 5% fetal bovine serum (PAA), 5% bovine calf serum (PAA), and 1% Penicillin/Streptomycin (Gibco-Invitrogen). The cells were maintained in incubators at 37ºC with 5% CO_2. P19 cells were aggregated in 150 mm Petrie dishes (VWR) and treated (early) 1 day and again 2 days after plating and left for three more days. Treatments were: no treatment (control), co-treatment of 1% dimethylesulfoxide DMSO (Sigma-Aldrich) with RA (Sigma-Aldrich), co-treatment of 1% DMSO with RA and with curcumin (Sigma-Aldrich) or co-treatment of 1% DMSO with RA and C646 (Gift from Dr. David Meyers). After 4 days of aggregation, cells were allowed to attach to 100 mm tissue culture dishes (Corning) and 22X22 mm coverslips (VWR) coated with 0.1% gelatin and grown for another 5 days, after which they were harvested and stained. P19 cells were also treated at late stages of differentiation. Cells were aggregated in 150 mm Petrie dishes and treated (early) 1 day and again 2 days after plating. Treatments were: no treatment (control), 1% DMSO, co-treatment of 1% DMSO with RA or bexarotene (Purchased from LC Laboratories). After 4 days of aggregation, cells were transferred to tissue culture dishes and coverslips coated with 0.1% gelatin. Then cells were treated with curcumin for three days and left to grow for 2 more days without treatment, after which they were harvested and stained.

C2C12 myoblasts (Purchased from the American Type Culture Collection-ATCC) were grown and maintained in Dulbecco's Modified Eagle Medium (D-MEM) (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (HyClone/Thermo Scientific) and 1% Penicillin/Streptomycin. C2C12 cells were differentiated in D-MEM supplemented with 2% horse serum (Gibco/Invitrogen) and 1% Penicillin/Streptomycin. The cells were maintained in incubators at 37ºC with 5% CO_2. For differentiation, C2C12 cells were grown in 100 mm tissue culture dishes for 1, 2 and 3 days treated with and without curcumin. At each time point, cells were harvested and stained.

Immunofluorescence microscopy

Cells were differentiated and grown on 0.1% gelatin coated coverslips in 6-well tissue culture vessels (VWR) as described above. Following aggregation and treatments, the cells were fixed with cold methanol for 20 minutes at -20°C, air dried, rehydrated with PBS for 15 minutes at room temperature, and incubated with the appropriate *primary antibodies overnight at 4°C. On the following day, cells were washed three times with PBS and incubated with the corresponding fluorescent *secondary antibodies in PBS for two hours at room temperature with protection from light. After washing another three times with PBS, the cells were incubated for 5 minutes in PBS with 25ng/ml of Hoechst stain (Molecular Probes). Finally, after another three washes, the coverslips were mounted on slides (VWR) with 50% glycerol for microscopy. Microscopic analysis was performed with the Zeiss Axiovert 200 M microscope. Cells were observed through a Zeiss 20X objective and image acquisition was taken with the AxioCam HRM monochrome camera (Zeiss). Differentiation was determined as the

percentage of cells stained positive for skeletal markers out of a total percentage of cells. Images captured through different fluorescence filters (488 and 594) were processed and merged by the Zeiss AxioVision Rel 4.8 software.

*The primary antibodies used were:

1- anti-Myosin Heavy Chain MF20 (Developmental Studies Hybridoma Bank)

2- anti-MyoD (M-318, Santa Cruz)

3- anti-Myf5 (C-20, Santa Cruz)

4- anti-p300 (N-15, Santa Cruz)

*The secondary antibodies used for staining were Alexa Flor®488 goat anti-mouse (Invitrogen) and/or Alexa Flor®594 donkey anti-mouse (Invitrogen).

Western Blotting

At the specific time points, cells were washed with PBS and harvested. Cell pellets were lysed by incubating with the whole cell extract buffer (10% glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% NP-40) for 30 minutes shaking on ice at 4ºC. Whole lysates were centrifuged at 14,000 rpm for 20 minutes and the supernatant was collected. Protein concentrations were quantified by Bradford method (Biorad by manufacture's recommendation) using Bio-Rad Protein Assay Dye Reagent (Bio-Rad), Multiscan Spectrum Photospectrometer (Thermo) and the Multiscan (Thermo Scientific) software at reading absorbance 595 nm. Equal amounts of protein were diluted in 2x Laemmli buffer (25% glycerol, 125 mM Tris-HCl pH 6.8, 4% SDS, 10% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 5 minutes at 95˚C, resolved on (6%, 8%, 10% or 12%) SDS-polyacrylamide gel and transferred overnight onto an Immun-Blot PVDF membrane (Bio-Rad). Membranes were then blocked for 30

minutes to 1 hour with 5% non-fat milk in PBST (PBS with 1% Tween). Membranes were incubated overnight, rotating in the appropriate primary antibody* diluted in 1% non-fat milk in PBST at 4˚C. After that, they were washed three times with PBST, incubated with the corresponding secondary antibody against mouse or rabbit IgG (GE Healthcare UK Limited) for 30-60 minutes, and then washed again three times with PBST. Protein expression was then visualized using Western Lightning Chemiluminescence (Perkin Elmer) reagents. To reprobe the membranes, they were stripped using stripping buffer (2% SDS, 62.5 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol) at 50ºC for 30 minutes and then blocked with 5% non-fat milk in PBST for 30 minutes again, after which they were reprobed (as described above).

*Primary antibodies used were:

1- anti-p300 (N-15, Santa Cruz)

2- anti-MyoD (C-20, Santa Cruze)

3- anti-Myf5 (C-20, Santa Cruze)

4- anti-myogenin (From F5D hybridoma)

5- anti-β-tubulin (From E7 hybridoma)

Quantitative reverse-transcriptase PCR (RT-PCR)

At the indicated time points, cells were washed with PBS and harvested. Total RNA was isolated using Total RNA Kit I (Omega) according to the manufacturer's protocol, and eluted with 50 µl of DEPC-treated water. RNA was quantified at reading absorbance 260 nm using the NanoDrop Spectrophotometer (ND-1000). Reverse transcription to cDNA was performed with 1 µg of RNA using a High Capacity cDNA Reverse Transcription kit (ABI). Quantitative Real Time Polymerase Chain Reaction (RT-PCR) was performed with the MX300P platform (Strategene) qPCR Instrumentation using the SYBR green quantification method and ROX normalization. Quantification was analyzed as fold changes relative to the day 1 differentiated condition after being normalized to GAPDH. Primers used are listed in Table 3.

Chromatin Immunoprecipitation (ChIP)

Cells were differentiated as described above. At the indicated time points, cells were fixed using 1% formaldehyde (VWR) shaking at room temperature for 30 minutes. Then, the crosslinking was quenched with glycine for a final concentration of 125 M. Cells were washed twice with ice-cold PBS and resuspended in ChIP lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) supplemented with protease inhibitor Cocktail Set lll, EDTA-Free (Calbiochem), and kept on ice for 10 minutes. Cell lysates were sonicated using the Bioruptor system (Diagenode) for a total of 45 minutes, 30-second pulses (40 second rest between pulses) at high setting, and then lysates were cleared by centrifugation at 14,000 rpm for 15 minutes at 4^{o}C. To quantify DNA, they were incubated with elution buffer (50 mM Tris, 10mM EDTA and 1% SDS) overnight

at 65ºC for reverse crosslinking. Then, SDS was diluted with 50 mM Tris, 10 mM EDTA and supplemented with RNase A (Sigma) and incubated for 1 hour at 37ºC. After that, proteinase K (Roche) and $CaCl_2$ were added to the lysates and incubated for 30 minutes at 65ºC. The samples were then purified using Omega Bio-tek Cycle Pure Kit (Omega) according to the manufacturer's protocol, and diluted in 50 µl of kit's elution buffer. DNA was then quantified using the nanodrop system at absorbance 260 nm and the amount of DNA to be used was calculated accordingly. The DNA was pre-cleared by incubation with dynabeads protein-A (Invitrogen) for 2 hours at 4ºC. Input samples were set aside at -80^{o}C. For immunoprecipitation, the appropriate *antibody was added to samples in dilution buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA and 10% Triton (Promega)) supplemented with protease inhibitor Cocktail Set lll, EDTA-Free, rotating overnight at 4ºC. Rabbit IgG antiserum or mouse IgG antiserum was used as a negative control antibody.

*The antibodies used were:

1- anti-p300 (N-15, sc-584, Santa Cruz Biotechnology)

2- anti-PCAF (H-369, sc-8999, Santa Cruz Biotechnology)

3- anti-H3K9ac (ab4441 – Abcam)

4- anti-H3K27ac (ab7429 – Abcam)

5- anti-H3K4me (ab8895 - Abcam)

Following an overnight incubation with the appropriate antibody, the immunoprecipitates were captured by addition of dynabeads protein-A for 2 hours at 4ºC. The beads were then washed for 10-minute washes in each of the following buffers: low-salt (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 150 mM NaCl), high-salt (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0, 150 mM NaCl) and finally LiCl (1% NP-40, 1% sodium dioxycholate, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.25 M LiCl) at 4ºC. The immune complexes were then washed twice for 5 minutes in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Protein/DNA complexes were eluted from the beads using elution buffer (50 mM Tris, 10 mM EDTA and 1% SDS) rotating for 30 minutes at room temperature, and crosslinking were reversed for both samples and inputs in elution buffer overnight at 65^{o}C. The following day, SDS in all samples and inputs was diluted with (50 mM Tris and 10 mM EDTA) and supplemented with RNase A, followed by 1 hour incubation at 37ºC. Then, proteinase K and $CaCl_2$ were added and samples were incubated for 30 minutes at 65ºC. DNA was purified using Omega Bio-tek Cycle Pure Kit. PCR was performed using HotStarTaq® DNA polymerase PCR kit (Qiagen), dNTP Mix (Promega), and specific primers*. The immunoprecipitated DNA was quantitated using qPCR employing the SYBR green and ROX chemistry on MX300p instrumentation (Strategene). Each sample was used in triplicate PCR reactions. For each time point and each biological replicate, an aliquot of the “input” starting chromatin material was used to create a dilution curve in order to express target DNA abundance in the immunoprecipitated samples as percentage of input in the starting material (percent of input values).

*Primer pairs used for amplification of genes can be found in Table 4.

III. Results

Curcumin inhibits lineage specification at early stage of myogenesis

In this study, P19 cells were used to investigate the role of p300 HAT activity on *MyoD* expression during myogenesis. To examine the effect of curcumin on the determination and differentiation of skeletal muscles, cells were aggregated for 4 days and treated with 1% DMSO + 10 nM RA with or without 10 µM C646 or 10 µM curcumin (early stage treatments). Then, they were allowed to attach to tissue culture dishes for five more days without any treatment. On day 9 of differentiation, cells were co-stained for myosin heavy chain (MHC) and Myf5, and for MHC and MyoD. Microscopic analysis shows that cells were able to differentiate into bipolar and elongated skeletal muscles when treated with 1% DMSO + 10 nM RA (Figure 4A). However, cells treated with 10 µM curcumin showed more than 82% decline in the formation of skeletal muscles and 80% inhibition in MyoD and Myf5 expression (Figure 4B). In order to assess the expression of the muscle marker, myogenin, cells were harvested on day 9 of differentiation, and protein was extracted to be assessed by western blot technique. Cell extracts were probed for myogenin and p300. Figure 4C shows that curcumin and C646 were able to inhibit myogenin expression compared to cells co-treated with 1% DMSO + 10 nM RA. This observation suggests that curcumin treatment at early stage of differentiation has the ability to inhibit the specification of stem cells into skeletal muscles. This might be through the ability of curcumin to inhibit the HAT activity of p300, which is required for Myf5 and MyoD expression. It also supports the notion that Myf5 and MyoD regulate myogenin expression (Kablar et al., 1997).

However, p300 expression was maintained during the early stage of myogenesis (Figure 4D), which suggests that curcumin does not inhibit p300 protein expression.

Since MyoD is a late transcription factor and not expressed during early stage of myogenesis, we sought to examine its expression profile when cells are treated at the late stage of differentiation, in order to examine the involvement of p300 HAT activity in *MyoD* expression at late stages of myogenesis.

Figure 4

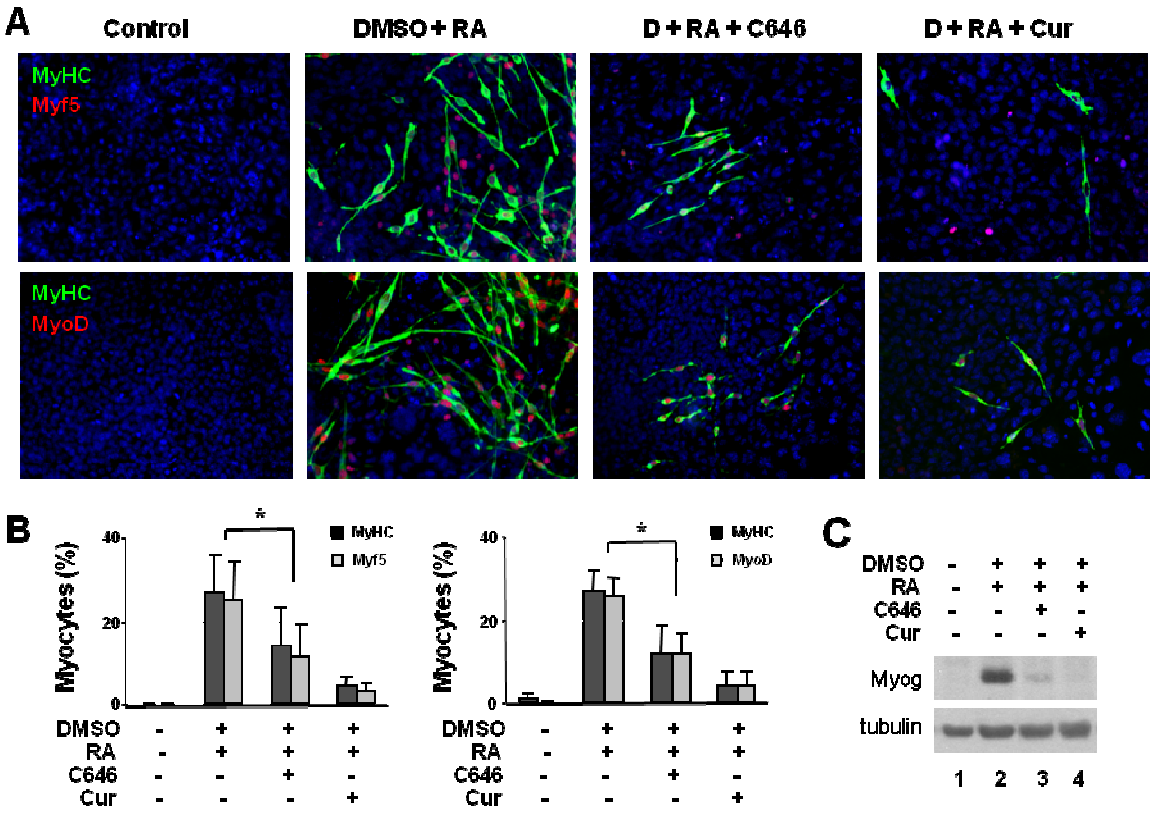


Figure 4: Curcumin inhibits commitment of skeletal myogenesis. P19 cells were treated early with curcumin and C646 for 4 days. **(A)** Cells were probed for MHC (green), Hoechst (blue) and Myf5 (red) or MyoD (red). **(B)** Quantification (%) of cells expressing MHC, Myf5 and MyoD (mean ± SD, n = 3). *P<0.05. **(C)** Myogenin protein expression on day9 of differentiation was assessed by western blot. **(D)**. p300 protein expression was assessed on day4 of differentiation with β-tubulin as a loading control.

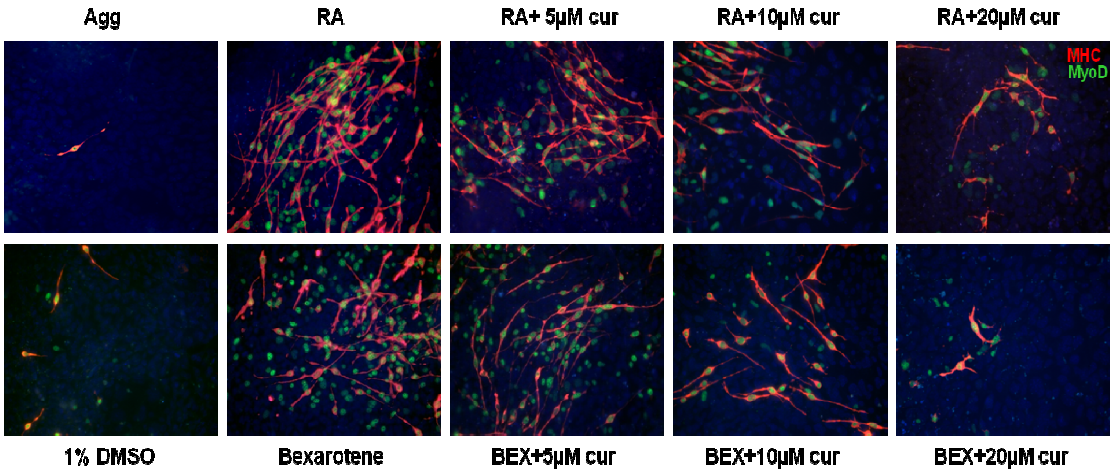
Curcumin late treatment inhibits myogenic differentiation

To assess the expression profile of MyoD at P19 late stage of differentiation, cells were allowed to aggregate for 4 days with 1% DMSO + 10 nM RA or 1% DMSO + 100 nM bexarotene. Thereafter, cells were allowed to attach to tissue culture dishes and were treated for 4 days with 5 µM, 10 µM and 20 µM curcumin (late stage treatment). On day 9 of differentiation, cells were co-stained for MHC and MyoD (Figure 5A). As seen in Figure 5A, cells treated with curcumin tend to have less skeletal muscle than cells treated with 1% DMSO + RA or 1% DMSO + bexarotene. Furthermore, 20 µM curcumin treatments inhibited skeletal muscle formation by about (83%), 5 µM curcumin by (27%) and 10 µM curcumin by (43%) (Figure 5B). Moreover, curcumin inhibited the MyoD expression in P19 cells with 5 µM, 10 µM and 20 µMtreatment by about 27%, 37% and 74% respectively (Figure 5B), suggesting that MyoD is still expressed at late stages of differentiation and treatment with curcumin was sufficient to inhibit skeletal muscle formation and MyoD expression that might be again through inhibiting the p300 HAT activity. This will allow us to study the role of p300 on MyoD regulation during myogenesis

However, on day 9 of differentiation, cells were co-stained for MHC and Myf5 and immunofluorescence showed that Myf5 is also expressed in P19 cells at this stage (data not shown). Therefore, we decided to move on to use myoblast C2C12 cells to better define the mechanism of p300 HAT activity on *MyoD* regulation in the absence of Myf5. In addition, the C2C12 cell line gives a higher percentage of skeletal muscle differentiation compared to P19 cells, since C2C12 cells are already committed to becoming skeletal muscle cells.

Figure 5

A



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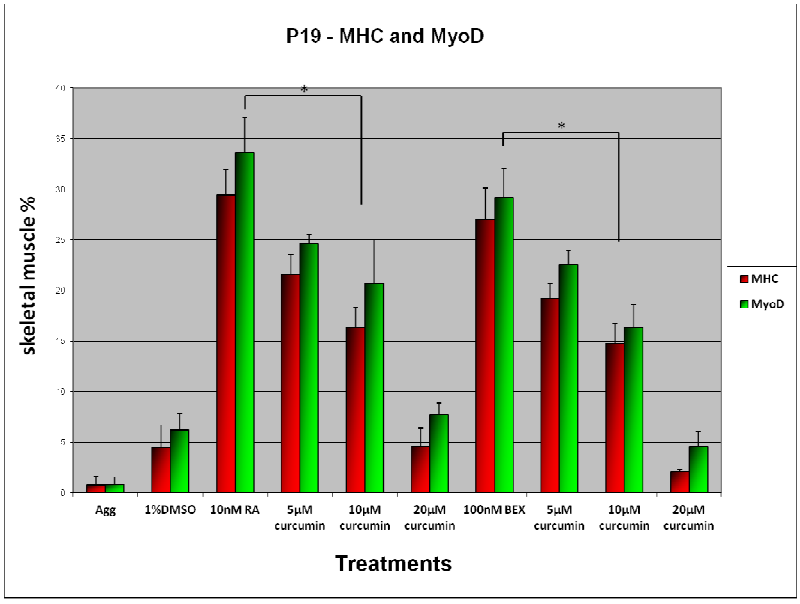

Figure 5: Curcumin inhibits myogenic differentiation and MyoD expression. P19 cells were treated with curcumin on the last 4 days of differentiation (day 5-9). **(A)** On day 9, cells were stained for MHC (red), Hoechst (blue) and MyoD (green). **(B)** Quantification (%) of cells expressing MHC and MyoD (mean ± SD, n = 3). *p < 0.05

Curcumin treatment inhibits the development of skeletal myocytes

To further investigate the expression profiles of Myf5 and MyoD during skeletal myogenesis, C2C12 cells were maintained in growth medium with 10% heat inactivated fetal bovine serum (10% HI-FBS) in DMEM. Cells were allowed to differentiate for 1, 2 and 3 days in DMEM supplemented with 2% horse serum (HS). C2C12 cell were then treated with curcumin for 1, 2 and 3 days, and then collected at each time point. After that, cells were co-stained for MHC and MyoD (Figure 6A), and co-stained for MHC and p300 (Figure 6B). As seen in (Figure 6), curcumin inhibited C2C12 cells differentiation at the three different time points. Apparently, there were more myocytes after 3 days than after 1 and 2 days of differentiation (Figure 6C). After 2 days of differentiation, 5 µM, 10 µM and 20 µM curcumin inhibited the differentiation by 17%, 38% and 97% respectively.

C2C12 cells were collected at each time point and protein was extracted for western blot to examine Myf5 and myogenin expression. Cell extract was then probed for Myf5 and myogenin. As seen in figure 6D, Myf5 has disappeared during differentiation while still being expressed in undifferentiated cells, which supports the finding that Myf5 is an early marker during muscle specification (Kablar et al., 1998; Tapscott, 2005). Moreover, myogenin protein expression increased from day 1 to day 2 of differentiation. This observation is in agreement with the finding of Dedieu and colleagues (Dedieu, Mazeres, Cottin, & Brustis, 2002). Furthermore, curcumin inhibited myogenin expression with all three concentrations of 5 µM, 10 µM and 20µM (Figure 6D).

The ability of C2C12 to produce up to 60% of differentiated cells along with the presence of MyoD and disappearance of Myf5 expression makes C2C12 a good cell line to investigate the mechanism of p300 HAT activity on MyoD regulation.

Figure 6

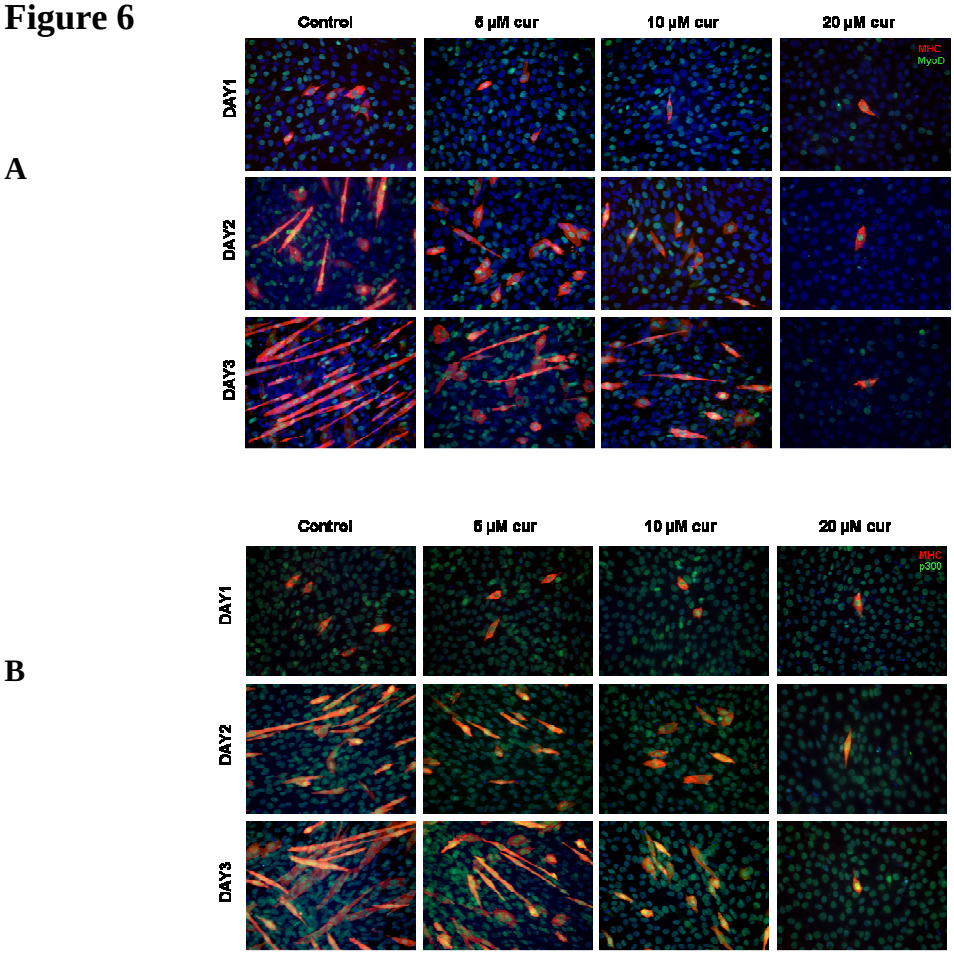

Figure 6: Curcumin inhibits C2C12 cell differentiation. C2C12 cells were treated with curcumin for 1, 2, and 3 days. At each time point, cells were co-stained for MHC (red), Hoechst (blue), **(A)** MyoD (green) and **(B)** p300 (green). **(C)** Quantification (%) of cells expressing MHC (mean ± SD, n = 3). *$p < 0.05$. **(D)** Myf5 and myogenin proteins expression was assessed by western blot after 1, 2 and 3 days of differentiation with and without curcumin, and β-tubulin used as a loading control.

Curcumin treatment does not affect p300 occupancy at *MyoD* core enhancer region

To examine the MyoD expression profile during skeletal myogenesis and to check if curcumin was able to affect p300 expression, C2C12 cells were collected at each time point and protein was extracted for western blot. Cell extract was then probed for p300 and MyoD. We found that p300 protein expression was not affected during differentiation. However, MyoD protein expression decreased gradually from day 1 to 2 of differentiation (Figure 7A). This result is in agreement with the *in vivo* findings that showed that MyoD expression decreased in p300 acetyltransferase mutant mice, (J. F. Roth et al., 2003). These results indicate that curcumin was sufficient to inhibit the expression of MyoD which led to the inhibition of cell differentiation probably by inhibiting the HAT activity of p300.

To determine at which transcription level curcumin affects MyoD expression, we analyzed MyoD mRNA levels during C2C12 differentiation. Due to the fact that MyoD declined from day 1 to 2 of differentiation and that 20 µM curcumin might kill some of the cells, we decided to perform RT-PCR after 1 day of differentiation with and without 5 µM and 10 µM curcumin to assess MyoD transcript levels. Cells were differentiated and mRNA was isolated after 1 day of differentiation with and without curcumin. Following that, total RNA was reverse transcribed and amplified by Real Time PCR. Figure 7D shows a significant reduction, more than 45%, in MyoD mRNA levels in cells treated with 10 µM curcumin as compared to day 1 differentiated cells. This observation is in accordance with the decrease of MyoD protein expression in curcumin treated cells (Figure 7A).

Next, we performed Chromatin Immunoprecipitation (ChIP) to assess the p300 occupancy. Since core enhancer region (CER) is essential for *MyoD* expression, we sought to examine the p300 occupancy at this genomic location. C2C12 cells were treated with 5 µM and 10 µM curcumin for 1 day. Then cells were cross-linked with 1% formaldehyde and immunoprecipitated (IP) with p300 antibody. IgG was used as a negative control. We found that p300 occupancy was about more than 3 folds higher in day 1 differentiated cells compared to undifferentiated cells (Figure 7E). However, both curcumin concentrations (5 µM and 10 µM) did not affect p300 occupancy at the *MyoD* CER (Figure 7E). To address our hypothesis that p300 directly regulates *MyoD* expression through histone acetylation at the CER, we next sought to examine the histone acetylation profiles at different lysines during myogenesis.

Figure 7

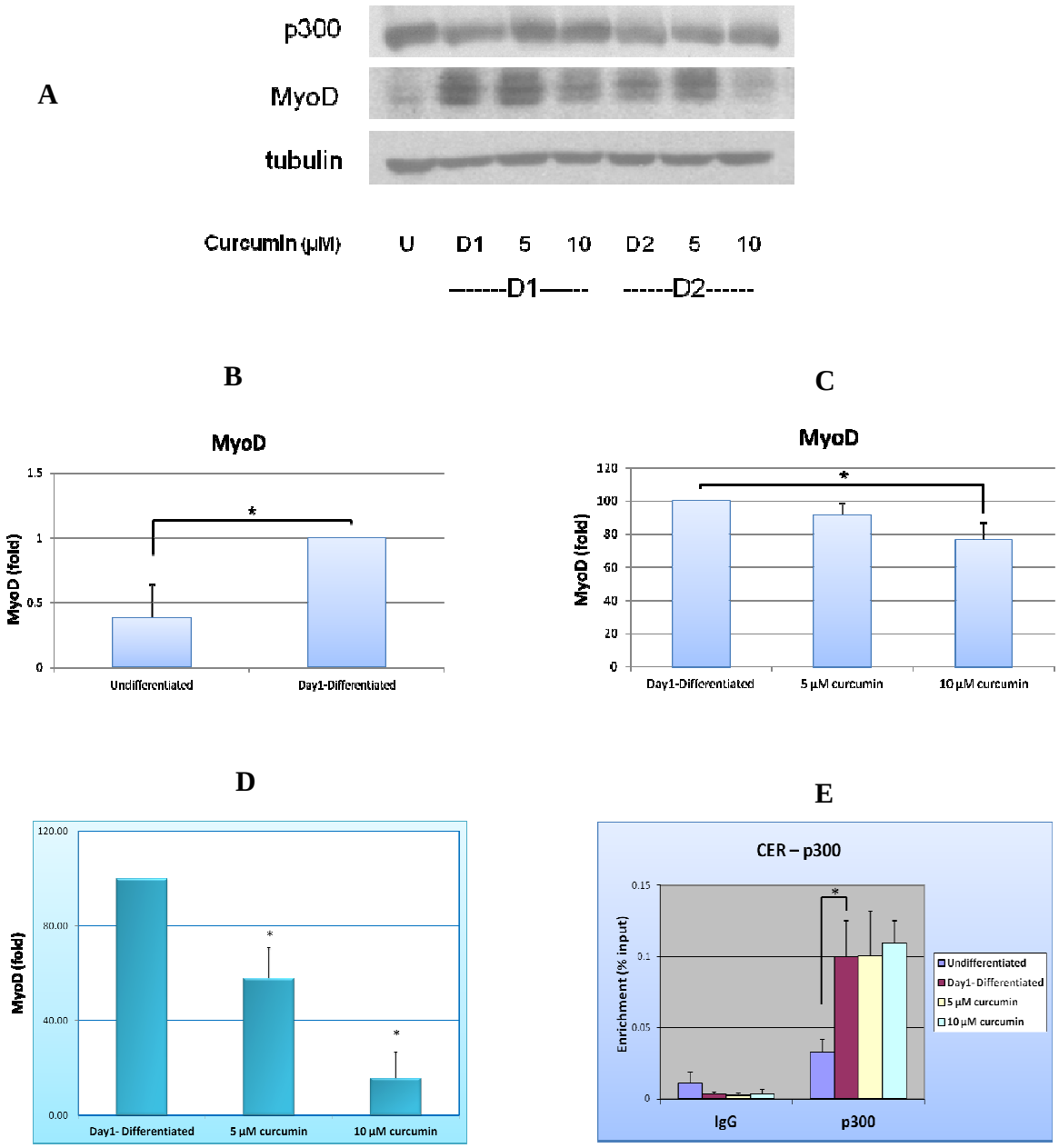

Figure 7: Curcumin has no effect on p300 occupancy at *MyoD* CER. C2C12 cells were treated with 5 μM and 10 μM curcumin for 1 day. **(A)** p300 and MyoD proteins expression was assessed by western blot after 1 and 2 days of differentiation, with β-tubulin as a loading control. (**B**) Quantification of MyoD undifferentiated and untreated cells. *P<0.05. (**C**) Quantification of MyoD curcumin treated and untreated cells. **(D)** The level of MyoD mRNA after 1 day of differentiation was determined by quantitative RT-PCR and plotted as the fold change relative to the untreated control after being normalized to GAPDH. Cells were cross linked with 1% formaldehyde and subjected to ChIP analysis using antibody against p300. **(E)** Immunopurified DNA was quantified by qPCR at *MyoD* CER for undifferentiated cells and day 1 differentiated cells treated with and without 5 μM and 10 μM curcumin (mean ± SD, n = 3).

Acetylation of H3K27 and H3K9 at CER

To examine the histone acetylation during myogenesis, C2C12 cells were harvested and processed for ChIP as mentioned above. H3K27ac and H3K9ac were immunoprecipitated. IgG was used as a negative control. Figure 8A shows that H3K27 acetylation is significantly higher than H3K9 acetylation at the CER region after 1 day of differentiation. In addition, after 1 day of differentiation, H3K27 acetylation was about 20% higher than in undifferentiated cells. This result is in accordance with Jin Qihuang who demonstrated that p300 is specifically required for H3K27 acetylation in cells (Jin et al., 2011). Moreover, figure 8A shows that H3K27 acetylation level was reduced by 30% in both curcumin concentration (5 μm and 10 μm) treated cells compared to untreated cells. This result suggests that p300 HAT activity is important for H3K27 acetylation and curcumin might have the ability to inhibit p300 HAT activity which led to inhibition of H3K27 acetylation.

Since other histone acetyltransferases can play a role in skeletal muscle formation, we sought to examine the level of PCAF histone acetyltransferase. Figure 8B shows that PCAF occupancy is significantly low as compared to p300. Furthermore, the level of PCAF is maintained in both undifferentiated and differentiated cells. Also, PCAF occupancy is unchanged in curcumin treated and untreated cells. These observations suggest that PCAF was not affected by curcumin and hence we can exclude the involvement of PCAF in *MyoD* regulation during myogenesis. IgG was used as a negative control.

Furthermore, we examined the level of H3K4 methylation (an active enhancer marker) as a control for our curcumin treatment, as curcumin only inhibits histone acetylation not histone methylation. Figure 8C shows that there is no difference in the level of H3K4 methylation in undifferentiated and day 1 differentiated cells treated and untreated with curcumin, validating the effect of curcumin on histone acetylation. IgG was used as a negative antibody.

Figure 8

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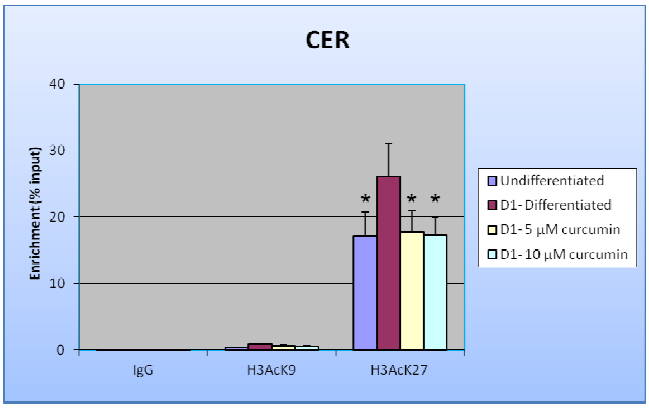

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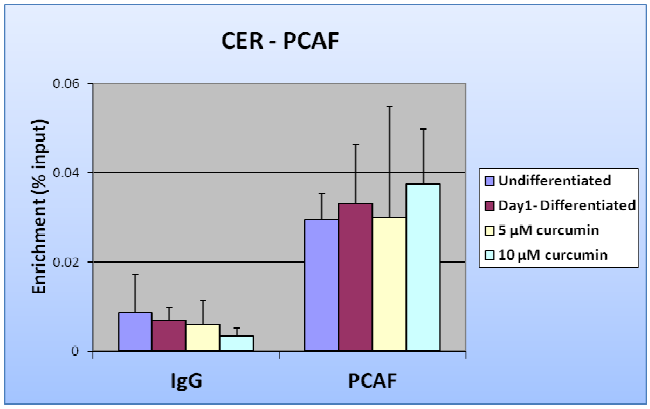

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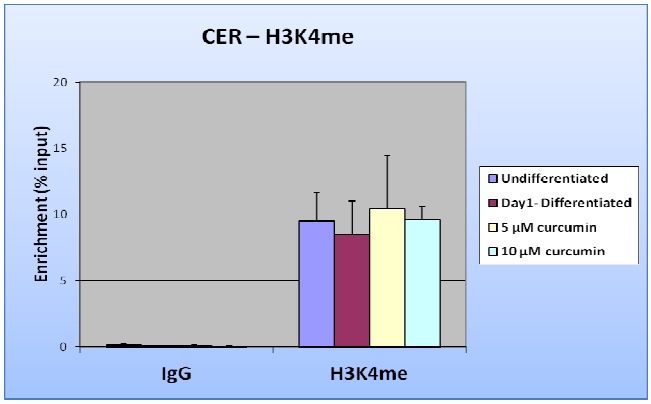

Figure 8: H3K27 is acetylated at the *MyoD* CER. C2C12 cells were differentiated and treated with 5 µM and 10 µM curcumin for 1 day. Cells were then cross linked with 1% formaldehyde and subjected to ChIP analysis using antibody against H3K27ac, H3K9ac, PCAF and H3K4me. Immunopurified DNA from undifferentiated cells and day 1 differentiated cells treated with and without 5 µM and 10 µM curcumin was quantified by qPCR at *MyoD* CER. **(A)** H3K27 and H3K9 acetylation. *P<0.05. **(B)** PCAF occupancy and **(C)** H3K4 methylation (mean ± SD, n = 3).

IV. Discussion

p300 is required for *Myf5* regulation at the early epaxial enhancer

During myogenesis, MRFs (Myf5, MyoD, myogenin and Mrf4) play crucial roles in skeletal myogenic specification and differentiation, in which Myf5 is the first MRF to be expressed. Myf5 marks the commitment of the skeletal muscle lineage (Francetic & Li, 2011). We have shown previously that p300, a transcriptional co-activator, is directly involved in *Myf5* regulation at the early epaxial enhancer. Furthermore, we have shown that the acetylation of H3K27 is highly associated with p300 HAT activity during the regulation of *Myf5* (Francetic T. et al., 2012). Therefore, we were interested to study the role of p300 HAT activity in *MyoD* regulation, a later expressed gene, during skeletal myogenesis.

p300 is essential for stem cell specification and differentiation

We have shown the presence of p300 at the *MyoD* core enhancer region. P19 and C2C12 cell lines have been used as models for our study of the role of p300 in *MyoD* regulation. Previous studies proved the validity of using P19 and C2C12 cell systems for the purpose of studying the regulation of MRFs expression (Armour, Garson, & McBurney, 1999; Dedieu et al., 2002; Furutani, Umemoto, Murakami, Matsui, & Funaba, 2011; Skerjanc, Slack, & McBurney, 1994). Furthermore, inhibition of the HAT activity of p300 was a useful system to study skeletal myogenesis which resembles the inhibition of p300 acetyltransferase activity by a mutation in mouse embryonic stem cells (J. F. Roth et al., 2003). In our study, we chemically inhibited p300 HAT activity using

curcumin, which has been known for its effective inhibition of p300 HAT activity during myogenesis. Curcumin inhibited P19 cell specification as well as cell differentiation when added at early and late stages of myogenesis, which have been shown by the reduction in skeletal muscle formation and Myf5 and MyoD expression. Since p300 is known to regulate Myf5 and MyoD, we speculate that curcumin was able to inhibit the HAT activity of p300 when added at early and late stages of differentiation, which led to the suppression of Myf5 and MyoD expression, and eventually inhibition of skeletal muscle formation. However, previous studies demonstrated that curcumin is not a p300 specific inhibitor. Curcumin is also able to inhibit the HAT activity of CBP (Balasubramanyam et al., 2004). Nevertheless, it has been shown that CBP is not essential for Myf5 or MyoD expression (J. F. Roth et al., 2003). Hence, the suppression of Myf5 and MyoD expression is expected to be due to the inhibition of p300 HAT activity. However, it would be interesting to assess the CBP occupancy at *MyoD* CER. In our study, we also used C2C12 cell system to study skeletal myogenesis. Since Myf5 is still expressed in P19 cells during skeletal myogenesis, we used C2C12 cell line to exclude the involvement of Myf5 role in *MyoD* regulation through p300 HAT activity, as Myf5 disappeared during C2C12 differentiation. C2C12 cells were also used for their extensive and rapid differentiation within a few days of serum withdrawal (Blau et al., 1983). C2C12 results were similar to P19 cells, in which they both showed a decline in skeletal muscle formation when cells were treated with curcumin. Moreover, MyoD mRNA level was declined in curcumin treated cells after 1 day of differentiation. This observation is in accord with the Roth study where there was a reduction in MyoD transcript in p300 acetyltransferase mutant cells (J. F. Roth et al., 2003). Furthermore,

presence of Myf5 in C2C12 undifferentiated cells is in agreement with the finding of Braun and colleagues that Myf5 is expressed in myoblasts (Braun et al., 1989).

Role of p300 at the core enhancer region of *MyoD*

As mentioned above, MyoD is activated around 2.5 days after Myf5 expression during myogenesis (Tajbakhsh & Buckingham, 2000). Along with myogenin, MyoD marks the development of skeletal myocytes (Zhang et al., 1999; Kennedy et al., 2009; Le May et al., 2011). We have shown that p300 is present at the *MyoD* core enhancer region, and occupies the CER in both undifferentiated and day 1 differentiated cells (Figure 7E). However, the occupancy of p300 at the *MyoD* CER significantly increases in day 1 differentiated cells as compared to undifferentiated cells (Figure 7E). Because MyoD has been found to be acetylated in proliferating myoblasts, further mechanisms of MyoD activation are to be elucidated (Polesskaya et al., 2000).

Histone acetylation is an important function of p300. We here show that H3K27 acetylation at the *MyoD* core enhancer region is higher in day 1 differentiated cells than in undifferentiated C2C12 cells (Figure 8A). This result is in agreement with the p300 occupancy at the CER, implying the requirement of p300 during differentiation. However, H3K27 acetylation level in curcumin treated cells returns back to the same acetylation basal level as in undifferentiated cells. Since p300 is required for H3K27 acetylation in cells, we suggest that the increase in H3K27 acetylation in day 1 differentiated cells is mediated by p300 (Figure 8A). Moreover, we speculate that the decrease in H3K27ac level in cells treated with curcumin is due to the ability of curcumin to inhibit the HAT activity of p300. We were also able to show that the H3K27

acetylation level is high as compared to H3K9 acetylation at the *MyoD* core enhancer region, and hyper-acetylation usually is a common landmark of transcriptionally active chromatin (Eberharter & Becker, 2002). On the other hand, we suggest that the presence of PCAF at the core enhancer region is for maintaining the basal level of histone acetylation. Since previous studies illustrated the requirement of PCAF for H3K9 acetylation (Jin et al., 2011), we suggest that the presence of PCAF (Figure 8B) is to acetylate H3K9 (Figure 8A) which might also be important for myogenesis. Additionally, we were able to validate the curcumin inhibitory effect on histone acetylation by assessing the H3K4 methylation level, which was found to be unchanged in curcumin treated and untreated cells (Figure 8C).

In summary, our results show that p300 is present at the *MyoD* core enhancer region in day 1 differentiated cells more than in undifferentiated cells. Moreover, the presence of p300 in undifferentiated cells, suggests that the basal level of activation is maintained in the *MyoD* core enhancer region. We also suggest that the presence of p300 at day 1 differentiated cells is to acetylate histones. In addition, curcumin decreases H3K27 acetylation level, which also indicates the ability of p300 to acetylate histones at specific lysines. These results support our hypothesis that p300 is directly involved in *MyoD* regulation through histone acetylation.

The fact that p300 is present at the *MyoD* core enhancer region in C2C12 cells indicates that p300 may also be recruited to this enhancer to have a role in the transcriptional regulation of *MyoD*.

Role of p300 in myogenesis

We have discussed the role that p300 may have in *MyoD* transcription. However, the role of p300 in skeletal myogenesis extends beyond just *MyoD* transcription. p300 is also known as a co-activator of both *Myf5* and *MEF2C* (Francetic T. et al., 2012; Sartorelli et al., 1997). We have shown here that p300 is involved in histone acetylation at the core enhancer region of *MyoD*. In addition, we have previously shown that p300 is also involved in *Myf5* transcription (Francetic T. et al., 2012). Hence, it is expected that p300 is also involved in regulation of other genes during myogenesis. However, it is noteworthy that p300 is not required for every gene involved in myogenesis. For instance, Unlike Myf5 and MyoD, Pax3 expression is not dependent on p300 HAT activity during myogenesis (Francetic T. et al., 2012; J. F. Roth et al., 2003). Taking into consideration that the role of a co-activator is not specific to only a set of genes or a particular DNA motif (Vo & Goodman, 2001), one can speculate the involvement of p300 in several regulatory events during myogenesis.

Functional redundancy of histone acetyltransferases during skeletal myogenesis

Previous studies have shown that p300 and CBP share some functional homology and sequence similarity (Vo & Goodman, 2001) (Figure 3). However, CBP seems to not have the same function as p300 in skeletal muscle differentiation program. Roth and colleagues have demonstrated that the HAT activity of p300, not of CBP, is essential for Myf5 and MyoD expression (J. F. Roth et al., 2003). In development, however, CBP HAT activity appears to only partially substitute for p300 HAT activity. For instance, a mutation in the p300 HAT domain causes a more severe defect in the lung, small

intestine, and cardiovascular system than the defect caused by an equivalent mutation in CBP HAT domain (Shikama et al., 2003). Similarly, loss of CBP HAT activity causes less severe deficiency of skeletal muscle formation than the one caused by p300 HAT mutation (J. F. Roth et al., 2003). Furthermore, previous studies suggested that CBP, unlike p300, is more associated with transcriptional repression rather than transcriptional activation (Ramos et al., 2010). A double mutation of CBP and p300 HAT activity is required to fully characterize if CBP and p300 HATs share any functional redundancy during skeletal myogenesis. Beside p300 and CBP, PCAF also participates in skeletal muscle differentiation program (Kuninger et al., 2006; Dyda et al., 2000; X. J. Yang et al., 1996). PCAF is a histone acetyltransferase that is also known to acetylate MyoD and histones, and function in the same pathway as p300 (Sartorelli et al., 1999; Polesskaya et al., 2000; Puri, Sartorelli et al., 1997; Sartorelli et al., 1997; Yuan et al., 1996), suggesting that PCAF also shares some functional redundancy with p300 during skeletal myogenesis. Nevertheless, PCAF on its own can only stimulate muscle differentiation by potentiating *MyoD* function through p300 association (Puri, Sartorelli et al., 1997). Furthermore, PCAF on its own may not be essential for skeletal muscle differentiation, whereas it may be fully compensated by p300 or other acetyltransferase since PCAF null mice show a normal phenotype (Xu et al., 2000; Yamauchi et al., 2000). Hence, double mutation of PCAF and p300 HAT domains would show if PCAF could function normally in the absence of p300.

Curcumin is a p300 HAT inhibitor

Curcumin was used in our study to inhibit the HAT activity of p300. It resulted in inhibition of MyoD protein and mRNA expression, and hence, skeletal muscle formation. However, curcumin, as expected, did not decrease the p300 protein expression and occupancy at the core enhancer region. Inhibition of p300 HAT activity with curcumin caused a decline in the H3K27 acetylation level, suggesting that curcumin might be able to inhibit the HAT activity of p300, which led to a decrease of histone acetylation at the core enhancer region. Nevertheless, the decrease in histone acetylation in curcumin treated cells was not mediated by PCAF, as curcumin does not inhibit the HAT activity of PCAF (Balasubramanyam et al., 2004). It would also be interesting to examine the CBP occupancy at the CER to exclude its involvement in *MyoD* regulation.

Recruitment of p300 to the *MyoD* locus during myogenesis

As mentioned before, p300 is crucial for myogenesis as it plays a critical role in Myf5 and MyoD activation at early and late stages of myogenesis (J. F. Roth et al., 2003; Shiama, 1997; Yao et al., 1998). Given that p300 cannot directly bind to DNA, it needs to be recruited to the enhancer region by transcription factors, which bind the DNA to activate myogenic genes expression. It has been previously demonstrated that the transcription factor MyoD recruits p300 to the DNA in order to activate transcription of myogenic specific genes, since p300 directly interacts with MyoD at its binding site and plays a crucial role in MyoD-dependent transcription activation during myogenesis (Eckner et al., 1996; Puri et al., 1997; Sartorelli et al., 1997; X. J. Yang et al., 1996). Furthermore, the Six family of transcription factors act upstream of MRFs and is required

for myogenic differentiation. Since Six-1 protein cooperates and functions in parallel to MRFs during myoblast differentiation (Liu, Chu, Chakroun, Islam, & Blais, 2010); it would be of interest to study the role of Six-1 in p300 recruitment to *MyoD* gene. To date, little is known regarding the recruitment of p300 to the enhancer/promoter regions. Therefore, the transcription factors and mechanisms that are involved in p300 recruitment to the gene remain to be characterized.

Conclusion

We have shown that curcumin inhibits P19 cell specification and differentiation into skeletal muscles when the cells are treated at both early and late stages of differentiation. Moreover, our data shows that curcumin is able to inhibit MyoD expression and hence the differentiation of C2C12 cells into myocytes. These results suggest that the inhibition of MyoD expression might be due to the negative effect of curcumin on p300 HAT activity. Furthermore, curcumin did not decrease p300 occupancy at the CER, but it decreased histone acetylation at the locus, indicating the direct involvement of p300 HAT activity in the early activation of *MyoD* gene expression.

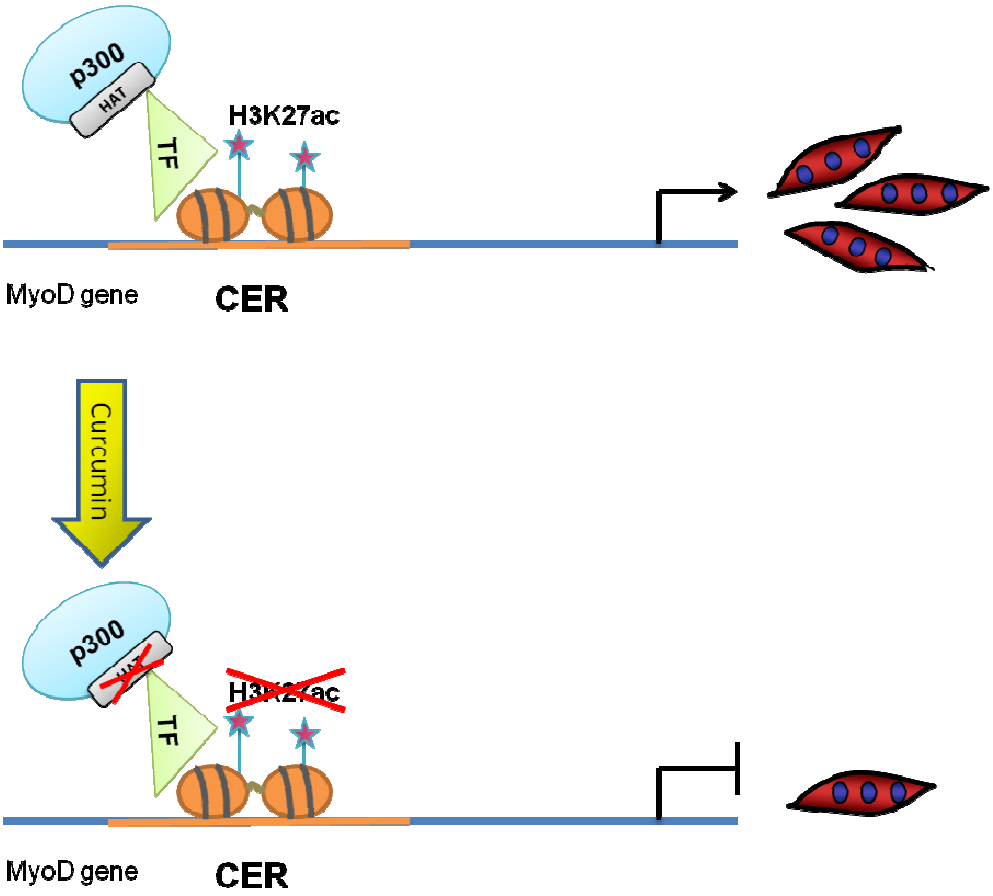

Figure 9: Role of p300 in *MyoD* regulation: p300 occupancy in undifferentiated cells is less than day1 differentiated cells at the CER. After 1 day of differentiation, p300 seems to be recruited to the CER by unknown transcription factors to acetylate histones. Treatment with curcumin does not affect p300 occupancy whereas it decreases H3K27 acetylation at this locus. Therefore, we hypothesize that p300 is directly involved in the regulation of *MyoD* expression through histone acetylation at the core enhancer region.

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Appendices

Supplementary Table 1. Reagents and suppliers

Reagent	Cat. No.	Supplier
P19 cells	CRL-1825	ATCC
Minimum Essential Medium α	12000-022	Gibco/Invitrogen
Dulbecco's Modified Eagle Medium	12800-017	Gibco/Invitrogen
Fetal Bovine Serum for P19 cells	095150	Multicell-Wisent
Fetal Bovine Serum for C2C12 cells	SH30396.03	Hyclone
Bovine Calf Serum	074150	Multicell-Wisent
Penicillin G	P-7794	Sigma
Streptomycin Sulfate	11860-038	Gibco/Invitrogen
DMSO	D2650	Sigma
All-trans Retinoic Acid	R2625	Sigma-Aldrich
Curcumin	C7727-500MG	Sigma-Aldrich
Glycerol	4750	OmniPur/EMD
Bexarotene	B-2422	LC Laboratories
horse serum	16050-130	Gibco/Invitrogen
Hoechst 33258 pentahydrate	H-21491	Molecular Probes
Bio-Rad Protein Assay	500-0006	Bio-Rad
β-mercaptoethanol	O3446I100	Fisher Scientific
Precision Plus Protein Dual Color Standards (MW ladder)	161-0374	Bio-Rad

Immun-Blot PVDF Membrane	162-0177	Bio-Rad
Western Lightning™ Chemiluminescence	NEL105	Perkin Elmer
NP-40	9036-19-5	Bio Basic Inc
Tween 20	BP337-500	Fisher Scientific
Total RNA Kit I	R 6834-01	Omega
dNTP Mix	PRU1515	Promega
High Capacity cDNA Reverse Transcription Kit	4368841	ABI
Cycle Pure Kit	D 6492-02	Omega
Formaldehyde	BDH 0500_1LP	BDH
RNase A	R-4875	Sigma-Aldrich
Protease Inhibitor Cocktail Set III, EDTA-Free	539134	Calbiochem
Proteinase K	03115879001	Roche
Triton X-100	H5142	Promega
dynabeads	201402	Invitrogen
ROX	04914139001	Roche
SYBR green	S7563	Invitrogen
HotStarTaq DNA Polymerase kit	203205	Qiagen
MF20 hybridoma cells	N/A	Developmental Studies Hybridoma Bank

Supplementary Table 2. Antibodies

Antibody	Catalogue No.	supplier
anti-Myosin Heavy Chain MF20	-	Lab grown
anti-MyoD (M-318)	sc-760	Santa Cruz
anti-Myf5 (C-20)	sc-302	Santa Cruz
anti-p300 (N-15)	sc-584	Santa Cruz
anti-MyoD (C-20)	sc-304	Santa Cruz
anti-myogenin (From F5D hybridoma)	-	Lab grown
anti-β-tubulin (From E7 hybridoma)	-	Lab grown
anti-PCAF (H-369)	sc-8999	Santa Cruz
anti-H3K9ac	ab4441	Abcam
anti-H3K27ac	ab7429	Abcam
anti-H3K4me	ab8895	Abcam
Alexa Flor®488 goat anti-mouse	A11001	Invitrogen
Alexa Flor®594 donkey anti-mouse	A21203	Invitrogen
Anti-rabbit IgG horseradish peroxidase conjugate 2º Ab	NA9340	GE Healthcare UK limited
Anti-mouse IgG horseradish peroxidase conjugate 2º Ab	NA931	GE Healthcare UK limited

Supplementary Table 3. Primers used for Real-Time RT-PCR

Gene	Forward Primer	Reverse Primer
MyoD	TGCCTTCTACGCACCTGGA	ATCATGCCATCAGAGCAGTTGG
GAPDH	TCGGTGTGAACGGATTTG	GGTCTCGCTCCTGGAAGA

Supplementary Table 4. Primers used for ChIP Assay

Gene	Forward Primer	Reverse Primer
MyoD - CER	TGCTTCTTTCGGCCAAGTAT	CCAACTGGCTGTGTTGTGAG