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# **The role of p300 in regulation of *Myf5* expression**

Tanja Francetić

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Cellular and Molecular Medicine

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## ABSTRACT

The commitment of cells to skeletal muscle differentiation is regulated by the myogenic regulatory factors Myf5, MyoD, myogenin and MRF4. Myf5 is the earliest of the MRFs expressed in an embryo. An array of transcriptional factors and signals present in dermomyotome and myotome such as Wnt, Shh, Six1/4, Eya1/2 and Pax3/7 regulate the expression of *Myf5*. Also, the HAT activity of coactivator p300 is also needed for *Myf5* expression. However, the exact function of the p300 HAT activity that is required for expression of *Myf5* has not been determined. The spatio-temporal expression of *Myf5* is also regulated by a large number of enhancers spread over 140 kb upstream of the transcription start site. The early epaxial enhancer regulates the expression at the earliest time point known. We hypothesized that HAT activity of p300 may be involved in direct regulation of *Myf5*. We used the embryonal carcinoma P19 cells to study skeletal myogenesis and the chemical inhibitor curcumin to study the role of p300 HAT activity. Curcumin was able to inhibit commitment into skeletal myogenesis by downregulating expression of *Myf5* and *MyoD*. Furthermore we show that p300 is present at the early epaxial enhancer and that the function of p300 there may be histone acetylation. Therefore we provide evidence that p300 may be directly involved in regulation of *Myf5* expression.

*Dedicated to everlasting search for the truth*

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## LIST OF ABBREVIATIONS

APC	Axin-adenomatous polyposis coli
AcH3	Acetylated histone H3
AR	Androgen receptor
bHLH	Basic helix-loop-helix
BMP	Bone morphogenic protein
cAMP	Cyclic adenosine monophosphate
CBP	CREB binding protein
CH	Cysteine-histidine
CREB	cAMP response element-binding protein
Dach	Dachshund
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
EE	Early epaxial

Eya	Eyes absent
FAT	Factor acetyltransferase
FGF	Fibroblast growth factor
Gli	Glioma-associated oncogene homolog
GSK3 $\beta$	Glycogen synthase kinase 3 $\beta$
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
JNK	c-Jun N-terminal kinase
Kb	Kilo basepair
MHC	Myosin heavy chain
MPC	Muscle precursor cells
mRNA	Messenger RNA
MRF	Myogenic regulatory factor
PBS	Phosphate buffered saline
PCAF	p300/CBP associated factor
PMSF	Phenylmethylsulfonyl fluoride
RA	All- <i>trans</i> retinoic acid

RAR	Retinoic acid receptor
RARE	Retinoic acid response element
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
Shh	Sonic hedgehog
Six	Sine oculis
TE	Tris-EDTA buffer
TRABS	Transcription balancing sequences
TTNPB	4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)- 1-propenyl]benzoic acid
Wt	Wild type

## INTRODUCTION

Embryonic development has been a research interest for a very long time. The perfect execution of immensely complex process that leads from a single cell to a fully formed newborn individual captured the minds of scientists and led to the accumulation of vast knowledge. Yet there are still many questions waiting to be answered. Understanding how an organism develops is not the only reason we study development. We study it also to better understand some functions of adult organism and especially to understand the congenital disorders. In this work we turn our attention to the process by which skeletal muscle arises. Skeletal muscle is rather important to survival of the organism. Functions such as locomotion, feeding, reproduction and even breathing would be impossible without skeletal muscle. Here we study the differentiation of skeletal muscle at the molecular level. Our interest is the function that a coactivator protein p300 plays in the regulation of expression of *Myf5*, a gene which induces skeletal muscle differentiation (Braun et al. 1989).

### **Somitogenesis**

In the mammalian embryo the skeletal muscles of trunk, limbs, diaphragm and tongue develop from the somites, while the craniofacial muscles develop from prechordal, presomitic as well as somitic paraxial mesoderm (Christ, Brand-Saberi, and Grim 1992; Borycki et al. 1999). In a mouse embryo, somites are generated in rostro-caudal direction by segmentation of paraxial mesoderm on both sides of neural tube. The segmentation unfolds by the “clock and wavefront” model originally proposed by Cooke and Zeeman (1976). The current model is very similar to the one originally proposed. The cells of paraxial mesoderm express genes in a cyclical pattern governed by a time

delay negative feedback loop. The cyclical expression represents the clock, and in mice it involves genes belonging to Wnt, Notch and FGF pathways. The wave is generated by a gradient of FGF-Wnt-retinoic acid signaling. When cells are in the permissive stage of the cycle and the gradient of the wave reaches a threshold and segmentation occurs (reviewed by Dequéant and Pourquié 2008). Segmentation starts from embryonic day 8 (E8.0). After segmentation, somites give rise to the epithelial dermomyotome on the dorsal side and mesenchymal sclerotome on the ventral side. The sclerotome later forms the cartilage and the bone of spine and ribs and dermomyotome gives rise to dermatome which forms dermis of the back and myotome which forms the skeletal musculature (Christ and Ordahl 1995). The myotome is formed by involution of cells from dermomyotome. This process occurs in two waves. First, so called “pioneer” cells from the dorsomedial lip delaminate and position themselves underneath the dermomyotome. These cells will orient themselves rostro-caudally as they differentiate to myofibers and cover the underside of the entire dermomyotome. A second phase involves cells delaminating from all four lips of dermomyotome. Cells from the dorsomedial and ventrolateral lip migrate to the rostral and caudal lip where they enter the myotome, differentiate and take a direction parallel to already present cells from first wave (Cinnamon, Kahane, and Kalcheim 1999; Kahane, Cinnamon, and Kalcheim 1998; Kahane, Cinnamon, and Kalcheim 1998). The back muscles are subsequently derived from the epaxial myotome and the body wall and limb muscles from the hypaxial myotome (Christ and Ordahl 1995). Hypaxial somite contains migratory muscle precursor cells (MPCs) which delaminate to the limb bud where they form muscles of the limb. Migration of the Pax3 positive muscle precursor cells from the hypaxial

demomyotome is regulated by c-Met and Lbx1 genes. c-Met controls delamination and migration, where as Lbx1 regulates expression of genes that interpret cues that guide the migrating cells. Once these cells have reached the limb myogenic regulatory genes are expressed and the cells differentiate (reviewed by Vasyutina and Birchmeier 2006) . The migratory cell precursors are also present in occipital and cervical somites. These progenitor cells will however, give rise to hypoglossal cord and eventually tongue and pharyngeal muscles (Mackenzie, Walsh, and Graham 1998).

### **Genetics of myogenesis**

Development of skeletal muscle is regulated by four myogenic regulatory factors (MRFs): *MyoD* (Davis, Weintraub, and Lassar 1987), *Myf5* (Braun et al. 1989), *myogenin* (Edmondson and Olson 1989; Wright, Sassoon, and Lin 1989) and *MRF4* (Miner and Wold 1990; Rhodes and Konieczny 1989; Braun et al. 1990). The MRFs were first discovered because of their ability to transform fibroblast cell lines to muscle. All four MRFs belong to the basic helix-loop-helix (bHLH) family of transcription factors (Davis, Weintraub, and Lassar 1987; Braun et al. 1989; Edmondson and Olson 1989; Wright, Sassoon, and Lin 1989; Miner and Wold 1990; Rhodes and Konieczny 1989; Braun et al. 1990). The bHLH domain is DNA binding and also responsible for dimerisation with E protein family (Hu, Olson, and Kingston 1992; Murre et al. 1989; Parker et al. 2006). The MRF-E protein heterodimers bind E-box elements found in the promoters of muscle specific genes and induce the myogenic program (Rudnicki and Jaenisch 1995). MRFs can positively regulate their own transcription and the transcription of each other creating positive auto- and cross-regulation loops which ensures that myogenesis proceeds in a

failsafe manner (Braun et al. 1989; Thayer et al. 1989). In the mouse embryo, the earliest MRF to be expressed is *Myf5*. It is first expressed in the dorsomedial lip of dermomyotome at embryonic day 8 (E8.0), which soon after forms the epaxial myotome (Ott et al. 1991). *Myf5* is expressed in the hypaxial myotome as well (Tajbakhsh et al. 1997). Myogenin is expressed after *Myf5* at E8.5 and *MRF4* at E9. The last one to be expressed in the somite is *MyoD* at E10.5 (Sassoon et al. 1989; Bober et al. 1991). The cells which migrate to the limb bud do not express MRFs until they have reached the limb bud (Tajbakhsh and Buckingham 1994).

### **Mouse knockout models of MRFs**

The mouse models of MRF null mutations have been invaluable in studying the function of MRFs *in vivo*. Introduction of *MyoD* null mutation in mice surprisingly did not have a negative effect on skeletal muscle development. *MyoD*<sup>-/-</sup> mice were viable and exhibited normal physiology and morphology of the skeletal muscle, although an increased and prolonged expression of *Myf5* was observed (Rudnicki et al. 1992). The skeletal muscle of *Myf5*<sup>-/-</sup> mice also appeared morphologically normal and there was no change in levels of *MyoD*, *myogenin* or *MRF4* compared to wild type (wt) mice. The *Myf5* knockout mice however, died perinatally due to the loss of distal part of the ribs and inability to breathe. The only abnormality in skeletal muscle development in *Myf5*<sup>-/-</sup> mice was a delayed appearance of myotomal cells possibly until *MyoD* is expressed. (Braun et al. 1992). The lack of muscle deficiency in *MyoD*<sup>-/-</sup> or *Myf5*<sup>-/-</sup> mice was rather surprising, so a theory of their functional redundancy arose. The evidence for it was first observed in both *MyoD*<sup>-/-</sup> and *Myf5*<sup>-/-</sup> mice. More specifically, prolonged and increased

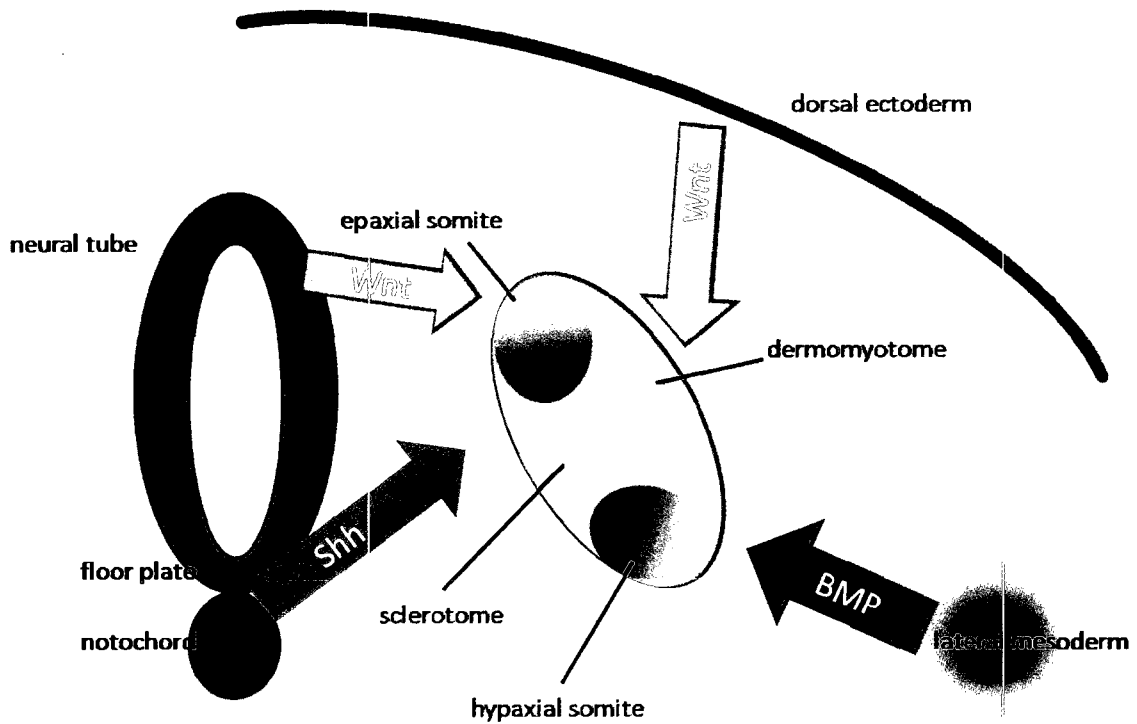
expression of *Myf5* in *MyoD*<sup>-/-</sup> mice and a delay in myotome formation in *Myf5*<sup>-/-</sup> mice, presumably until *MyoD* is expressed (Rudnicki et al. 1992; Braun et al. 1992). A double *MyoD*<sup>-/-</sup>;*Myf5*<sup>-/-</sup> knockout model gave a clear phenotype indicating the redundancy between the two genes. Mice deficient in both *MyoD* and *Myf5* displayed a complete loss of all the skeletal muscle and loss of skeletal muscle specific mRNAs (Rudnicki et al. 1993). *Myogenin* null mice also displayed a severe deficiency in skeletal muscle, however they were able to form myoblasts which mainly fail to fuse into myotubes. The *myogenin* null mice die perinatally and only a few myofibers are observed at birth (Hasty et al. 1993; Venuti et al. 1995; Nabeshima et al. 1993). Lack of myofibers implicated *myogenin* in later stages of differentiation and put it hierarchically after *MyoD* and *Myf5* (Rawls et al. 1995). The three *MRF4* knockout mice had range of phenotype from viable with no muscle defects, to lethal phenotype with some muscle defects (Braun and Arnold 1995; Patapoutian et al. 1995; Zhang, Behringer, and Olson 1995; Olson et al. 1996). An increase in *myogenin* expression and some deficiencies in myotomal myogenesis and deep back muscle, or intercostal muscle formation in *MRF4* knockout mice (Patapoutian et al. 1995; Braun and Arnold 1995) led to the conclusion that *MRF4* may have a function in terminal differentiation similar to *myogenin*.

More recently the role of *MRF4* was reinvestigated and *MRF4* was shown to have a function in commitment as well. Since *Myf5* and *MRF4* are adjacent to each other on the same chromosome, if one is knocked out there is, in most cases, a cis effect by which the expression of the other is also decreased or lost (Olson et al. 1996; Kassam-Duchossoy et al. 2004; Braun and Arnold 1995). In the light of this, new *MyoD/Myf5* double mutations were made in such manner that *MRF4* expression was still present. The

remaining *MRF4* expression was enough to support both epaxial and hypaxial differentiation thus showing the function of *MRF4* as a commitment gene (Kassar-Duchossoy et al. 2004). The function of *MyoD* was also reevaluated. A double null mutant of *MyoD* and *MRF4* showed almost identical phenotype to that of *myogenin* null mutants. This indicated that *MyoD*, along with *myogenin* and *MRF4*, plays a role in terminal differentiation of myoblasts (Rawls et al. 1998).

### **Regulation of MRF expression**

During somitogenesis the tissues surrounding the somites produce signals that direct myogenesis. Factors from the notochord, floor plate, neural tube, dorsal ectoderm and lateral mesoderm all affect the expression of myogenic regulatory factors (Figure 1). Sonic hedgehog (Shh) from the notochord and floor plate induces both *Myf5* and *MyoD* in epaxial myotome but not in hypaxial myotome. A number of studies indicated that the effect of Shh on *Myf5* is direct and on *MyoD* indirect. Loss of Shh lead to loss of *Myf5* but not *MyoD* (Chiang et al. 1996). Furthermore, Shh was shown to activate *Myf5* expression through a Gli binding site in the epaxial enhancer of *Myf5* (Gustafsson et al. 2002). Also, Shh cannot activate *MyoD* in absence of *Myf5* (Borycki et al. 1999; McDermott et al. 2005). The Wnt factors also take part in specification of somites. It was found that Wnt1 expressed by neural tube preferentially activates *Myf5*, and Wnt7a expressed by dorsal extoderm activates *MyoD*. Wnt4, Wnt5a and Wnt6 have an intermediate effect in activation of both *MyoD* and *Myf5* (Tajbakhsh et al. 1998). The effect of Wnt signaling on epaxial expression of *Myf5* is direct, through a  $\beta$ -catenin binding site in extended epaxial enhancer of *Myf5* (Borello et al. 2006).



**Figure 1. Myogenesis in the somite is regulated by signaling molecules from neighboring tissues.** Epaxial myogenesis is positively regulated by Wnt factors from neural tube and Shh from floor plate and notochord. Wnt signals from dorsal ectoderm induce myogenesis in hypaxial myotome, whereas BMP from lateral mesoderm is inhibitory.

Bone morphogenic proteins (BMPs) from lateral plate mesoderm were found to have a negative effect on expression of *MyoD* and *Myf5*. BMP4 inhibits *MyoD* expression in lateral somite (Hirsinger et al. 1997). Different levels of BMPs also have a different effect. Low levels of BMP2, 4 and 7 maintain Pax3 expression in proliferative populations in the limb, whereas high levels inhibit myogenic differentiation (Amthor et al. 1998). Noggin, a BMP antagonist, is produced in the lateral somite and inactivates BMP4 signals. Noggin expression in lateral somite is induced by Wnt1 from the neural tube and possibly Shh from notochord (Hirsinger et al. 1997). Noggin is expressed by the notochord and dorsal neural tube in caudal regions of chick embryo and in somites of more rostral regions. In somite Noggin is first lateral regions of epithelial somite Later

Noggin is expressed in the dorsomedial lip where *Myf5* is also first expressed (Reshef, Maroto, and Lassar 1998).

The expression of MRFs is also regulated by factors expressed by the somitic cells themselves. Pax3, Pax7 (paired box proteins 3 and 7), together with Six family of proteins and their cofactors, the Eya proteins play a significant role in myogenesis and regulation of MRF expression (Grifone et al. 2005; Franz et al. 1993; Grifone et al. 2007).

### **Role of Pax3 and Pax7 in expression of MRFs**

*Pax3* is expressed in unsegmented paraxial mesoderm and throughout newly formed somite. The expression of *Pax3* later becomes increased and restricted to the dermomyotome and downregulated in ventral somite that is to become sclerotome. As the somite matures further, *Pax3* expression becomes downregulated epaxially and further restricted ventrolaterally (Dietrich, Schubert, and Gruss 1993; Pourquie et al. 1995). Signals from notochord, surface ectoderm and lateral mesoderm maintain *Pax3* expression in the dermomyotome and later increase its expression laterally (Pourquie et al. 1996; Dietrich et al. 1998; Fan and Tessier-Lavigne 1994; Pourquie et al. 1995; Dietrich, Schubert, and Lumsden 1997). *Pax3* is involved in development of both epaxial and hypaxial muscle, as defects in both can be observed in *Pax3* deficient mice. For example, Splotch mice carrying a *Pax3* null mutation show a deficit in limb and shoulder muscles as well as a reduction in dorsal axial muscle (Grifone et al. 2007; Franz et al. 1993; Daston et al. 1996). However, while deep back muscles in Splotch mice are only reduced and loosely organized, some hypaxial muscles completely fail to develop,

especially distant muscle formed by the migratory populations, thus showing *Pax3* is more crucial to hypaxial muscle (Tremblay et al. 1998). *Pax3* has a role in survival of muscle precursor cells (MPCs) in hypaxial dermomyotome (Borycki et al. 1999). Severe loss of limb muscle in *Pax3* null mice was determined to be a consequence of loss of *c-Met* and *Lbx1* which regulate migration of MPCs to the limb (Grifone et al. 2005; Alvares et al. 2003; Epstein et al. 1996; Dietrich et al. 1999; Baldt et al. 1995). *Pax3* affects differentiation of limb muscle not only through migration but also by regulating expression of one of the MRFs. Migrating MPCs do not express myogenic regulatory factors until they reach the limb bud when *MyoD* and *Myf5* are expressed (Tajbakhsh and Buckingham 1994). The expression of *Myf5* is directly regulated by *Pax3* through the limb bud enhancer of *Myf5* (Bajard et al. 2006). *Pax3* and *Myf5* were determined to be hierarchically above *MyoD*, since *MyoD* cannot be correctly expressed in *Pax3/Myf5* double mutant mice (Tajbakhsh et al. 1997). Although, *MyoD* is activated normally in the hypaxial somite of *Pax3* mutant mice, there is evidence that *Pax3* is required for *MyoD* activation by signals from dorsal exoderm (Tajbakhsh et al. 1997; Borycki et al. 1999). *Pax7* is initially expressed in central dermomyotome only, but then takes the same expression pattern as *Pax3* in the myotome (Relaix et al. 2004; Relaix et al. 2005). *Pax7* <sup>-/-</sup> mice exhibit no change in dermomyotome and myotome formation compared to wt mice and in adult *Pax7* <sup>-/-</sup> mice no reduction in thickness of muscle or individual muscle fibers was observed (Oustanina, Hause, and Braun 2004; Mansouri et al. 1996). However, loss of both *Pax3* and *Pax7* in a double mutant mouse produces a more severe phenotype than that observed in the *Pax3* null mice. Somite development is more severely affected. The muscles of the trunk are compromised and there is a continued

apoptosis of MPCs whereas in *Pax3* null mice it stops after E11.5. This reflects the redundancy between *Pax3* and *Pax7* functions in skeletal myogenesis (Relaix et al. 2005). The redundancy between *Pax3* and *Pax7* is also seen when *Pax7* is inserted in *Pax3* locus, it is able to successfully replace *Pax3* in functions performed in neural tube, neural crest and somite development. However, *Pax7* is not able to replace the functions of *Pax3* necessary for delamination, migration and differentiation of migrating MPCs. This is a result of inability of *Pax7* to induce *c-met* expression (Relaix et al. 2004). *Pax3* and *Pax7* also have a function in marking a population of muscle precursor cells that in adult mice become satellite cells (Relaix et al. 2005). Also, it was found that *Pax7* may have a function in renewal and propagation of satellite cells (Oustanina, Hause, and Braun 2004).

### **Six and Eya proteins and regulation of MRFs**

*Sine oculis* genes were first discovered in *Drosophila* where they play a significant role in development of the eye (Serikaku and O'Tousa 1994; Cheyette et al. 1994). *Six* genes are homologues of *sine oculis* and in vertebrates there are six paralogues. Six proteins have a Six domain and a Six-type homeobox domain (Seo et al. 1999). Both *Six1* and *Six4* genes are expressed in the somite (Ozaki et al. 2001; Oliver et al. 1995). While a null mutation in *Six4* gene produces no overt muscle phenotype a null mutation of *Six1* gene in mice resulted in deficiencies or even absence of mainly hypaxial muscle but also some epaxial muscles (Ozaki et al. 2001; Laclef et al. 2003). Hypaxial extension of dermomyotome and expression of *MyoD* and *myogenin* in the limb bud were compromised in *Six1*<sup>-/-</sup> mice (Laclef et al. 2003). A double null mutation of *Six1* and

*Six4* in mice produced an increased severity of muscle hypoplasia compared to *Six1*<sup>-/-</sup> mice indicating a redundancy of function between the two genes. The majority of muscular defects in *Six1/Six4* double mutant came from inability to express *Pax3* in hypaxial myotome or epaxial myotome. Epaxial differentiation however, appears to progress normally in *Six1/Six4* double null mutants except for down regulation of *MRF4* (Grifone et al. 2007; Grifone et al. 2005). Loss of *Pax3* in hypaxial myotome however, led to unsuccessful migration of MPCs to the limb bud due to deficiency of expression of *c-Met* and *Lbx1* (Grifone et al. 2005). The regulation of *Pax3* expression in hypaxial myotome by *Six1* and *Six4* proteins is direct since both *Six1* and *Six4* bind and positively regulate the hypaxial promoter of *Pax3* (Grifone et al. 2007). *Six1* and *Six4* proteins also directly regulate expression of *Myf5* in the limb bud by binding the limb bud enhancer of *Myf5* (Giordani et al. 2007), which is also known to be regulated by *Pax3* (Bajard et al. 2006). In the hypaxial myotome of *Six1/Six4*, mutant mice expression of *MyoD*, *myogenin* and *MRF4* is also compromised (Grifone et al. 2007; Grifone et al. 2005). *Six1* and *Six4* proteins are also known to directly regulate *myogenin* promoter (Spitz et al. 1998). *Eyes absent (eya)* was also first identified in *Drosophila* where together with *sine oculis* and *dachshund (Dach)* regulates eye development (Mardon, Solomon, and Rubin 1994; Shen and Mardon 1997; Chen et al. 1997; Bonini et al. 1997; Pignoni et al. 1997). While only one *eyes absent* gene is identified in *Drosophila*, there are four *Eya* mouse homologues (Borsani et al. 1999; Xu et al. 1997). *Pax3*, *Eya2*, *Six1* and *Dach2* share a common expression pattern in the chick somite. All four are expressed in strongly in dorsal somite. In a more mature somite *Pax3* and *Dach2* are expressed more strongly in lateral dermomyotome and *Eya2* and *Six1* become more restricted to

myotome (Heanue et al. 1999). Eya proteins are cofactors of Six proteins (Tootle et al. 2003) and they seem to share roles with Six1 and Six4 proteins in skeletal muscle differentiation. *Eya1/Eya2* double knockout mice phenotype is almost identical to that of *Six1/Six4* double knockout, with a similar loss of *Pax3* expression. However, Eya proteins do not regulate expression of Six proteins in myotome or vice versa (Grifone et al. 2007). (Chen et al. 1997; Shen and Mardon 1997; Mardon, Solomon, and Rubin 1994). *Pax3* is activated by *Dach2* in the chick somites. Reciprocally, *Dach2* can also be activated by *Pax3*. *Eya2* and *Six1* cannot be induced by *Pax3* or *Dach2*. However, *Dach2* synergizes with *Eya2* and *Eya2* synergizes with *Six1* in regulation of myogenesis (Heanue et al. 1999).

### **Retinoic acid and skeletal myogenesis**

Retinoic acid also plays an important role in skeletal myogenesis. Retinoic acid regulates transcription through binding to the retinoic acid receptors (RARs) which have three types ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). They heterodimerize with retinoid X receptors (RXRs) which also exist in three types ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) (Mollard et al. 2000; Mangelsdorf and Evans 1995; Gronemeyer and Moras 1995; Giguere 1994; Chambon 1994). The heterodimers bind the retinoic acid response elements (RAREs). In the repressed state there is no ligand bound to RAR/RXR heterodimer. Co-repressors bind the heterodimer and recruit methyltransferases and histone deacetylases (HDACs) which make chromatin inaccessible. Binding of ligand to the RAR/RXR heterodimer creates a conformational change causing a disassociation of corepressors and recruitment of transcriptional activators (reviewed by Niederreither and Dolle 2008).

A very large number of genes were reported to be regulated by retinoic acid. This explains diverse and numerous roles retinoic acid plays in development, one of which is in segmental patterning and bilateral symmetry of somites. In absence of retinoic acid signaling, only a few somites are formed normally before there is a discord in somitogenesis on left and right side. Less somites are formed one side resulting in loss of symmetry (Kawakami et al. 2005; Sirbu and Deuster 2006; Vermot and Pourquie 2005; Vermot et al. 2005). Retinoic acid also plays a role in skeletal muscle differentiation. In zebrafish, chemical inhibition of retinoic acid synthesis caused a decrease in expression of *MyoD* and *myogenin* in the somite, whereas treatment with exogenous retinoic acid had the opposite effect (Hamade et al. 2006). Retinoic acid treatment is also known to enhance expression of *MyoD* and induce differentiation in C2C12 myoblasts cell line and in cultured satellite cells (Albagli-Curiel et al. 1993; Halevy and Lerman 1993). Treatment with retinoic acid during aggregation is also known to induce skeletal myogenesis in P19 cells (Edwards and McBurney 1983). Recently retinoic acid was shown to enhance skeletal myogenesis in both P19 and embryonic stem cells by increasing expression of *Wnt3a*, *Pax3* and *Meox1*. Moreover, RARs were shown to bind the regulatory regions of these genes (Kennedy et al. 2009).

### **Regulation of *Myf5*/*MRF4* locus**

*Myf5* and *MRF4* genes are found on mouse chromosome 10 approximately 8.8kb apart (Hadchouel et al. 2000). The link between *Myf5* and *MRF4* genes is conserved in birds (Saitoh et al. 1993), mice (Patapoutian et al. 1993) and humans (Braun et al. 1990). The transcription regulatory elements of *Myf5* and *MRF4* span a 140 kb region upstream

of the *Myf5* start site (Carvajal et al. 2001) and have been well characterized (Patapoutian et al. 1993; Zweigerdt, Braun, and Arnold 1997; Hadchouel et al. 2000; Hadchouel et al. 2003; Summerbell et al. 2000; Teboul et al. 2002; Carvajal et al. 2001; Zammit et al. 2004; Buchberger, Nomokonova, and Arnold 2003). The large number of enhancer elements in this locus allows for complex regulation of expression. The equilibrium between enhancer elements, minimal promoters and transcription balancing sequences (TRABS) further fine tunes the spatiotemporal expression (Carvajal, Keith, and Rigby 2008).

### **Enhancer elements of *Myf5***

Expression of *Myf5* in epaxial dermomyotome of the somite is regulated by the early epaxial (EE) enhancer. This enhancer regulates expression of *Myf5* at the earliest known time point and is located immediately downstream of the *MRF4* gene (Summerbell et al. 2000; Teboul et al. 2002). Epaxial enhancer by itself is activated by Shh through a Gli binding site (Gustafsson et al. 2002). When a 195kb upstream regulatory region is present together with *Myf5* minimal promoter, only the maintenance of *Myf5* in epaxial dermomyotome appears to be dependant on Gli site in EE enhancer (Teboul, Summerbell, and Rigby 2003). The extended epaxial enhancer which includes sequence 5' to epaxial enhancer, is positively regulated by Wnt signaling via Lef/Tcf sites found immediately upstream of the early epaxial enhancer (Borello et al. 2006). Once *Myf5* is expressed, the cells of dorsomedial dermomyotome delaminate to form epaxial myotome (Denetclaw and Ordahl 2000; Ordahl and Le Douarin 1992). The expression of *Myf5* here is regulated by an element located -57/-56.5kb from the *Myf5*

start site (Hadchouel et al. 2003). A region within -23kb also regulates expression in a subdomain of epaxial myotome (Hadchouel et al. 2000). The early hypaxial expression of *Myf5* is regulated by an intragenic enhancer overlapping with *Myf5* coding region (Summerbell et al. 2000). Hypaxial expression is further regulated by region between -53.3 and -48kb (Hadchouel et al. 2003) and a distant element located at -140/-88.2 (Carvajal et al. 2001). As the somite matures, *Myf5* expression in both hypaxial and epaxial somite is regulated by an element located at -57.5/-57kb (Hadchouel et al. 2003) and, a region between -88.2kb and -63kb ensures the maintenance of *Myf5* expression in axial muscles after E11.5 (Carvajal et al. 2001).

Expression in limbs is directed by elements located between -58kb and -48kb (Hadchouel et al. 2003; Hadchouel et al. 2000; Summerbell et al. 2000). The element located at -57.5/-57kb region regulates expression in both fore and hind limbs (Hadchouel et al. 2003) and it is under direct control of Pax3, Six1 and Six4 proteins (Bajard et al. 2006; Giordani et al. 2007). A second element located between -53.3kb and -48kb regulates *Myf5* expression preferentially in the hind limbs (Hadchouel et al. 2003).

Expression in the branchial arches is initiated by two elements; an intragenic element overlapping with the coding region of *Myf5* and the proximal arch element immediately upstream of *Myf5* transcription start site (Summerbell et al. 2000). Negative arch element in the intragenic region of *MRF4* downregulates the early expression in branchial arches (Summerbell et al. 2000). The effect of the negative branchial arch element is overcome by more distal hyoid (-45/-23kb) and mandibular arch (-88.2/-63kb) elements (Carvajal et al. 2001a). The expression of *Myf5* in the hypoglossal cord is

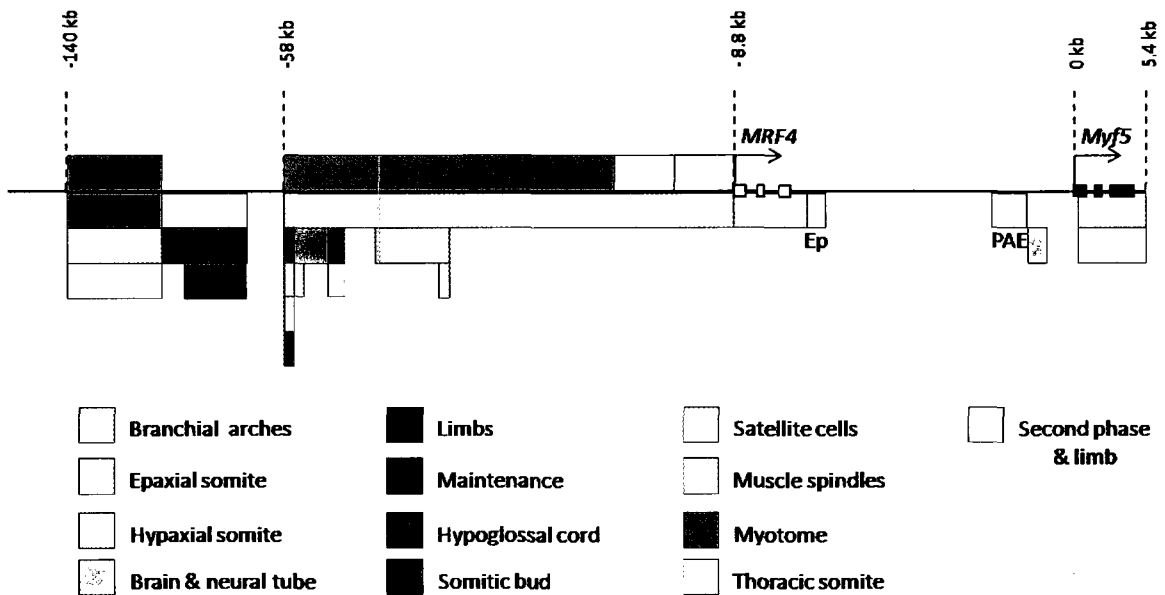
directed by two elements located at -57.5/-57kb and (Hadchouel et al. 2003) -81/-63kb (Carvajal et al. 2001b).

Expression of *Myf5* in central nervous system is regulated by two elements. The more proximal element located just 294bp upstream of *Myf5* transcriptional start site regulates expression in neural tube (Summerbell et al. 2000). The more distal element located at -56.6/-53.7 directs expression in brain and in neural tube (Hadchouel et al. 2003).

*Myf5* expression in the adult mice is regulated by two regulatory regions. Expression in the satellite cells is regulated by an element located in -140/-88kb region whereas, expression in muscle spindles is regulated by region from -59kb to -8.8kb (Zammit et al. 2004).

Expression of *MRF4* gene is regulated by a set of enhancers that overlap with those of *Myf5*. In both dorsal and ventral region of caudal and rostral somites, *MRF4* expression is regulated by an element located between -58.6kb and -17.3kb (Carvajal et al. 2001a). The region between -17.3kb and -15.3kb regulates early *MRF4* expression in central myotome of thoracic somites (Pin et al. 1997). The expression in the ventral myotome of thoracic somites is regulated by an element located at -140/-88kb. The expression in the limb and the second phase of *MRF4* expression are regulated by an element between -15.3kb and -8.8kb (Patapoutian et al. 1993). Another level of regulation of *Myf5/MRF4* locus is the equilibrium formed between enhancer sequences and minimal promoters of *Myf5* and *MRF4* and TRABS. Carvajal et al. (2008) showed that in absence of *Myf5* minimal promoter, the enhancer elements of *Myf5* can drive transcription from *MRF4* promoter and also from alternative transcription start sites or

cryptic promoters; and in some cases from both *MRF4* and cryptic promoter at the same time. Furthermore some enhancers can also interact nonproductively with the cryptic promoters, thus showing that enhancer elements in *MRF4/Myf5* locus are not simply in on or off interaction with their respective promoters or cryptic promoters, but rather exist in equilibrium between the two. These cryptic promoters were termed transcription balancing sequences (Carvajal, Keith, and Rigby 2008).



**Figure 2. Enhancer elements of *Myf5* and *MRF4*.**

*Myf5* and *MRF4* genes are both located on chromosome 10 about 8.8kb apart. Elements regulating their expression span the region 140kb upstream of *Myf5* start site and the intragenic region of *Myf5*. Regulatory elements of *Myf5* are shown below the line representing DNA and regulatory elements of *MRF4* above the line.

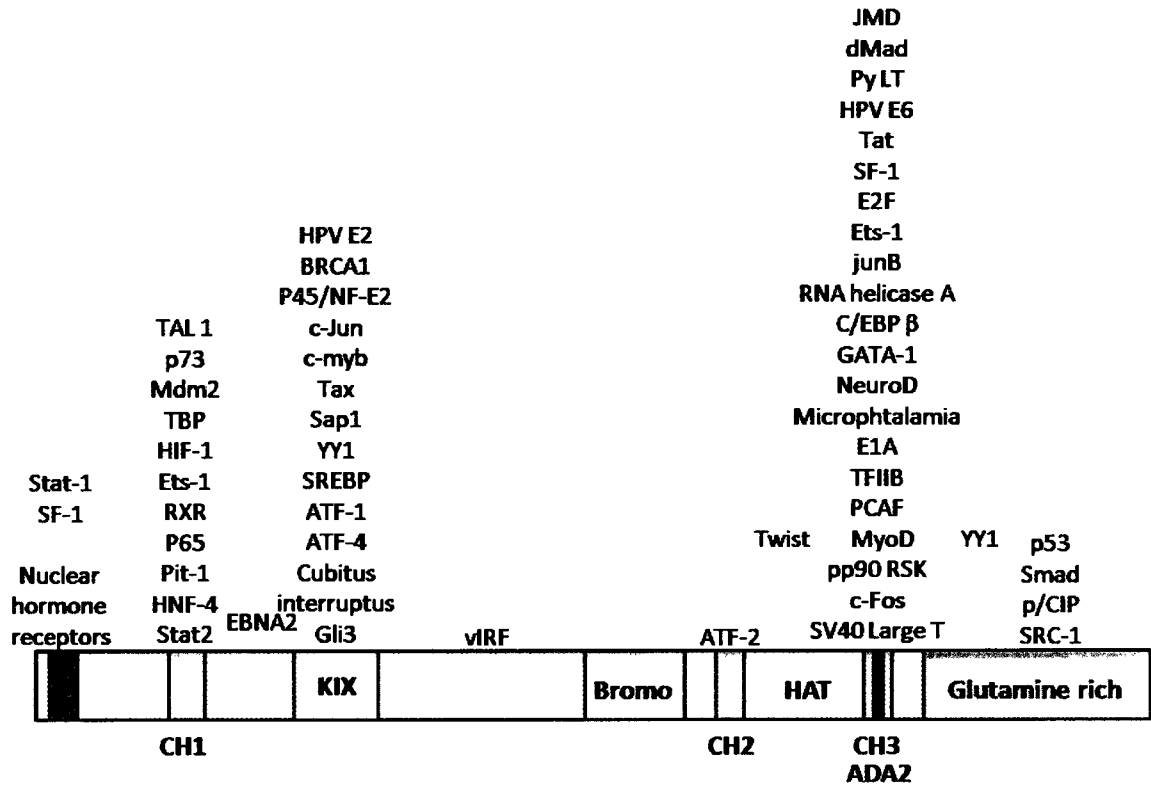
The study by Carvajal et al. (2008) shed additional light on previously made *Myf5* and *MRF4* knockout mice. The three *MRF4* knockout mice had a range of phenotype from viable with no muscle defects, to lethal phenotype with some muscle defects (Braun and Arnold 1995; Patapoutian et al. 1995; Zhang, Behringer, and Olson 1995; Olson et al. 1996). The strength of the promoter used to drive a selection gene, the direction of

transcription and the amount of deletion of original *MRF4* promoter all caused a greater or lesser interaction of *Myf5* enhancer elements to promoter of the selection marker, and thus a greater or lesser loss of *Myf5* expression (Olson et al. 1996, Kassar-Duchossoy et al. 2004). A similar effect was shown in a *Myf5* knockout study that used three different alleles which inhibited *MRF4* expression to a different extent (Kassar-Duchossoy et al. 2004).

### **Transcriptional coactivators p300 and CBP**

p300 and CREB binding protein (CBP) are closely related coactivator proteins. p300 was first identified as E1A binding protein (Eckner et al. 1994; Stein et al. 1990; Whyte et al. 1989) and it was noted for playing a part in functions of E1A such as cell cycle and transcriptional regulation (Eckner et al. 1994). CBP was identified, as its name implies, as a protein interacting with cAMP response element-binding protein (CREB) (Chrivia et al. 1993). CBP was determined to take part in transcription regulation by CREB as a coactivator (Kwok et al. 1994; Arias et al. 1994). p300 and CBP share 63% amino acid sequence similarity and are functionally homologous (Lundbald et al. 1995; Arany et al. 1995; Arany et al. 1994; Iyer, Ozdag, and Caldas 2004) . The functional domains of p300 and CBP are also the most conserved regions and consist of three cysteine-histidine rich zinc-finger domains (CH1-CH3), KIX domain, a bromodomain, a histone acetyltransferase (HAT) domain, ADA2 homology domain and a C-terminus glutamine rich domain (Vo and Goodman 2001; Arany et al. 1994). The N- and C-terminus activate transcription and interact with a large variety of factors (Figure 3). The

middle of the protein contains the HAT domain and performs the acetylation function of p300 and CBP (Chan and La Thangue 2001).

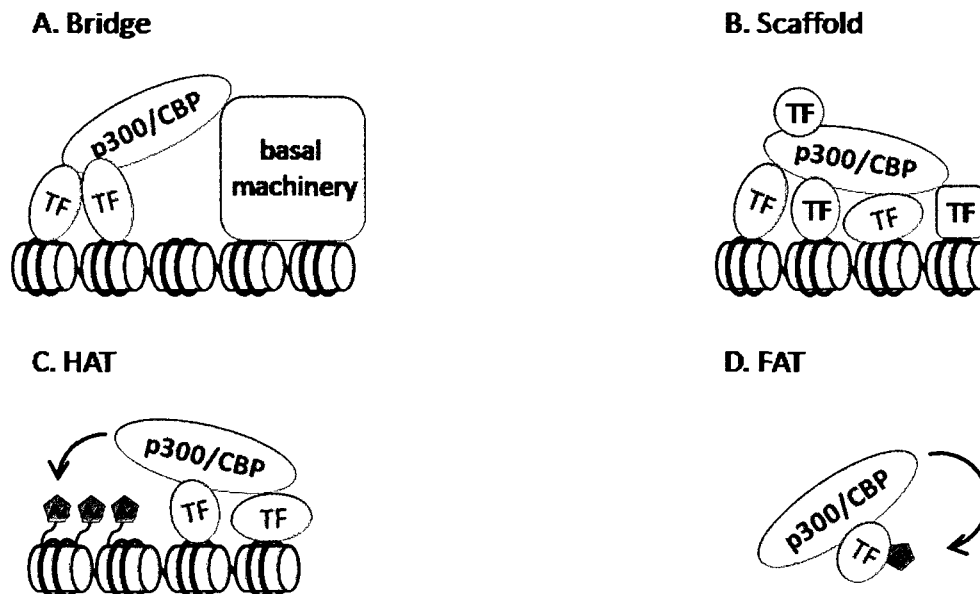


**Figure 3. Domains and binding sites of p300/CBP.**

p300 and CBP have modular structure consisting of three cysteine-histidine zinc finger domains (CH1-3), bromodomain, KIX, HAT, ADA2 homologous domain and glutamine rich domain. Both p300 and CBP interact with a large variety of factors, some of which are shown above.

p300 and CBP are very versatile in the ways that they are involved in transcriptional regulation. One way p300/CBP regulates transcription is by acting as a bridge between specific transcription factors and the basal transcriptional machinery

(Figure 4A). For example, at the interferon- $\beta$  promoter an enhanceosome is formed with AFT2/c-JUN, interferon regulated factor 1 and p50/p56 of NF- $\kappa$ B. The enhanceosome recruits RNA polymerase II holoenzyme complex through interaction with CBP (Kim, H, and Maniatis 1998; Yie et al. 1999; Wathelet et al. 1998). The ability of p300 and CBP to bind a large variety of factors allows them to act as scaffolds for transcription factor binding (Figure 4B).



**Figure 4. Modes of transcription regulation by p300/CBP.**

p300 and CBP regulate transcription by acting as (A) bridges providing a connection between transcriptional factors and basal transcriptional machinery; (B) scaffolds for various transcriptional factors; (C) histone acetyltransferases (HATs) and (D) factor acetyltransferases (FATs).

The scaffolding of transcriptional factors enables protein-protein and protein DNA interactions that are necessary for activation of transcription. p300 was suggested to perform a scaffolding function at the TNF $\alpha$  promoter by providing a surface for ATF-2, c-jun, Ets, Sp1 and Egr1 to interact with (Barthel et al. 2003; Chan and La Thangue 2001). The discovery of histone acetyltransferase activity of p300 and CBP shed more light on functions that p300 and CBP perform in transcriptional regulation (Figure 4C). p300 and CBP can acetylate all four core histones (Bannister and Kouzarides 1996; Ogryzko et al. 1996). Acetylation of lysine residues on histone tails neutralizes the charge of the residue and thus decreases the interaction of histone tails with DNA. This allows the DNA wrapping to loosen around histones and make transcription binding sites more available. Histone acetylation is generally considered to be a hallmark of gene activation (Kalkhoven 2004; Clayton, Hazzalin, and Mahadevan 2006). Aside from acetylating histones, p300 and CBP also act as factor acetyltransferases (FATs) (Figure 4D). Both p300 and CBP acetylate a number of transcription factors such as c-myb, HMGI(Y), TFIIE $\beta$ , TFIIF, GATA1, GATA4, MyoD, MEF2C, p53, HIV-1Tat, dTCF and EKLF, and thus affect activity of their targets (Imhof et al. 1997; Gu and Roeder 1997; Boyes et al. 1998; Munshi et al. 1998; Waltzer and Bienz 1998; Zhang and Bieker 1998; Ott et al. 1999; Martinez-Balbas et al. 2000; Tomita et al. 2000; Takaya et al. 2008; Poleskaya et al. 2000; Sartorelli et al. 1999; Ma et al. 2005). In some rare cases p300 can act as a repressor of transcription. For example, expression of *c-Myc* gene is repressed by a complex of p300 with YY1 and HDAC3 (Sankar et al. 2008). Also, at the *survivin* promoter, exchange of CBP for p300 is associated with repression (Ma et al. 2005).

## Common and distinct functions of p300 and CBP

Upon discovery of p300 and CBP as factors interacting with E1A and CREB respectively (Eckner et al. 1994; Stein et al. 1990; Whyte, Williamson, and Harlow 1989; Chrivia et al. 1993), it was also determined that CBP can interact with E1A and p300 with CREB (Lee, Zhang, and Shi 1996; Arany et al. 1995). Subsequent studies showed that both p300 and CBP act as coactivators to a number of transcriptional factors, which led to the belief that they are interchangeable. With time, however, the evidence that certain functions are performed only by p300 or CBP accumulated (Goodman and Smolik 2000; Vo and Goodman 2001). For example, p300 but not CBP, is necessary for retinoic acid (RA) induced differentiation of F9 cells and upregulation of cell-cycle inhibitor p21<sup>Cip1</sup> (Kawasaki et al. 1998). Similarly, only p300 is necessary for cell response to DNA damage by ionizing radiation (Yuan et al. 1999; Yuan et al. 1999). Mice knockout models for p300 or CBP also display some common but also some distinct features as well. Null mutation of *p300* and *CBP* are both lethal and both display failure to close the neural tube (Yao et al. 1998; Kung et al. 2000). *p300*<sup>-/-</sup> mice have heart defects (Yao et al. 1998) but this is not the case with *CBP*<sup>-/-</sup> mutant (Tanaka et al. 1997). Likewise, *CBP*<sup>+/-</sup> mice but not *p300*<sup>+/-</sup> mice have craniofacial abnormalities and hematological malignancies (Tanaka et al. 1997; Kung et al. 2000). Heterozygous loss of HAT activity of both p300 and CBP causes neonatal lethality. Loss of p300 HAT activity causes a more severe defect in heart, lung and small intestine, than loss of CBP HAT activity (Roth et al. 2003; Shikama et al. 2003). More importantly, p300 but not CBP HAT activity is important for skeletal muscle differentiation and expression of *Myf5* and *MyoD* (Roth et al. 2003).

### **p300 and myogenesis**

p300 plays an important part in skeletal muscle differentiation. For example, MyoD-dependant transcription and cell cycle arrest both require p300 (Puri et al. 1997). p300 is able to perform its MyoD related functions by acting as a coactivator for MyoD (Sartorelli et al. 1997; Yuan et al. 1996). It binds directly to MyoD (Sartorelli et al. 1997; Yuan et al. 1996), but it can also form a complex with both MyoD and PCAF (Puri et al. 1997). Furthermore, the acetyltransferase activity of p300 is also important for MyoD regulated transcription. p300 acetylates MyoD and in turn acetylation was shown to increase activity of MyoD and DNA binding (Polesskaya et al. 2000; Sartorelli et al. 1999). Although, *in vitro* upon binding with MyoD, p300 acetylates histones after which PCAF is recruited to acetylate MyoD (Dilworth et al. 2004). p300 is also a coactivator for MEF2C factor (Sartorelli et al. 1997). Proteins from the MEF2 family interact with MRFs cooperatively to activate transcription (Molkentin et al. 1995). Similar to MyoD, p300 can acetylate MEF2C as well, and this acetylation increases the binding of MEF2C to DNA (Ma et al. 2005; Angelelli et al. 2008). Mutation of p300 which leads to inhibition of HAT activity causes skeletal muscle deficiencies in mice embryos and decrease both *MyoD* and *Myf5* expression. This finding puts p300 hierarchically upstream of *MyoD* and *Myf5*. The expression of *Pax3* however, is not affected by this loss of p300 HAT activity. Interestingly, loss of the HAT activity of CBP, has no detrimental effect on skeletal muscle differentiation (Roth et al. 2003). The acetylation target of p300 that is needed for *Myf5* to be expressed is not known. We speculate that p300 is required to acetylate histones in the regulatory regions of the *Myf5* gene, more specifically, at the early epaxial enhancer of *Myf5*. We chose to study the early epaxial enhancer of *Myf5*

because it regulates *Myf5* expression at the earliest time point (Summerbell et al. 2000). Furthermore, extended epaxial enhancer of *Myf5* is regulated by  $\beta$ -catenin (Borello et al. 2006). p300 interacts with  $\beta$ -catenin as a coactivator (Hecht et al. 2000) so we can predict one possible binding target for p300 at the EE enhancer.

### **Curcumin, a p300/CBP HAT activity inhibitor**

Curcumin (diferuloylmethane) is a natural chemical produced by *Curcuma longa*. It has a long tradition of use as a spice and food colouring. Besides its use in food preparation, curcumin was shown to have much more valuable properties as an anticancer, antiinflammatory and antioxidant agent (Huang et al. 1988; Huang et al. 1994; Aggarwal et al. 2007; Ruby et al. 1995; Reddy and Lokesh 1992; Reddy and Lokesh 1994). Curcumin exerts these properties by modulating expression and activity of numerous genes and their products. On a molecular level, targets of curcumin also include a large variety of receptors, transcriptional factors, kinases, growth factors and inflammatory cytokines. Curcumin also inhibits carcinogen activation by suppressing certain cytochrome P450 isozymes or activating phase II carcinogen detoxifying enzymes (Iqbal et al.; Thapliyal and Maru 2001). Curcumin suppresses activation of NF- $\kappa$ B by inhibiting activity of I $\kappa$ B $\alpha$  kinase (IKK) (Jobin et al. 1999; Singh and Aggarwal 1995). Also, the activation of signal transducer and activator of transcription (STAT) proteins 3 and 5 is suppressed by curcumin by inhibition of their phosphorylation (Chen et al. 2004; Bharti, Donato, and Aggrawal 2003). Interestingly, curcumin interacts with the DNA binding site of AP1 and by occupying it inhibits activity of AP1 (Bierhaus et al. 1997). Curcumin also inhibits  $\beta$ -catenin/TCF signaling. Curcumin decreases the amount of

nuclear  $\beta$ -catenin in a manner independent of degradation induced by phosphorylation by Axin-adenomatous polyposis coli (APC)-glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) (Park et al. 2005). Curcumin also inhibits  $\beta$ -catenin by inducing caspase-3 mediated degradation of  $\beta$ -catenin (Jaiswal et al. 2002). Further more, curcumin modulates activity or expression of JNK, ERK, PPAR- $\gamma$ , AR, EGR1, MMP (reviewed by (Shishodia, Chaturvedi, and Aggarwal 2007; Aggarwal et al. 2007).

For our purpose however, we use curcumin for its activity as a specific inhibitor of HAT activity of p300 and CBP. Concentration of 100 $\mu$ M curcumin was shown to almost fully inhibit the HAT activity of p300 and CBP, but not affect HAT activity of PCAF. Furthermore, curcumin inhibited acetylation of p53 by p300 *in vivo* (Balasubramanyam et al. 2004). Radiolabeled curcumin forms a covalent bond with p300. Curcumin inhibits the HAT activity of p300 and CBP by a Michael reaction. The  $\alpha$  and  $\beta$  unsaturated carbonyl groups in the side chain of curcumin, act as acceptors in a Michael reaction. Curcumin also increases proteasome-mediated degradation of p300 and CBP (Marcu et al. 2006). The ability of curcumin to affect many factors and genes make it a less than ideal chemical to study HAT activity. However, curcumin is also the most specific inhibitor of p300 HAT activity available commercially.

### **P19 cells and skeletal myogenesis**

P19 cells are a mouse pluripotent embryonal carcinoma cell line. They were derived in 1982 by injection of 7.5 day old embryos into testis of mice. Injected embryos formed teratocarcinomas which contained a variety of tissues such as neuronal and epithelial tissues, cartilage, bone, smooth and striated muscle and undifferentiated cells

from which P19 cell line was derived (McBurney and Rogers 1982). P19 cells, like teratocarcinomas from which they were created, maintained pluripotency. P19 cells were able to differentiate into a variety of cell types such as neuronal cells, astroglia, microglia, oligodendrocytes, skeletal and cardiac muscle and other mesodermal and endodermal cell types (McBurney 1993). P19 cells therefore provide a great tool to study the differentiation into these cell types. In order to differentiate into skeletal muscle, P19 cells require aggregation and treatment with 0.75% - 1.25% dimethyl sulfoxide (DMSO). This treatment also induces differentiation to cardiomyocytes (McBurney 1993). The mechanism by which DMSO induces differentiation is not understood. However, it is known that DMSO treatment increases intracellular stores of calcium in a variety of cells including P19 cells (Morley and Whitfield 2005). Also DMSO treatment is known to induce genome wide epigenetic changes by altering DNA methylation (Iwatani et al. 2006). Aggregation of P19 cells with treatment of 10 to 100 nM retinoic acid, in absence of DMSO, is also known to induce skeletal myogenesis. However, concentrations of retinoic acid higher than 100 nM are known to induce neuronal differentiation (Edwards, Harris, and McBurney 1983). More recently, a combined treatment of P19 cells with DMSO and retinoic acid was shown to enhance skeletal myogenesis by inducing expression of *Wnt3a*, *Pax3* and *Meox1* (Kennedy et al. 2009). Skeletal and cardio myogenesis in P19 cells is also affected by unknown factors in serum. Namely, it was shown that different lots of fetal calf and calf serum have varying ability to support skeletal and cardio myogenesis (Wilton and Skerjanc 1999).

Expression of genes regulating specification and skeletal muscle differentiation in P19 cells occurs in a order similar to that observed in mouse somite. P19 cells induced to

differentiate to skeletal muscle first express mesodermal markers such as Brachyury T, Wnt3a and Wnt5b, followed by genes expressed in MPCs like Pax3, Meox1, Six1, Eya2 and Gli2 and finally MRFs such as MyoD and myogenin (Ridgeway, Wilton, and Skerjanc 2000; Ridgeway and Skerjanc 2001). Wnt signaling is known to activate expression of *Myf5* and *MyoD* in explants cultures (Tajbakhsh et al. 1998). Similarly, Wnt signaling was shown to be necessary and sufficient to induce skeletal myogenesis in P19 cells. Exogenous expression of Wnt3a or activated  $\beta$ -catenin induced skeletal myogenesis in P19 cells by activating expression of Pax3, Gli2, Meox1 and Six1; where as expression of dominant negative  $\beta$ -catenin inhibited expression of these factors (Petropoulos and Skerjanc 2002). Pax3 was also shown to be necessary for skeletal myogenesis in P19 cells. Expression of Pax3 induced expression of Six1, Eya2 and Meox1, followed later by expression of MyoD. However, exogenous expression of a dominant negative Pax3 completely inhibited skeletal myogenesis and expression of same genes which are upregulated by wild type Pax3 (Ridgeway and Skerjanc 2001). Exogenous expression of Gli was shown to induce expression of Pax3 and Meox1, where as expression of Meox1 was able to upregulate expression of Gli in P19 cells (Petropoulos et al. 2004). Overall, regulation of skeletal myogenesis in somite is recapitulated to a degree in P19 cells. The similarity with skeletal myogenesis *in vivo* and ease of manipulation, make P19 cells a valuable system for study of skeletal myogenesis.

## **Hypothesis and significance**

Regulatory regions of *Myf5* have been characterized in great detail, and a lot is known about the role of each enhancer element in the spatio-temporal regulation of *Myf5* expression in a mouse embryo (Patapoutian et al. 1993; Zweigerdt, Braun, and Arnold 1997; Hadchouel et al. 2000; Hadchouel et al. 2003; Summerbell et al. 2000; Teboul et al. 2002; Carvajal et al. 2001; Zammit et al. 2004; Buchberger, Nomokonova, and Arnold 2003). We have discussed above the signals and transcription factors regulating commitment to skeletal myogenesis and expression of the *Myf5* gene. However, very little is known about how these signals are integrated to induce *Myf5* expression from each enhancer element. There is limited information about the factors regulating enhancers of *Myf5*. Our research aims to understand more completely regulation of *Myf5* transcription at the level of the enhancer and therefore presents a step further in understanding the process of signal integration. Our research also aims to give novel, more mechanistic insight into the role of p300 and its HAT activity in *Myf5* transcription.

We have mentioned above the requirement of p300 HAT activity for the expression of *Myf5*. p300 is a very general coactivator and we assumed that the most direct effect p300 can have on *Myf5* is as a coactivator of the expression of *Myf5*. We choose to investigate the epaxial enhancer because of it regulates the expression of *Myf5* at an earliest time point (Summerbell et al. 2000) and because it is regulated by  $\beta$ -catenin (Borello et al. 2006) which represents a plausible binding partner of p300. Our hypothesis is as follows:

**p300 is involved in regulation of expression of *Myf5* directly by acetylating histones within the regulatory regions of the *Myf5* gene.**

We have addressed this hypothesis by studying skeletal muscle differentiation in a P19 cell system. P19 cells are embryonal carcinoma cells that can be induced to differentiate into myocytes (van Der Heyden and Defize 2003). We used curumin to chemically inhibit HAT activity of p300 and then assessed the occupancy and histone acetylation profiles at the epaxial enhancer.

## **METHODOLOGY**

### **Cell culture and differentiation**

P19 cells were grown in Minimum Essential Medium  $\alpha$  ( $\alpha$ -MEM) (Gibco-Invitrogen) with 5% fetal bovine serum (PAA), 5% donor bovine serum (PAA), 1% Penicillin/Streptomycin (Gibco-Invitrogen). The cells were grown at 37°C with 5% CO<sub>2</sub>. At the beginning of differentiation (day 0), cells were plated in 150mm Petri dishes (VWR) to induce aggregation. Cells were treated with 1% dimethylsulfoxide (DMSO) or cotreated with 1% DMSO and 1pM – 10nM TTNPB (Sigma-Aldrich) or 1% DMSO and 100pM-1 $\mu$ M all-*trans* retinoic acid (RA) (Sigma-Aldrich) during the four days the cells were aggregated. After aggregation cells were grown attached to tissue culture dishes or coverslips coated with 0.1% gelatin for 5 more days. We determined that 10nM RA enhances skeletal muscle differentiation and in the following experiments we used 1% DMSO or 1% DMSO with 10nM RA treatment for the first four days of differentiation. To inhibit p300 HAT activity the cells were treated with 10 $\mu$ M curcumin (Sigma-Aldrich) during the first 4 days of differentiation, or during the whole 9 days of differentiation. P19 cells were a gift from Dr. Ilona Skerjanc.

### **Immunofluorescence**

Cells were differentiated and grown on coverslips as described above. The cells were then fixed with cold methanol on ice. Following fixing, cells were air dried and rehydrated in PBS at room temperature and then incubated with primary antibody in PBS overnight. The cells were washed three times with PBS, then incubated with secondary antibody in PBS for 30-60min at room temperature. The cells were washed again three

times with PBS and stained with 0.1 µg/ml Hoechst (Molecular Probes) for 5min, then washed another three times with PBS. The stain was visualized using Axiovert 200M microscope (Zeiss), AxioCam HRM camera (Zeiss) and AxioVision Rel 4.6 software (Zeiss). The percentage of differentiated cells was determined as the fraction of fields of view occupied by positively stained cells out of total fields of view occupied by all the cells. The primary antibodies used were anti-MHC (1:10 dilution, homemade from MF 20 hybridoma cell line), anti-MyoD (1:200, M-318 Santa Cruz) and anti-Myf5 (1:200, C-20 Santa Cruz). The secondary antibodies used were Alexa Fluor®488 goat anti-mouse (1:200, Invitrogen), Alexa Fluor®488 goat anti-rabbit (1:200, Invitrogen) and Alexa Fluor®594 donkey anti-mouse (1:200, Invitrogen).

### **Western blotting**

At the indicated time points cells were washed with PBS and harvested. The cells were lysed by incubation in 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 min on ice. The concentration of proteins in the whole cell extract was determined by Bradford assay using a Bio-Rad Protein Assay Dye Reagent (Bio-Rad) and Multiscan Spectrum photospectrometer (Thermo). 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) and boiled for 5min at 95°C then resolved on 6% SDS-polyacrylamide gel. The proteins were then transferred overnight onto Immun-Blot PVDF membrane (Bio-Rad). Upon transfer the membranes were blocked for 1h with 5% non fat milk in PBST (1% Tween in PBS). The membranes were then incubated

overnight at 4°C with primary antibody diluted with 1% milk in PBST. Following that, membranes were washed three times with PBST and incubated with secondary antibody for 30-60min at room temperature, and then washed again three times in PBST. The proteins were visualized using Western Lightning™ Chemiluminescence (Perkin Elmer) reagents. In order to reprobe the membranes, they were stripped by incubation with stripping buffer (2 % SDS, 62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol) at 50°C for 30min and blocked again with 5% milk in PBST for 1 hour. The membranes were then reprobed as described above. The primary antibodies used were: anti-p300 (N15, Santa Cruz) in 1:500 dilution, anti-MHC (homemade from MF20 hybridoma cell line) in 1:100 dilution and anti-β-tubulin (homemade from E7 hybridoma cell line) in 1:100 dilution. Both MF20 and E7 cell lines were a gift from Dr. Alexandere Blais. The quantification of results was done using Scion Image software (Scion Corporation).

### **Real Time RT PCR**

At the indicated time point cells were washed in PBS and harvested. Cells were harvested from 150mm Petri dish or 100mm tissue culture dish. A small fraction of total cells was used for RNA isolation. Total RNA was isolated using RNeasy Mini kit (Qiagen) or Total RNA Kit I (Omega) according to the manufacturer's protocol. The reverse transcription reaction was performed with 2μg of RNA per reaction using a High capacity cDNA Reverse Transcription kit (ABI). Real Time RT PCR was performed using a Power SYBR® Green PCR Master mix (ABI) on Applied Biosystems 7500 Fast Real-Time PCR System. The primers used are listed in the supplementary table 3. Results were normalized to GAPDH or β-tubulin4 and analyzed by threshold cycle (Ct)

comparative method. The  $2^{-\Delta\Delta Ct}$  value was calculated, where  $\Delta Ct = Ct_{Myf5} - Ct_{\beta-tubb4}$ , and  $\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$ .

### **Chromatin Immunoprecipitation**

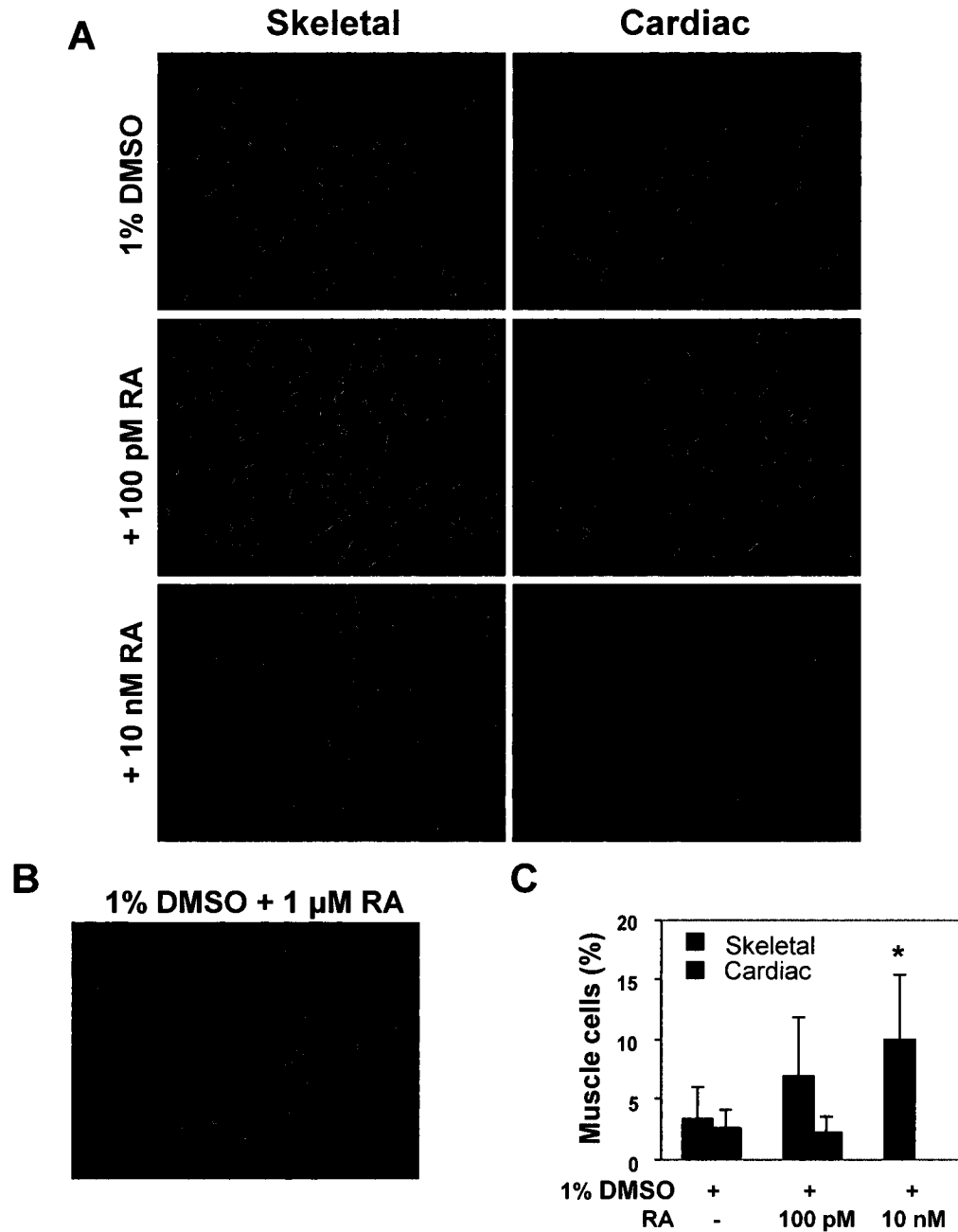
Cells were induced to differentiated as described above. At the indicated time point, cells were fixed with 1% formaldehyde for 15min at 37°C. The crosslinking was quenched with 200mM glycine. The cells were washed twice with ice-cold PBS. The cells were lysed using ChIP Lysis Buffer (50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 1%SDS, 1X protease inhibitors (Roche), 1mM DTT, 1mM PMSF, 20mM NaButyrate) for 10min on ice. The lysate was sonicated for 50min with Bioruptor system (Diagenode), using a 30s on/off cycle at high setting. The lysate was then pelleted by centrifugation at 13,000rpm for 10min. The supernatant was precleaned by incubation with DNA-blocked protein A-agarose beads (Upstate) for 1-2h at 4°C. The samples were then quantified by reading absorbance at 260nm. Equal amounts were diluted in ChIP Dilution Buffer (20mM Tris-HCl pH8.0, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 1X protease inhibitors (Roche), 1mM DTT, 1mPMSF, 20mM NaButyrate). Diluted chromatin was incubated with 2 $\mu$ g anti-p300 (Santa Cruz, C-20) or anti-AcH3 (Anti-Ac-Histone H3 (Lys 9/14), Santa Cruz) antibodies over night at 4°C. The chromatin-antibody complexes were pulled down by 2h incubation with DNA-blocked agarose protein A beads (Upstate). The beads were then washed with Washing Buffer A (20mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 150mM NaCl), Washing Buffer B (20mM Tris-HCl pH 8.0, 2mM EDTA pH 8.0, 0.1% SDS, 1% Triton X-100, 500mM NaCl), Washing Buffer C (20mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 1%

NP-40, 1% sodium dioxycholate, 0.25 M LiCl) and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) for 15min each at 4°C. The chromatin was eluted from the beads with Elution Buffer (100mM NaHCO<sub>3</sub>, 1% SDS) by agitation at room temperature for 30min. The eluted samples were digested with proteinase K (Roche) and reverse crosslinked at the same time by incubation overnight at 65°C. The DNA was purified using Cycle Pure Kit (Omega). The sequence of extended epaxial enhancer was amplified by a PCR reaction using equal amounts of elutes. The PCR reaction was performed using GoTaq® flexi DNA polymerase PCR kit (Promega) and dNTP mix (Promega) and primers specified in supplementary table 4. PCR product were resolved on a 2% agarose gel, stained with ethidium bromide and visualized by AlphaImager™ (Alpha Innotech).

## RESULTS

### **Retinoic acid enhances skeletal myogenesis**

The P19 cell system has been used extensively to study skeletal muscle differentiation. Aggregation, together with 0.75% - 1.25% DMSO treatment, is known to induce differentiation of P19 cells into skeletal myocytes. This treatment also induces differentiation to cardiac myocytes (Edwards, Harris, and McBurney 1983). In our experiments we aggregated the P19 cells and treated them with 1% DMSO for four days, after which the cells were allowed to attach to the surface of the dish or coverslip to grow attached for another five days with no treatment. The cells from the aggregates spread out on the coverslip or dish, and also continued to divide so that the total number of cells increased during differentiation procedure. The differentiated myocytes expressed myosin heavy chain (MHC) which is commonly used as a marker to detect skeletal muscle cells. However, cardiac muscle cells also express MHC. Fortunately, skeletal and cardiac myocytes can be distinguished easily by their morphology. Skeletal muscle cells have a bipolar shape with two long processes, whereas the cardiac muscle cells are more rounded in shape (Skerjanc 1999). At the end of the nine day differentiation procedure, cells were fixed and MHC expression was detected using immunofluorescence. This allowed us to visualize P19 cells that differentiated into both skeletal and cardiac myocytes and distinguish them based on their morphology. We found that aggregation and treatment with 1% DMSO induced on average 3.5% of cells to differentiate to skeletal myocytes and 3% into cardiac myocytes (Figure 5A and 5C). A recent report showed that about 2% of P19 cells differentiated into skeletal myocytes when induced with 1% DMSO (Kennedy et al. 2009), which is very close to the 3.5% which we



**Figure 5. All-trans retinoic acid enhances skeletal myogenesis.** (A) P19 cells were aggregated while treated with 1% DMSO or 1% DMSO with different concentrations of RA. After aggregation the cells were grown attached to plate or coverslip surface without any treatment for five more days. The cells were then fixed and probed for MHC (green) and Hoechst (blue). Two types of cells stained positive, skeletal muscle cells, bipolar in shape, and cardiac cells. (B) The highest concentration of both RA inhibits differentiation of both cardiac and skeletal muscle. (C) Quantification of differentiation induced by RA respectively. The bars represent standard deviation of five experiments (\*  $P < 0.05$ ).

observed. The differentiation of P19 cells into cardiac myocytes can be induced at a much higher rate (10-25%) using the same protocol (Skerjanc 1999). However, differentiation of P19 cells into skeletal and cardiac myocytes is affected by the unknown factors in the serum (Wilton and Skerjanc 1999). In our experiments we used the serum that supports skeletal myogenesis; thus, the rate of cardiac differentiation that we observed is relatively low.

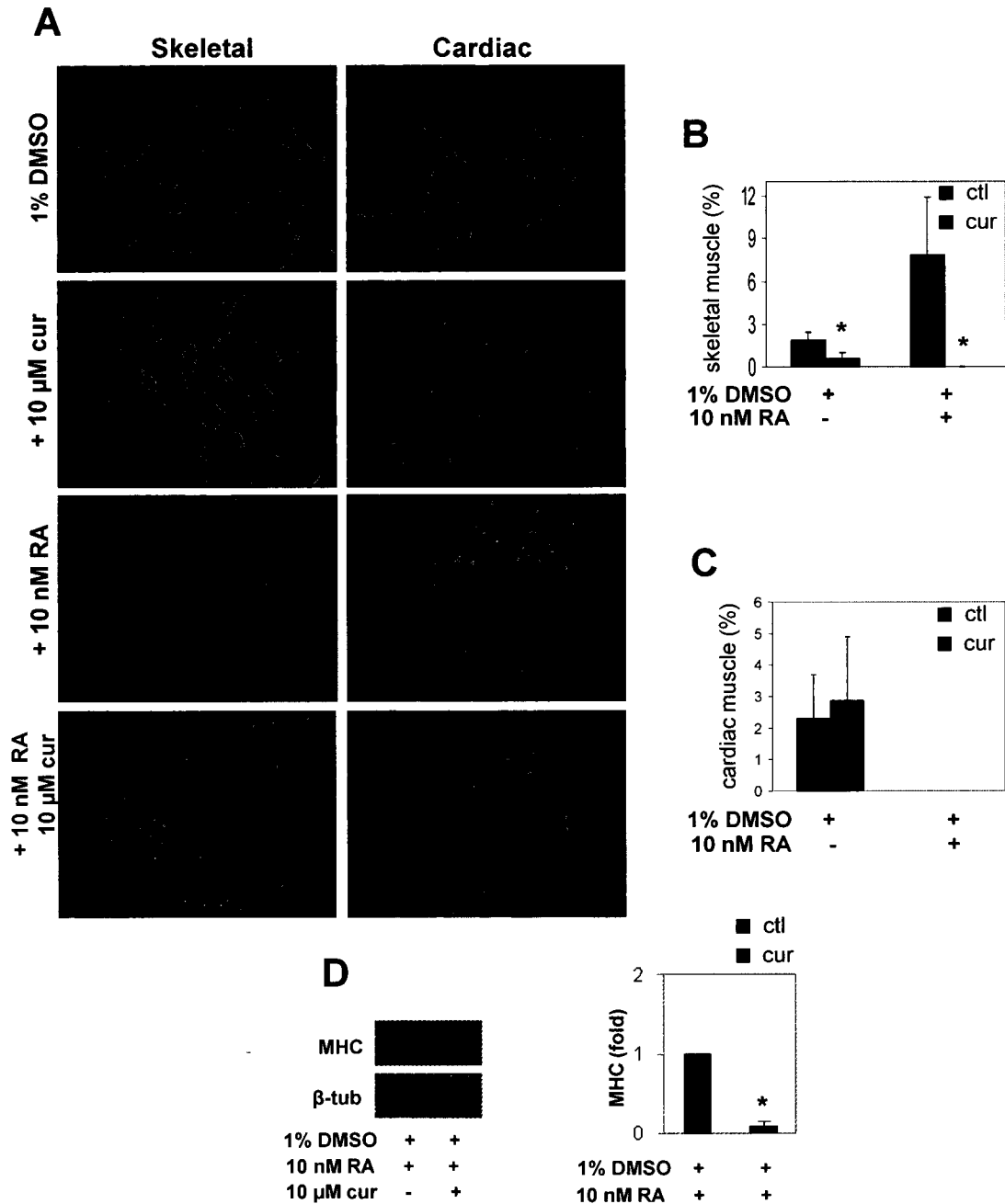
The P19 cell system is very valuable and it is used extensively. However, it is obvious that it is not a very efficient system for skeletal muscle differentiation. For this reason we sought to enhance the percentage of skeletal myocytes in the differentiated population. Aside from DMSO, treatment with retinoic acid during aggregation is also known to induce skeletal myogenesis in P19 cells (Edwards and McBurney 1983). We wanted to test the ability of cotreatment with 1% DMSO and all-*trans* retinoic acid (RA) to induce skeletal muscle differentiation compared to 1% DMSO alone. P19 cells were treated with 1% DMSO alone or in combination with a range of concentrations of RA during aggregation. Upon aggregation the treatment with DMSO or DMSO and RA was withdrawn and the cells were grown for another 5 days attached to the coverslip surface. The efficiency of skeletal and cardiac muscle differentiation was assessed by immunofluorescent staining with MHC and the nuclei of cells were detected with Hoechst stain. Three concentrations of RA were tested ranging from 100 pM to 1  $\mu$ M. The two lower concentrations, 100 pM and 10 nM, induced on average 7% and 10% of the cells to differentiate to skeletal myocytes respectively (Figure 5A and 5C). The highest concentration of 1  $\mu$ M however, entirely inhibited skeletal muscle differentiation (Figure 5B). Concentration of 10 nM in cotreatment with 1% DMSO enhanced the

skeletal muscle differentiation by nearly 3 fold, the most out of the tested concentrations. The same concentration of RA was also reported to induce skeletal muscle differentiation in P19 in absence of DMSO (Edwards and McBurney 1983; Wobus et al. 1994). Another study showed that a concentration of 30 nM RA with DMSO enhanced skeletal myogenesis of P19 by 4 fold (Kennedy et al. 2009) which concurs with our results. Cardiomyogenesis is also known to be affected by RA. The concentration of 1nM which we did not test, was reported to induce cardiac muscle differentiation (Edwards and McBurney 1983), higher concentrations of RA however, inhibited cardiomyogenesis (Skerjanc and McBurney 1994; Kennedy et al. 2009). We found that cardiomyogenesis was not affected by 100 pM RA, however the concentration of 10 nM inhibited the cardiac muscle differentiation entirely (Figure 5A and 5C). Treatment with RA was shown to decrease expression of BMP4 and increase expression of BMP inhibitor Tob1 in P19 cells which is the likely mechanism by which RA inhibits cardio myogenesis (Kennedy et al. 2009). The highest concentration (1 $\mu$ M) of RA, which we show to inhibit both cardiac and skeletal muscle, is in the range of concentrations of RA known to induce neuron and astroglia differentiation (Edwards and McBurney 1983). The concentration of 10 nM RA in cotreatment with 1% DMSO proved to be the most efficient in enhancing skeletal myogenesis and we continued to use it in further experiments.

## **Curcumin treatment inhibits skeletal myogenesis**

We have outlined before the role of p300 and its HAT activity in skeletal myogenesis, more specifically in MyoD regulated transcription and in expression of *MyoD* and *Myf5* (Roth et al. 2003; Yuan et al. 1996; Sartorelli et al. 1997). The HAT activity of p300 is necessary for expression of *MyoD* and *Myf5* in embryonic stem cells. Also, loss of HAT activity of p300 in mice caused a decrease in muscle size and perinatal death due to the inability to breathe. Moreover the expression of *Myf5* and *MyoD* is also decreased in these mice (Roth et al. 2003). The specific role of p300 HAT activity in expression of *Myf5* or *MyoD* is not known. Our focus is on expression of *Myf5* specifically and the role of p300 HAT may have in it. We speculated that p300 and its HAT activity are involved directly in regulation of *Myf5* expression. To address our hypothesis, a tissue culture system was chosen over a mouse model because it was readily available, easy to manipulate, and faster to work with. An embryonic stem cell lines harboring mutation in p300 or CBP HAT domain created by Roth et al. (2003) would have made an excellent system, however we were unable to obtain them. The same mutations could have been introduced to P19 cell lines but the preparation of these cell lines requires time, so a chemical inhibition of p300 HAT activity was chosen instead.

Curcumin, inhibits the HAT activity of p300 and CBP both *in vitro* and *in vivo*. However, it has no effect on the HAT activity of other acetyltransferases such as PCAF. In addition, it has been shown that curcumin inhibits the acetylation of the transcription factor p53 by p300 (Balasubramanyam et al. 2004). We aggregated P19 cells and treated them with 1% DMSO in the presence or absence of 10 nM RA followed by withdrawal of the treatments during the five days when the cells were grown attached. Cells were



**Figure 6. Curcumin inhibits skeletal myogenesis.** (A) P19 cells were differentiated with 1% DMSO and 10 nM RA. Cells were also treated with 10  $\mu$ M curcumin during the entire differentiation after which they were probed for MHC (green) and Hoechst (blue). Efficiency of skeletal (B) and cardiac (C) muscle differentiation was quantified. Error bars represent standard deviation of five independent experiments. (D) Expression of MHC in cells differentiated with 1% DMSO and 10 nM RA was examined by Western blotting on day 9.  $\beta$ -tubulin was used as loading control. Error bars represent standard deviation of three independent experiments. (\*  $P < 0.05$ ).

treated with curcumin during and after aggregation for the full nine days of differentiation. We initially treated the P19 cells with 100 $\mu$ M of curcumin, which is the concentration known to inhibit 90% of p300 HAT activity (Balasubramanyam et al. 2004). This concentration however, proved to be toxic to the P19 cells (data not shown). We then tested a range of concentrations of curcumin and found that 10  $\mu$ M is the highest concentration which was not toxic to differentiating P19 cells (data not shown). This concentration is expected to inhibit approximately 30% of HAT activity of p300 (Balasubramanyam et al. 2004). In order to determine exactly how much of p300 HAT activity is lost by treatment with 10  $\mu$ M curcumin in P19 cells we attempted to do an *in vitro* fluorescence based HAT assay. However, this assay was not successful, possibly because of low sensitivity. The P19 cells were continuously treated with 10  $\mu$ M curcumin for the 9 days of differentiation. The continuous treatment caused almost a complete inhibition of skeletal myogenesis, both in cells induced to differentiate with 1% DMSO alone, and in cells induced with 1% DMSO with 10 nM RA (Figure 6A and 6B). Surprisingly, the inhibition of myogenesis appears to be more dramatic in cells differentiated with RA. To observe the effect of curcumin on skeletal myogenesis in another way we also studied the change in MHC expression by Western blotting. Cells were induced to differentiate with 1% DMSO and 10 nM RA and treated continuously with curcumin. Cells treated with RA do not differentiate into cardiac muscle (Figure 5 and 6, (Kennedy et al. 2009)) so the MHC detected by Western blotting is expressed only by the skeletal myocytes. We found that there was a complete loss of MHC expression in cells treated with curcumin (Figure 6D). This shows a loss of skeletal muscle differentiation due to curcumin treatment, which is consistent with the

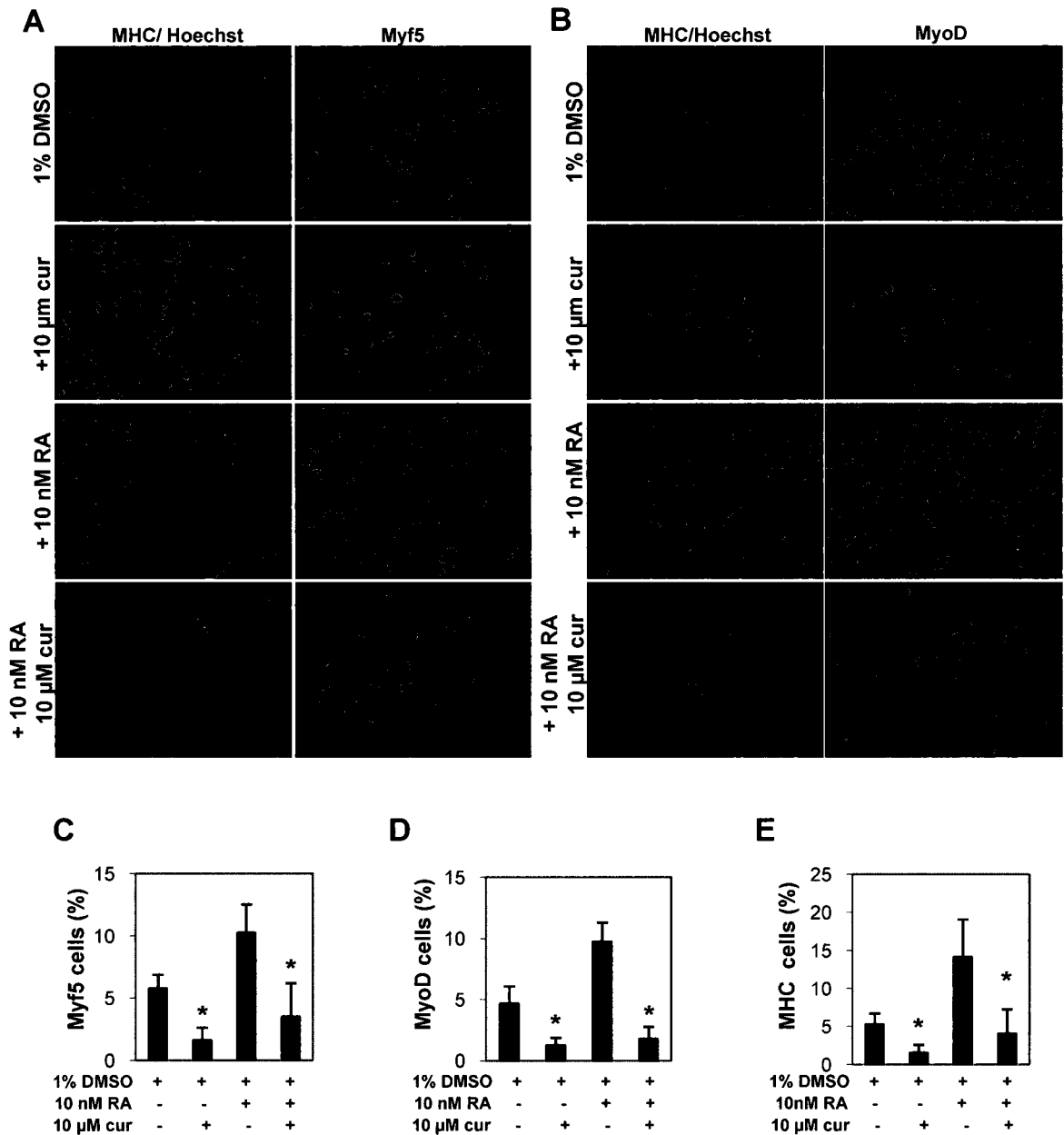
immunofluorescence results. We also observed the effect of curcumin on cardiomyogenesis. Cells induced with 1% DMSO and continuously treated with 10  $\mu$ M curcumin differentiated into cardiomyocytes with 2.9% efficiency which is nearly the same as 2.3% observed in untreated cells (Figure 6A and 6C). Based on our results, curcumin has no effect on cardiac muscle differentiation in P19 cells.

We can conclude that a continuous nine day treatment with 10  $\mu$ M curcumin successfully inhibits skeletal muscle differentiation but has no effect on cardiac muscle differentiation. Although our interest is primarily on the role of p300 HAT activity in skeletal myogenesis, curcumin is an inhibitor of both p300 and CBP HAT activity (Balasubramanyam et al. 2004). CBP is expressed in P19 cells (data not shown). However, Roth et al (2003) have established that loss of HAT activity of CBP is not detrimental to skeletal muscle differentiation in embryonic stem cells. Based on these observations we speculate that the effect of curcumin on the differentiation of P19 cells is due to loss of p300 HAT activity. Therefore, our results suggest that the HAT activity of p300 may be necessary for skeletal myogenesis but dispensable for cardiomyogenesis in P19 cells. This is consistent with results of Roth et al. (2003), who showed that p300 HAT activity is required for skeletal myogenesis in embryonic stem cells. Curcumin treatment however, also inhibits canonical Wnt signaling by decreasing nuclear localization of  $\beta$ -catenin. At 10  $\mu$ M concentration curcumin decreased transcriptional activity of  $\beta$ -catenin by 30% in SW480 cells. However, in AGS and HCT116 cells transcriptional activity of  $\beta$ -catenin was increased 10-20% by 10  $\mu$ M curcumin (Park et al. 2005). Activity of  $\beta$ -catenin is necessary for skeletal myogenesis in P19 cells (Petropoulos and Skerjanc 2002). It is necessary to determine the effect of curcumin on

activity of  $\beta$ -catenin in P19 cells in order to address the possibility that curcumin inhibits skeletal myogenesis in P19 cells by inhibiting activity of  $\beta$ -catenin. We have therefore shown that curcumin successfully inhibits skeletal myogenesis in P19 cell. We speculate that the mechanism of inhibition by curcumin may be through inhibition of HAT activity of p300 and/or through inhibition of transcriptional activity of  $\beta$ -catenin.

### **Curcumin treatment during aggregation is sufficient to inhibit myogenesis**

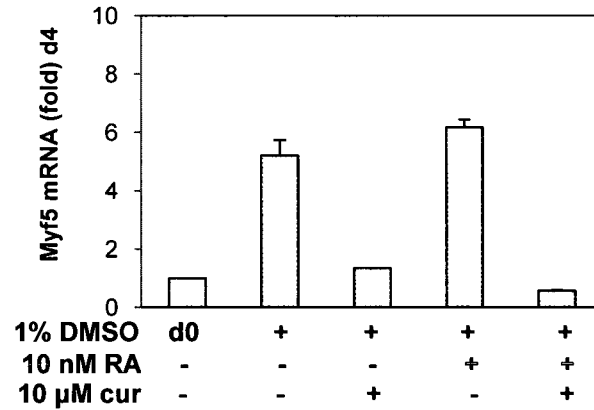
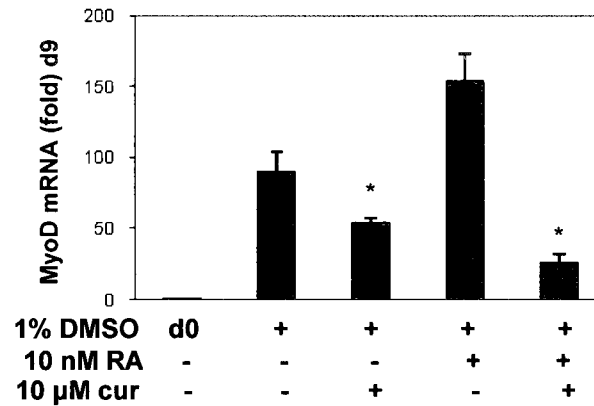
p300 performs various functions during skeletal myogenesis. Some of these functions are executed after expression of early MRFs and commitment. For example p300 is a coactivator for MyoD and MEF2C both of which are expressed in committed cells (Yuan et al. 1996; Sartorelli et al. 1997). p300 also plays a role before commitment, since p300 HAT activity is necessary for expression of *Myf5* and *MyoD* (Roth et al. 2003). We next wanted to determine if curcumin has an effect on myogenesis during the aggregation of P19 cells. The cells were induced to differentiate with 1% DMSO, with or without 10 nM RA, and treated with 10  $\mu$ M curcumin during the aggregation only. For the last five days all treatments were withdrawn and then the cells were probed for MHC expression by immunofluorescence. Curcumin treatment during aggregation only, was sufficient to decrease skeletal muscle differentiation of the cells induced to differentiate with 1% DMSO by 70%. Similarly skeletal muscle differentiation of cells induced with 1% DMSO and 10 nM RA, was decreased by 71% when exposed to curcumin treatment during aggregation (Figure 7A, 7B and 7E). Therefore, curcumin treatment during just the aggregation period is sufficient to inhibit skeletal muscle differentiation. This indicates the HAT activity of p300 during early stages of differentiation may be crucial for myogenesis in P19 cells. However, as discussed earlier, curcumin may be inhibiting skeletal myogenesis by inhibiting activity of  $\beta$ -catenin.



**Figure 7. Curcumin inhibits commitment to skeletal myogenesis.** P19 cells were differentiated as before and treated with curcumin during aggregation only. Cells were probed at the end of differentiation for MHC (red), Hoechst (blue) and Myf5 (green) (A) or MyoD (green) (B). Percent of cells expressing Myf5 (C), MyoD (D) was quantified. (E) The fraction of skeletal muscle cells expressing MHC was also determined. The error bars represent standard deviation of three or four independent experiments (\* P>0.05).

### **Curcumin inhibits commitment to skeletal myogenesis**

We established that curcumin decreases the number of skeletal muscle cells expressing MHC and therefore inhibits skeletal myogenesis both by a continuous treatment and by treatment only during aggregation (Figure 6 and 7). We next wanted to investigate if curcumin inhibited commitment to skeletal muscle lineage. For this purpose we studied how the expression of early MRFs, *Myf5* and *MyoD* is affected by curcumin treatment. We initially assessed the effect of curcumin on Myf5 and MyoD protein expression by Western blotting. We found that both Myf5 and MyoD protein expression increased at the end of differentiation (day 9). The treatment with curcumin had no effect on levels of Myf5 and MyoD protein (data not shown). However, we suspected the antibodies used, had poor specificity because they were detecting Myf5 and MyoD protein in undifferentiated cells and many non specific bands of varied molecular weights. For this reason we also determined the effect of curcumin on mRNA levels of Myf5 and MyoD by Real Time RT-PCR. We differentiated the cells as before and treated them with curcumin during aggregation only. Total RNA was isolated from undifferentiated cells and differentiated cells on day 4 and day 9. Following that, total RNA was reverse transcribed and amplified by Real Time PCR. A large induction was observed in Myf5 and MyoD mRNA levels in cells treated with 1% DMSO and 1% DMSO with 10 nM RA on day 9 compared to undifferentiated cells on day 0 (Figure 8B and data not shown). The same treatments induced on day 4 a low level of Myf5 expression. On day 4 Myf5 mRNA levels were roughly 3 fold less than on day 9. MyoD was not significantly induced on day 4 (Figure 8A and data not shown). The cells treated

**A****B**

**Figure 8. Curcumin inhibits expression of Myf5 and MyoD mRNA.** Cells were induced to differentiate with 1% DMSO or 1%DMSO with 10 nM RA treatment. Cells were also treated with 10 μM curcumin during aggregation. On day 4 the mRNA levels of Myf5 (**A**) and on day 9 the mRNA levels of MyoD (**B**) were determined by Real Time RT-PCR. Single representative experiment, of total three experiments, is shown.

with 10 nM RA showed an increase in MyoD mRNA less than 2 fold compared to cells treated with 1% DMSO alone. Curcumin treatment decreased the mRNA levels of both Myf5 on day 4 and day 9 and MyoD on day 9 when cells were induced with either 1% DMSO alone or 1% DMSO with RA (Figure 8A and 8B and data not shown).

We have observed with curcumin treatment a decrease in expression of Myf5 and MyoD mRNA but not protein. Since we suspected that Western blot results may not be representative we assessed the effect of curcumin on expression of Myf5 and MyoD proteins by immunofluorescence. The P19 cells were aggregated and induced to differentiate with 1% DMSO with or without 10 nM RA treatment during aggregation in presence or absence of 10  $\mu$ M curcumin. Upon staining with anti-Myf5 and anti-MyoD antibodies, the fraction of cells expressing Myf5 or MyoD was determined as described in Materials and Methods. The cells were also assessed for MHC expression by immunofluorescence. Cells expressing MHC in a skeletal muscle pattern also showed Myf5 or MyoD nuclear stain. The exception were a few cells expressing Myf5 or MyoD but not MHC. MHC is expressed after Myf5 or MyoD and these cells were likely myoblasts that had not yet terminally differentiated and expressed MHC. When P19 cells were induced with 1% DMSO approximately 5% of all cells expressed Myf5 or MyoD. A similar rate of skeletal muscle differentiation was observed with MHC staining (Figure 7). When cells were induced with DMSO and RA roughly 10% of the cells expressed Myf5 or MyoD and approximately 14% of cells showed a bipolar MHC stain (Figure 7). The difference in differentiation rate observed with Myf5 or MyoD staining and MHC stain can be attributed to the experimental variability. The treatment with curcumin during aggregation decreased the number of cells expressing Myf5 or MyoD by 66-82%

(Figure 7C and 7D). The number of skeletal myocytes stained with MHC was decreased by roughly 70% with curcumin treatment during aggregation (Figure 7E). Curcumin treatment inhibited the expression of MyoD and Myf5 proteins which confirmed that the Western blot (data not shown) results were not valid.

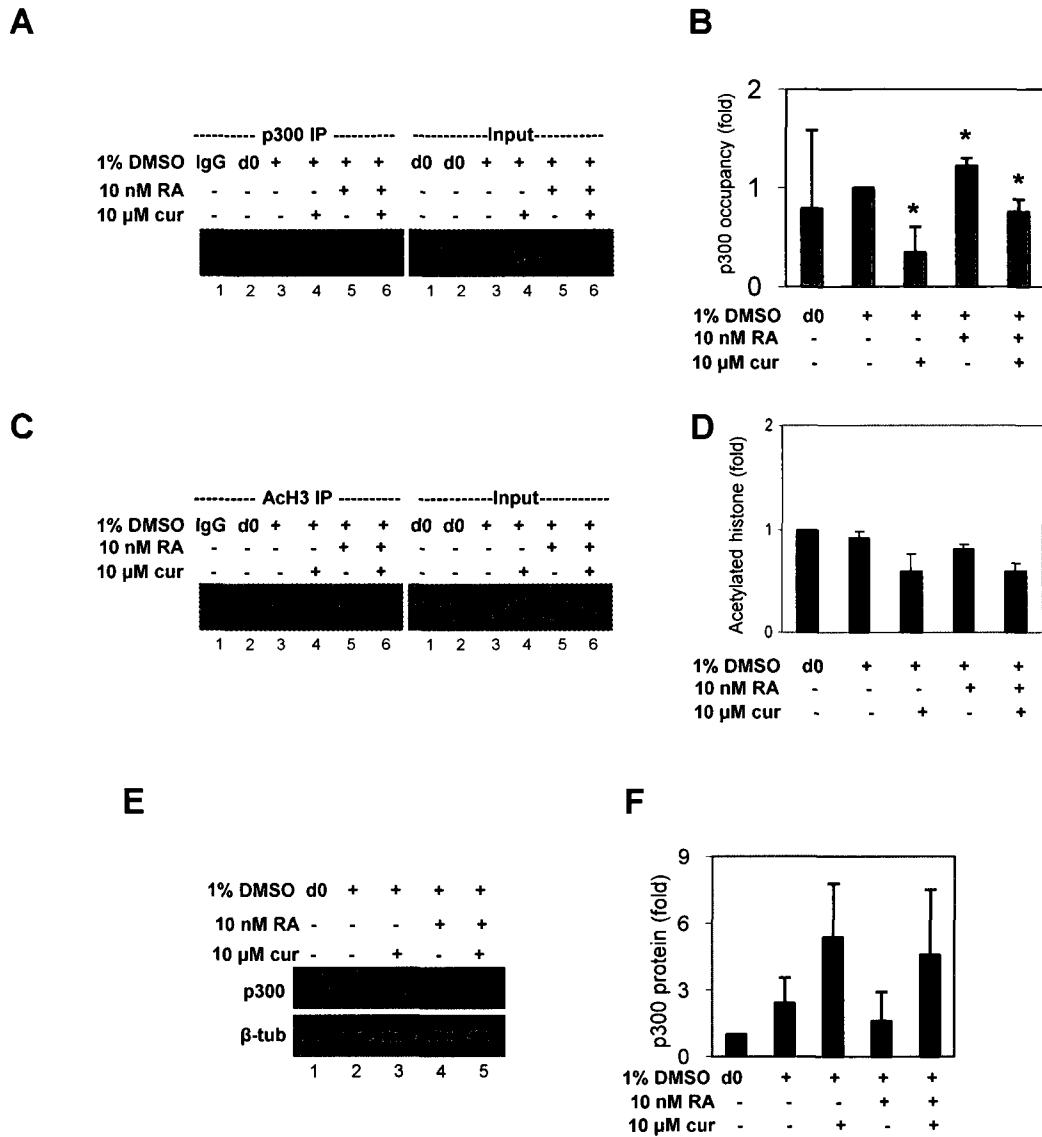
There is a discrepancy in the fold induction of Myf5 and MyoD expression with RA treatment observed with Real Time RT-PCR and with immunofluorescence. The cells treated with 1% DMSO and 10 nM RA showed an increase in Myf5 or MyoD mRNA on day 9 less than 2 fold (Figure 8 and data not shown) compared to cells treated with 1% DMSO alone. This is in discrepancy with 2-3 fold increase in number of Myf5, MyoD expressing cells with RA treatment (Figures 7). We observed an increase in levels of reference gene  $\beta$ -tubulin4 with RA treatment. Because the expression of the reference gene was increased with RA treatment, the correction of Myf5 and MyoD expression with reference gene decreased apparent levels of Myf5 and MyoD expression more than it should have. As a result we observed less than 2 fold induction of Myf5 and MyoD mRNA levels with RA treatment. No change in  $\beta$ -tubulin4 expression with curcumin treatment was observed. Reference gene, such as GAPDH, which does not change with chemical treatments and with differentiation should have been used.

Curcumin decreased expression of Myf5 and MyoD proteins and mRNA. Therefore curcumin treatment during the aggregation of P19 cells is sufficient to inhibit the commitment to the skeletal muscle differentiation. Since curcumin is an inhibitor of p300 HAT activity, it is possible that the decrease in *Myf5* and *MyoD* expression is due to lack of p300 HAT activity. This would agree with the findings of Roth et al (2003), that p300 HAT activity is required upstream of *Myf5* and *MyoD* expression in mouse

embryonic stem cells. However, more direct proof is necessary to show that in our system loss of p300 HAT activity is causing a decrease in *Myf5* and *MyoD* expression. As discussed earlier, curcumin may act also through inhibition of  $\beta$ -catenin activity. Furthermore, curcumin has an effect on many other proteins as well (Aggarwal et al. 2007), which could also create an inhibitory effect on skeletal myogenesis and expression of *Myf5* and *MyoD*.

### **p300 is present at the early epaxial enhancer of *Myf5***

Having shown that curcumin inhibits commitment to skeletal myogenesis which provides supporting evidence that the HAT activity of p300 may be needed for commitment, we next wanted to determine how p300 HAT activity may be involved in regulation of expression of *Myf5*. Roth et al (2003) have shown that p300 HAT activity is not required for the expression of *Pax3* which is expressed in muscle precursor cells (Borycki et al. 1999). We hypothesized that p300 is involved directly in regulation of *Myf5* expression. *Myf5* has a rather complex set of enhancers which regulate its spatio-temporal expression (Carvajal et al. 2001; Hadchouel et al. 2003; Zammit et al. 2004; Teboul et al. 2002; Hadchouel et al. 2000; Zweigerdt, Braun, and Arnold 1997; Summerbell et al. 2000; Patapoutian et al. 1993; Carvajal et al. 2001). Because curcumin exerts its effect during aggregation of P19 cells we investigated the potential role of p300 HAT activity in the regulation of the early expression of *Myf5*. The earliest expression of *Myf5* in the mouse is regulated by the early epaxial enhancer (Summerbell et al. 2000; Teboul et al. 2002). This enhancer is also regulated by Wnt signaling through  $\beta$ -catenin (Borello et al. 2006). Since p300 is one of the coactivators of  $\beta$ -catenin this presents a possibility that p300 is involved in the regulation of the early epaxial enhancer through interaction with  $\beta$ -catenin. To test the hypothesis that p300 is present at the early epaxial enhancer of *Myf5*, a chromatin immunoprecipitation (ChIP) assay was performed. We assessed the occupancy of p300 at the early epaxial enhancer in undifferentiated cells (day 0) and at the end of aggregation (day 4). Because curcumin treatment during aggregation only was sufficient to inhibit expression of *Myf5* (Figure 8) we investigated if p300 is involved in regulation of EE enhancer at the end of aggregation (day 4). p300



**Figure 9. Curcumin decreases p300 occupancy and histone acetylation at the early epaxial enhancer of *Myf5*.** Cells were aggregated and induced to differentiate with 1% DMSO and 10 nM RA. Cells were also treated with 10  $\mu$ M curcumin during the aggregation. Occupancy of p300 at early epaxial enhancer of *Myf5* at the end of aggregation (day4) was determined by ChIP assay (A) and quantified (B). The levels of histone acetylation at early epaxial enhancer of *Myf5* at the end of aggregation (day 4) were also determined by ChIP assay (C) and quantified (D). Error bars represent standard deviation of 2 (D) or 3 (B) independent experiments (\*  $P < 0.05$ ). (E) Protein expression of p300 was determined by Western blotting on day 4 and (F) and five independent experiments were quantified.

was found to be present at the early epaxial enhancer at a similar level in both undifferentiated cells and in aggregated cells treated with 1% DMSO on day 4 (Figure 9A and 9B). Although p300 occupancy in undifferentiated cells (day 0) was slightly lower than in cells treated with 1% DMSO on day 4, this difference was not statistically significant. It is surprising that p300 is present at the epaxial enhancer in both undifferentiated cells in which there is no expression of *Myf5* and in aggregated cells in which *Myf5* is expressed at a low level (Figure 8A). When cells were treated with 10 nM RA occupancy of p300 increased by 1.2 fold compared to cells treated with 1% DMSO alone. p300 occupancy at a promoter or enhancer is commonly associated with transcriptional activation (reviewed by (Vo and Goodman 2001) however, at the epaxial enhancer such a simple correlation cannot be made. The occupancy of p300 at EE enhancer may not be directly associated with increase in expression at these time points. We will further address the relationship between p300 occupancy and expression of *Myf5* in the discussion. However, based on our results we can conclude that p300 is present at the epaxial enhancer of *Myf5*.

### **Curcumin decreases p300 occupancy at early epaxial enhancer**

Treatment with curcumin caused a decrease in p300 occupancy at the early epaxial enhancer in aggregated cells treated with 1% DMSO with or without 10 nM RA (Figure 9A and 9B). It was expected that curcumin would affect only the HAT activity of p300 and not its occupancy at the epaxial enhancer. We investigated the possibility that curcumin caused a decrease in p300 protein levels. Western Blotting was used to determine the levels of p300 in undifferentiated cells and in aggregated cells. The level of p300 in undifferentiated cells (day 0) was roughly the same as in cells on day 4 of differentiation. The curcumin treatment caused an increase in p300 levels on day 4 of differentiation (Figure 9E and 9F). The increase observed, although not statistically significant, may reflect the attempt of cells to compensate for loss of the HAT activity of p300 by increasing levels of p300 protein. More importantly, it shows that the decrease in p300 occupancy at the early epaxial enhancer with curcumin treatment is not due to an overall decrease in p300 levels. Once the factor or complex that p300 binds to at EE enhancer is known it will be possible to shed further light on the mechanism by which curcumin causes a decrease in p300 occupancy at EE enhancer.

We have shown that p300 is present at similar levels at the early epaxial enhancer in both undifferentiated and aggregated P19 cells. In aggregated cells there is a low induction of *Myf5* expression and in undifferentiated cells there is none. Therefore, the occupancy of p300 at EE enhancer may not be directly associated with an increase in expression at these time points. Others have however shown that the HAT activity of p300 is necessary for expression of *Myf5* (Roth et al. 2003) and our results support this finding. The HAT activity of p300 may perform two functions at EE enhancer:

acetylation of histones and acetylation of transcriptional factors. Since the factors that p300 interacts with at early epaxial enhancer are not known we investigated the effect of loss of p300 occupancy due to curcumin treatment on histone acetylation. Histone acetylation at EE enhancer in undifferentiated and aggregated cells was assessed by a ChIP assay. The levels of acetylated histone H3 at EE enhancer were found to be the same in undifferentiated (day 0) and aggregated cells (day 4) induced with 1% DMSO with or without 10 nM RA (Figure 9C and 9D). An increase in histone acetylation is associated with activation of gene expression (Eberharter and Becker 2002). No change in histone acetylation is observed as a result of aggregation and treatment with 1% DMSO and RA. The curcumin treatment however, caused a hypoacetylation of histones at EE enhancer (Figure 9C and 9D). We have performed only two experimental repeats of ChIP assay probing for histone acetylation at EE enhancer, thus results presented (Figure 9C and 9D) are still preliminary and we can not make firm conclusions. However the data still indicates that there is a correlation between loss of p300 occupancy and decrease of histone acetylation. Therefore the function of p300 at EE enhancer may be acetylation of histones.

Since occupancy of p300 and histone acetylation was discrepant with expression of *Myf5* we wanted to verify if the epaxial enhancer of *Myf5* is active during aggregation stage in P19 cells. For this purpose we attempted to transiently transfect a reporter construct harboring the early epaxial enhancer with a tyrosine kinase promoter into P19 cells which were subsequently aggregated with 1% DMSO in the presence or absence of 10 nM RA. We also wanted to test if p300 can coactivate transcription from this construct. We transfected undifferentiated cells with epaxial enhancer reporter construct,

vectors expressing constitutively active  $\beta$ -catenin and p300. Unfortunately due to technical difficulties and time constraints we were unable to complete these experiments and we can not conclude whether the epaxial enhancer is active during aggregation of P19 cells nor can we confirm that p300 can activate transcription from the early epaxial enhancer.

## DISCUSSION

### Requirement of p300 HAT activity for myogenesis in the P19 cells

Our results suggest a new function of p300 in the regulation of *Myf5* expression. We have shown the presence of p300 at the early epaxial enhancer where its function may be maintenance of histone acetylation. We have used the P19 cell system to study the role of p300 in *Myf5* expression. This system was proven useful for our purpose of studying skeletal muscle differentiation and regulation of *Myf5* expression. The effects of inactivating the HAT activity of p300 by a mutation in E14 mouse embryonic stem cells (Roth et al. 2003) were very closely recapitulated by chemical inhibition of p300 HAT activity with curcumin. Curcumin treatment inhibited the differentiation of skeletal muscle and the expression of *Myf5* and *MyoD*. Because curcumin inhibits the HAT activity of p300 we speculated that the decrease in skeletal myogenesis and expression of *Myf5* and *MyoD* is due to loss of HAT activity of p300. Roth et al. (2003) have shown that the HAT activity of p300, but not CBP, is required for expression of *Myf5* and *MyoD*. This is also why we speculated that inhibition of HAT activity of p300 is the mechanism by which curcumin inhibits skeletal myogenesis and expression of *Myf5* and *MyoD*. However, curcumin also inhibits the HAT activity of CBP and the activity and expression of a wide range of transcriptional factors, kinases, signaling molecules and enzymes (Aggarwal et al. 2007). For this reason we can not claim with certainty that it is the loss of HAT activity of p300 that caused a decrease in skeletal myogenesis and expression of *Myf5* and *MyoD*, only that this is a possible mechanism.

### **Role of p300 at the early epaxial enhancer of *Myf5***

We have shown that p300 is present at the early epaxial enhancer. It occupies the EE enhancer in both undifferentiated and aggregated cells (day 4) (Figure 9A). It is unusual that p300 is found at the early epaxial enhancer in undifferentiated cells when there is no transcription of *Myf5*, since p300 occupancy is typically associated with activation of transcription (reviewed by Vo and Goodman 2001). Occupancy of p300 at the EE enhancer increases in aggregated cells (day 4) treated with 1% DMSO compared to undifferentiated cell (day 0) however, this increase is not significant (Figure 9). The same treatment induces moderate increase in mRNA levels of *Myf5* in aggregated cells (day 4) (Figure 8). There is a small increase in p300 occupancy with RA treatment compared to treatment with DMSO alone (Figure 9). We were also able to show that RA enhances skeletal muscle differentiation in P19 cells. However, on day 4 we observed only a marginal increase in *Myf5* expression with RA treatment. The occupancy of p300 at EE follows the general trend of *Myf5* expression. However, the changes in p300 occupancy are not significant enough to prove a clear increase in p300 occupancy with expression of *Myf5* (day 4 compared to day 0), or to indicate that there is activation of EE enhancer. Furthermore we cannot implicate directly p300 in regulation of *Myf5* expression. Because the increase in p300 occupancy on day 4 is not clear cut it may represent an onset of activation of EE enhancer.

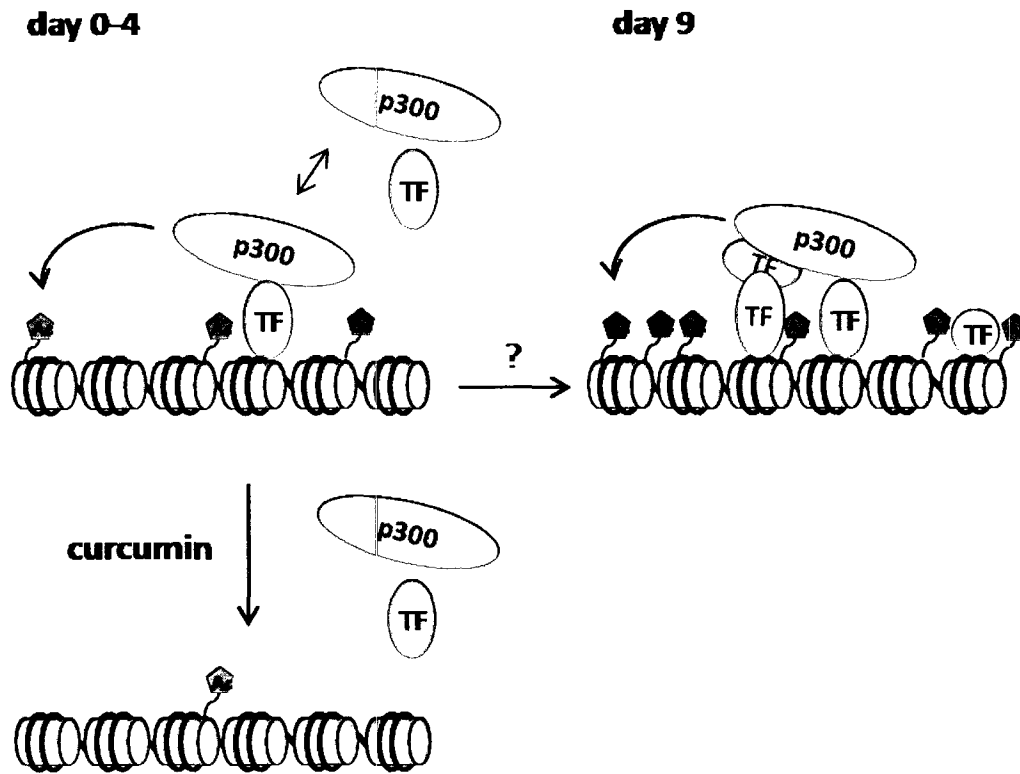
If, however, EE enhancer is only beginning to be activated on day 4 then, a different enhancer may be driving expression of *Myf5* at that time point. As mentioned earlier the regulatory region of the *Myf5* gene is large and complex and includes numerous enhancer elements (Carvajal et al. 2001; Hadchouel et al. 2003; Zammit et al.

2004; Teboul et al. 2002; Hadchouel et al. 2000; Zweigerdt, Braun, and Arnold 1997; Summerbell et al. 2000; Patapoutian et al. 1993; Carvajal et al. 2001). Although P19 cells provide a good system to study skeletal myogenesis, this system does not recapitulate all of the complexity of regulation observed in a mouse embryo. It is not known which enhancers or how many, drive the *Myf5* expression in P19 cells. A single enhancer with proximal promoter may be sufficient to drive *Myf5* expression. Therefore, it is possible that an enhancer other than EE is driving expression of *Myf5* on day 4. Furthermore, if EE enhancer is not active at these time points then we expect the observed p300 occupancy to represent a relatively low rate of association.

Acetylation of histone H3 at early epaxial enhancer is at the same level in undifferentiated cells (day 0) and in cells aggregated with 1% DMSO with or without RA (Figure 9). Hyperacetylation of histones is a common landmark of activation (Eberharter and Becker 2002) and we expected to see an increase in histone acetylation on day 4 much like we expected an increase in p300 recruitment. However, the acetylation of histones remained on day 4 at the same basal level observed on day 0 (Figure 9). Lack of increase in histone acetylation is observed possibly because the early epaxial enhancer may not be active on day 4 and transcription of *Myf5* may be driven from another enhancer element. However, if there is no activation of the early epaxial enhancer then what is the purpose of p300 recruitment to it? Treatment with curcumin caused dissociation of p300 from the early epaxial enhancer and hypoacetylation of histones (Figure 9). These results suggest that p300 may be necessary for maintenance of basal level of histone acetylation. Recruitment of p300 to EE enhancer in undifferentiated cells, when *Myf5* is not expressed (Figure 8 and 9), may suggest a role of p300 in repression.

There are a few known cases where p300 is involved in repression (Ma et al. 2005; Sankar et al. 2008). However, p300 appears to be acetylating histones at EE enhancer which is typical of activation. Also, a knockout of p300 or loss of its HAT activity in embryonal stem cells leads to a loss of *Myf5* expression (Roth et al. 2003), which indicates that p300 is needed for *Myf5* expression rather than repression. Dissociation of p300 from the EE enhancer and histone hypoacetylation (Figure 9) are correlated with a decrease in *Myf5* transcription (Figure 8). We previously stated that the EE enhancer may not be active on day 4 so repression of the EE enhancer by hypoacetylation is possibly not the cause of the decrease in *Myf5* expression. However, decrease of *Myf5* expression on day 4 with curcumin treatment indicates that curcumin inhibits activity of another enhancer. This may occur through inhibition of p300 HAT activity, but also through inhibition of other factors one of which could be  $\beta$ -catenin.

In summary our results suggest that p300 is present at the early epaxial enhancer at similar levels for the first four days of differentiation. We also suspect that the enhancer may not be driving transcription at this time and because of this we speculate that association of p300 with EE enhancer is at a low rate. However, increase in p300 occupancy with RA treatment as opposed to DMSO alone, may reflect an onset of activation of the EE enhancer. Curcumin causes a decrease in both p300 occupancy and histone acetylation which indicates that p300 may be acetylating the histones (Figure 10). We speculate that at a later time point (day 5-9) the early epaxial enhancer becomes active and drives transcription of *Myf5* in P19 cells. If this is the case we expect that there would be a significant increase in p300 occupancy and histone acetylation at the EE



**Figure 10. Model of p300 recruitment and histone acetylation at early epaxial enhancer.** In undifferentiated cells (day 0) and aggregated cells (day4), histone acetylation is at a similar level. At these time points (day 0-4), p300 is recruited to EE enhancer possibly at a low rate and may be maintaining histone acetylation. Curcumin treatment causes a decrease in p300 occupancy at the EE enhancer and a decrease in histone acetylation. At the end of the differentiation process (day 9) we propose that more activating transcriptional factors may be recruited to EE, p300 occupancy and acetylation level of histones may also increase.

enhancer. Also, at a later time point (day 5-9), the role of p300 may not be limited to histone acetylation but may include acetylation of transcriptional factors, scaffolding and bridging functions as well. In a developing mouse embryo the early epaxial enhancer drives the earliest expression of *Myf5* (Summerbell et al. 2000; Teboul et al. 2002). The fact that p300 is present at the EE enhancer in P19 cells indicates that p300 may be also recruited to this enhancer in a mouse embryo and may play a role in transcriptional regulation of *Myf5*.

The epaxial enhancer of *Myf5* is also regulated by  $\beta$ -catenin/LEF and Gli factors.  $\beta$ -catenin and *Gli1* can synergistically activate transcription from the extended epaxial enhancer, which includes three  $\beta$ -catenin/LEF sites immediately upstream from epaxial enhancer (Teboul, Summerbell, and Rigby 2003; Gustafsson et al. 2002; Borello et al. 2006). With p300 being a known coactivator of  $\beta$ -catenin (Hecht et al. 2000) it is natural to assume that they might interact at epaxial enhancer as well. However, it remains to be determined if the two occupy the extended epaxial enhancer at the same time during skeletal muscle differentiation, and how p300 affects the ability of  $\beta$ -catenin alone or  $\beta$ -catenin together with Gli factors to activate transcription from extended epaxial enhancer.

#### **p300 and regulation of *Myf5* expression**

The regulation of *Myf5* expression during mouse embryogenesis is a rather complex process. An array of signals and transcription factors are necessary for expression of *Myf5* (Punch, Jones, and Rudnicki 2009) and a very large number of enhancers ensures expression at specific locations and times in the developing embryo (Carvajal et al. 2001; Hadchouel et al. 2003; Zammit et al. 2004; Teboul et al. 2002; Hadchouel et al. 2000; Zweigerdt, Braun, and Arnold 1997; Summerbell et al. 2000; Patapoutian et al. 1993; Carvajal et al. 2001). Another level of regulation exists as the equilibrium between enhancers, proximal promoter and TRABs (Kassar-Duchossoy et al. 2004). Although quite a lot is known about how *Myf5* expression is regulated, very little is known about the regulation of individual enhancer elements. In fact, there is information only for epaxial enhancer and the limb enhancer, on the transcription factors directly involved in their regulation. The limb enhancer is regulated by Pax3 and Six1/4

(Bajard et al. 2006; Giordani et al. 2007) and the epaxial enhancer, together with immediate upstream sequence, by  $\beta$ -catenin/LEF and Gli factors (Borello et al. 2006; Gustafsson et al. 2002). We were able to show that p300 is also present at the epaxial enhancer, and that its function may be the acetylation of histones. Although we have yet to show that p300 recruited to the early epaxial enhancer is directly involved in transcriptional activation, our findings support this possibility. There is still much to be learned about the regulation of the epaxial enhancer and even more about the regulation of the rest of the enhancers of *Myf5*. In the P19 system we have yet to determine which enhancers drive the transcription of *Myf5* and at what time points. The binding of p300 along with chromatin modifications can be used successfully for prediction of potential enhancer elements (Heintzman et al. 2007). Therefore it is likely that p300 is involved at many of the enhancers of *Myf5*. In fact, the method developed by Heintzman et al. (2007) which uses histone acetylation and methylation pattern, along with p300 recruitment could be used to predict which of the *Myf5* enhancers are active in P19 cells and at the same time which ones recruit p300. Also, the function that p300 plays at the epaxial enhancer is not sufficient to explain the skeletal muscle deficiency and decrease of *Myf5* and *MyoD* expression in p300 HAT deficient mice. Deletion of the epaxial enhancer leads to loss of *Myf5* expression in the dorsomedial lip but not in other locations where *Myf5* is expressed (Teboul et al. 2002). Therefore, p300 may be coactivating transcription of *Myf5* in an embryo or in tissue culture from several different enhancer elements and at different time points. Loss of p300 or its HAT activity then causes inhibition of these enhancer elements and the added effect leads to an overall decrease in *Myf5* transcription.

## **Role of p300 in myogenesis**

We have discussed above the multiple roles that p300 may have in transcription of *Myf5*. In skeletal myogenesis however, the role of p300 does not stop at *Myf5* transcription. p300 is already known to be a coactivator for both MyoD and MEF2C (Sartorelli et al. 1999; Poleskaya et al. 2000; Angelelli et al. 2008; Sartorelli et al. 1997; Ma et al. 2005; Dilworth et al. 2004). Here we show that it may be involved in histone acetylation at the extended epaxial enhancer of *Myf5* and we have also discussed a possible role of p300 at other enhancers of *Myf5*. Transcription of *MyoD* is also affected by loss of HAT activity of p300 (Roth et al. 2003); thus, p300 may also be involved in the direct regulation of transcription of *MyoD*. In a similar manner it is possible that p300 plays a part also in regulation of other genes involved in myogenesis. However, not every gene required for myogenesis is regulated by p300. The expression of *Pax3* appears not to be dependant on p300 or its HAT activity (Roth et al. 2003). The nature of a general coactivator is that it is not specific to only a set of genes or particular DNA motif (reviewed by Vo and Goodman 2001) therefore it is possible that p300 is involved in regulation of many genes which are a part of regulatory cascade which induces skeletal myogenesis. Loss of expression of only a single gene may not cause a significant defect in the final outcome of differentiation. However, we speculate that many regulatory genes are inhibited by the loss of p300 HAT activity and the added effect leads to skeletal muscle deficiency and decrease of *Myf5* and *MyoD* expression. Therefore, the significance of p300 in myogenesis may be the transcriptional coactivation of a number of genes which induce the myogenic program.

### **Redundancy in function of histone acetyltransferases in skeletal myogenesis**

p300 and CBP share a degree of sequence similarity and functional homology (Vo and Goodman 2001). In the case for skeletal muscle differentiation it appears that p300 and CBP do not share the same functions. The HAT activity of p300 but not CBP, is necessary for expression of *Myf5* and *MyoD* (Roth et al. 2003). In development some functions appear to be performed primarily by p300 HAT activity and CBP can only partially substitute for p300. For example, a mutation in the HAT domain of either p300 or CBP causes impaired formation of the cardiovascular system, lung and small intestine but the deficiencies are more severe in p300 HAT mutants (Shikama et al. 2003). However, loss of HAT activity of CBP does not cause skeletal muscle deficiencies which are observed in p300 HAT mutant mice (Roth et al. 2003). Any possible functions performed by the CBP HAT activity may be compensated fully by p300 in the CBP HAT mutant. To fully discern if CBP HAT shares any functional redundancy with p300 HAT in skeletal myogenesis, a double mutation of CBP and p300 HAT domain is required.

p300 is not the only acetyltransferase enzyme involved in skeletal muscle differentiation. PCAF is a histone acetyltransferase also known to interact with MyoD, coactivate MyoD regulated transcription and acetylate it, as does p300 (Puri et al. 1997; Yuan et al. 1996; Sartorelli et al. 1997; Polesskaya et al. 2000), so PCAF and p300 already share some functional redundancy in skeletal muscle differentiation. PCAF null mice show a normal phenotype (Xu et al. 2000; Yamauchi et al. 2000), so PCAF on its own may not be crucial for skeletal muscle differentiation, or loss of PCAF may be fully compensated by p300. Redundancy between these two enzymes may be why the skeletal muscle phenotype is not extremely severe when only one is knocked out. Loss of the

HAT activity of both p300 and PCAF may show any additional overlap in the functions these two enzymes perform in skeletal myogenesis.

### **Curcumin as p300 HAT activity inhibitor**

Curcumin treatment in P19 cells showed the ability to inhibit skeletal myogenesis, MyoD and Myf5 protein and their mRNA expression. Unexpectedly, curcumin treatment also decreased recruitment of p300 to the epaxial enhancer. Curcumin was used primarily for its activity as a p300 HAT inhibitor and it was not expected to interfere with occupancy of p300. As mentioned earlier, curcumin could cause a decrease in p300 occupancy by creating steric hindrance or a conformational change which prevents p300 from binding to the other factors at the early epaxial enhancer. It is also possible that curcumin inhibited recruitment of transcriptional factors through which p300 is bound to the epaxial enhancer. For example, curcumin treatment is known to decrease nuclear localization of  $\beta$ -catenin and activation of Wnt regulated genes (Park et al. 2005). Furthermore curcumin also induces degradation of  $\beta$ -catenin by caspase-3 (Jaiswal et al. 2002).  $\beta$ -catenin is known to interact with p300 and it also regulates epaxial enhancer (Borello et al. 2006; Hecht et al. 2000). It is possible that curcumin decreases occupancy of p300 at the epaxial enhancer by reducing nuclear localization of  $\beta$ -catenin and its binding to the epaxial enhancer. A ChIP assay determining how occupancy of  $\beta$ -catenin and LEF at the epaxial enhancer changes with curcumin treatment could shed some light on this question. This is one example of how curcumin may affect transcription of *Myf5* and p300 occupancy at the epaxial enhancer independent of the loss of p300 HAT activity.

Curcumin is known to induce degradation of p300 by the proteasome (Marcu et al. 2006). However, we found that when cells were treated with curcumin during aggregation, on day 4 p300 protein expression was higher than in untreated cells (Figure 9). Although this increase is not statistically significant it shows that decrease in p300 occupancy is not due to p300 degradation. We speculate that possible increase in p300 expression may be an attempt of the cells to compensate for lack of HAT activity by increasing expression of p300. It is surprising that we did not observe a decrease in p300 protein levels. A difference in concentration and duration of treatment and cell line may account for this. Also curcumin is known to inhibit the activity of 20S core catalytic unit of the proteasome (Dikshit et al. 2006) which could contribute to the slight increase in p300 protein levels. Even though curcumin had an unexpected effect on occupancy of p300, rather than just inhibiting the HAT activity, we were still able to make an inference about the role of p300 HAT activity at the epaxial enhancer. A decrease in p300 occupancy correlates with histone hypoacetylation which indicates that the function of p300 at the epaxial enhancer may be histone acetylation.

### **Indirect effect of RA on *Myf5* transcription**

*All-trans* retinoic acid treatment caused an increase both on skeletal muscle differentiation and on Myf5 and MyoD protein and mRNA expression. The increase in mRNA expression was less than 2 fold whereas increase in skeletal myogenesis was 2-3 fold (Figures 6, 7, 8 and data not shown). Others have found that the RA treatment causes a four fold increase in skeletal myogenesis and more than 50 fold increase in MyoD expression (Kennedy et al. 2009). We did not observe such a large increase in MyoD or

Myf5 mRNA expression because RA treatment increased the expression of the reference gene. Recruitment of p300 to a promoter or enhancer element is generally associated with activation or increase in gene expression (reviewed by Vo and Goodman 2001). Interestingly, the treatment with RA did cause a small increase in p300 occupancy at the epaxial enhancer. However, there was no change in histone acetylation with RA treatment. We speculated that an increase in p300 occupancy on day 4 with RA treatment reflects onset of activation of the EE enhancer. However, because an increase in p300 recruitment presents the very beginning of activation, the increase in histone acetylation may not be pronounced enough to detect. The epaxial enhancer and the recruitment of p300 to it appear not to be under the direct control of RA signaling since only a small increase in p300 occupancy is observed after a four day treatment. A direct activation is expected to induce a faster and more pronounced activation of enhancer. Kennedy et al. (2009) have shown that *Pax3*, *Meox1* and *Wnt3a*, which are expressed prior to commitment, are upregulated by RA treatment. Therefore the effect of RA on expression of MRFs is indirect. Our findings agree with this. Since there is only a small increase in recruitment of p300 to epaxial enhancer with RA treatment after a prolonged treatment, this effect is not likely to be direct.

## CONCLUSION

We set out to study the mechanism by which p300 and its HAT activity regulate expression of *Myf5*. To this end, we chose a P19 cell system which proved to be useful in study of regulation of *Myf5* expression. Curcumin was used to inhibit the HAT activity of p300. Although it was successful at inhibiting skeletal myogenesis and expression of *Myf5* and *MyoD*, the shortcoming of curcumin is its pleiotopic effect. Because it has an effect on a large number of factors, the effect of curcumin treatment on skeletal myogenesis cannot be attributed with certainty to loss of p300 HAT activity. In further studies of the role of p300 HAT activity in *Myf5* expression a more specific inhibitor should be used, or the HAT activity of p300 could be inhibited by mutation.

We hypothesized that p300 acetylates the histones at the enhancer of *Myf5* and that it is directly involved in regulation of *Myf5* expression. We were able to show that p300 is present at the early epaxial enhancer of *Myf5*. We also showed preliminary data indicating that p300 acetylates histones at the early epaxial enhancer. However, it remains to be determined if p300 directly regulates expression of *Myf5*. In future studies it also needs to be determined if early epaxial enhancer is active during differentiation of P19 cells and at which time points. We also speculated that more than one *Myf5* enhancer may be active and may require p300 in P19 cells. It is necessary to determine if this is true in order to fully understand regulation of *Myf5* transcription in P19 cells. Even more important is the determination of transcriptional factors which regulate activity of *Myf5* enhancers in P19 cells and in the mouse embryo. Our research makes a step further in understanding regulation of one of *Myf5* enhancers. At this point, however, we show that

p300 is present at the epaxial enhancer of *Myf5* and that its role may be histone acetylation.

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## APPENDIX

**Supplementary Table 1. Reagents and suppliers**

<b>Reagent</b>	<b>Cat. No.</b>	<b>Supplier</b>
Minimum Essential Medium $\alpha$	12000-022	Invitrogen-GIBCO
Penicillin/Streptomycin	15140-122	Ivitrogen-GIBCO
Fetal Bovine Serum	A15-701	PAA
Donor Bovine Serum	B11-063	PAA
TTNPB	T3757	Sigma-Aldrich
All- <i>trans</i> Retinoic Acid	R2625	Sigma-Aldrich
Curcumin	C77727	Sigma-Aldrich
Bio-Rad Protein Assay Dye Reagent Concentrate	500-0006	Bio-Rad
Immun-Blot PVDF Membrane	162-0177	Bio-Rad
Western Lightning <sup>TM</sup> Chemiluminescence	NEL105	Perkin Elmer
RNeasy Mini kit	74104	Qiagen
Total RNA Kit I	R 6834-01	Omega
Cycle Pure Kit	D 6492-02	Omega
High Capacity cDNA Reverse Transcription Kit	4358841	ABI
Power SYBR® Green PCR Master Mix	4367659	ABI
Formaldehyde	BDH 0500 1LP	BDH
Complete Protease Inhibitor Cocktail Tablets	11697498001	Roche
Proteinase K	03115879001	Roche
GoTaq® flexi DNA polymerase PCR Kit	PRM8295	Promega
dNTP Mix	PRU1515	Promega
Hoechst 33258 pentahydrate	H-21491	Molecular Probes

**Supplementary Table 2. Antibodies**

Antibody	Cat. No.	Supplier
Anti-p300 (N-15)	sc-584	Santa Cruz
Anti-p300 (C-20)	sc-585	Santa Cruz
Anti-Ac-Histone H3 (Lys 9/14)	sc-8655	Santa Cruz
Anti-MyoD (M-318)	sc-760	Santa Cruz
Anti-Myf5 (C-20)	sc-302	Santa Cruz
Anti-mouse IgG horseradish heroxidase conjugate 2° Ab	NA931	GE Healthcare
Anti-rabbit IgG horseradish peroxidase conjugate 2° Ab	NA9340	GE Healthcare
Alexa Flour®488 goat anti-mouse 2° Ab	A11001	Invitrogen
Alexa Flour®488 goat anti-rabbit 2° Ab	A11008	Invitrogen
Alexa Flor®594 donkey anti-mouse 2° Ab	A21203	Invitrogen

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

Gene	Forward Primer	Reverse Primer
Myf5	CCTGTCTGGTCCCGAAAGAAC	GACGTGATCCGATCCACAATG
MyoD	CCCCGGCGGCAGAATGGCTAC G	GGTCTGGGTCCCTGTTCTGTGT
GAPD H	TCGGTGTGAACGGATTTG	GGTCTCGCTCCTGGAAGA
β-tubb4	TCTCGTCCAGAGCTAGAGCC	TCACTGTCCCCATGATAGTAGGT C

**Supplementary Table 4. Primers used for CHIP assay**

Enhancer	Forward Primer	Reverse Primer
EpExt	AGAAGCGGCACACGTTGTA	TGGAGAAGAGTGAACATCCTTG