C/EBPβ is a Negative Regulator of Skeletal Muscle Differentiation

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ABSTRACT

C/EBPβ is a bZIP transcription factor known to be involved in various physiological processes, including adipogenesis, osteogenesis and liver development. Previous studies in this laboratory revealed an inhibition of myogenesis and reduced myogenic protein expression in 5-azacytidine treated mesenchymal stem cells retrovirally transduced to overexpress C/EBPβ. The goal of this thesis was to evaluate the role of C/EBPβ in myogenic differentiation by overexpression in C2C12 myoblasts and primary myoblasts. We demonstrate reduced MyoD protein expression and subsequent downregulation of myogenic proteins during differentiation following C/EBPβ overexpression. We localized C/EBPβ to the quiescent $Pax7^{+}$ satellite cells associated with the muscle fiber. Upon satellite cell activation, we observed the downregulation of C/EBPβ protein expression prior to MyoD protein expression. Furthermore, the re-expression of C/EBPβ correlated with the loss of MyoD expression later in differentiation. Histological analysis of $C/EBP\beta^{-/-}$ mice revealed smaller fibers and a reduced $Pax7^{+}$ satellite cell population as compared to control animals. In this thesis, we propose that C/EBPβ is a negative regulator of skeletal muscle differentiation by inhibiting the expression of MyoD, thus impairing proper progression through the myogenic program. In addition, we propose a role for C/EBPβ in the maintenance of undifferentiatied satellite cells.

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LIST OF ABBREVIATIONS

5-azaC	5-azacytidine
AID	auto-inhibitory domain
APR	acute phase response
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
C/EBP	CCAAT/Enhancer binding protein
C/EBPβ	CCAAT/Enhancer binding protein beta
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
ChIP	chromatin immunoprecipitation
CRE	cAMP-response element
CREB	cAMP-response element binding
CypB	cyclophilinB
DI	differentiation index
DMEM	Dulbecco's Modified Eagle's Medium
DRR	distal regulatory region
EDL	extensor digitorum longus
FI	fusion index
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GCN5	general control of amino acid 5
HDAC1	histone deacetylase 1
HGF	hepatocyte growth factor
HI-FBS	heat inactivated fetal bovine serum
HI-HS	heat inactivated horse serum
HPV	human papillomavirus
HRP	horse radish peroxidase

IL-6	interleukin-6
LAP	liver activator protein
LIP	live inhibitory protein
MAPK	mitogen-activated protein kinase
MCK	muscle creatine kinase
MEF2	myocyte enhancer factor-2
MHC	myosin heavy chain
MPC	myogenic precursor cell
MRF	muscle regulatory factor
muSP	muscle-resident side population
NEAA	non-essential amino acids
NF-κB	nuclear factor-κB
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + Tween-20
PCAF	p300/CBP-associated factor
PDE	phosphodiesterase
PFA	paraformaldehyde
PIC	PW1^{+}/Pax7^{-} interstitial cells
PKA	protein kinase A
PKC	protein kinase C
PRR	proximal regulatory region
PVDF	polyvinylidene fluoride
RT-PCR	reverse transcription polymerase chain reaction
RSK	ribosomal s6 kinase
S1P	sphingosine-1-phosphate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SUMO	small ubiquitin-related modifier

TA	tibialis anterior
TNF-α	tumor necrosis factor-α
WT	wild type

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INTRODUCTION

Discovery of the satellite cell

Multinucleated myofibers make up the contractile units of skeletal muscle and since their terminally differentiated myonuclei are post-mitotic, growth and repair relies upon a population of quiescent muscle precursor cells, termed satellite cells (Collins, Olsen et al. 2005). These satellite cells are named for their anatomical position on the muscle fiber, occupying a niche between the sarcolemma and the basal lamina (Mauro and Adams 1961; Morgan and Partridge 2003). First discovered in 1961 by Alexander Mauro, he observed in the muscles of the frog that although these cells resided beneath the basal lamina, they were not fused with the muscle fiber itself. Mauro hypothesized that these cells could be "dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of the skeletal muscle fiber when the main multinucleate cell is damaged." (Mauro and Adams 1961). This discovery was the first piece of the puzzle to begin to elucidate the regenerative processes involved in post-natal skeletal muscle growth and repair.

Developmental origin of satellite cells

In the post-natal organism, satellite cells represent less than 5% of the total nuclei in the muscle, a significant decrease from approximately 30-35% at birth (Bischoff 1994). Armand et al. demonstrated using quail-chick chimeras that satellite cells are derived from the somites (Armand, Boutineau et al. 1983). These mesoderm-derived structures are formed along the sides of the neural tube and will form the sclerotome, myotome and dermatome, with the myotome eventually giving rise to the muscles of the

back, limbs and diaphragm (Ordahl and Le Douarin 1992). The dermomyotome represents the dorsal portion of the somites that will later differentiate to become the dermis and muscle (Christ and Ordahl 1995). Recently, it was demonstrated that satellite cells could also be derived from the mouse embryonic dorsal aorta (De Angelis, Berghella et al. 1999). In embryos null for satellite cells markers Pax3 and c-Met, myogenic cells isolated from the limb buds were shown not be derived from the somite, but rather arose from endothelial cells (De Angelis, Berghella et al. 1999). Pax3 null and c-Met null mice do not form limb muscles, due to impaired migration (Dietrich, Abou-Rebyeh et al. 1999) and a failure to delaminate from the dermomyotome, respectively (Tajbakhsh, Rocancourt et al. 1997). In addition, the expression of markers present in both endothelial and satellite cells, such as CD34 (De Angelis, Berghella et al. 1999; Beauchamp, Heslop et al. 2000) and Sca1 (Zammit and Beauchamp 2001; Mitchell, Mills et al. 2005), further support the possibility that endothelial cells and a subset of satellite cells may have a common developmental origin (paraxial mesoderm) or alternatively, that endothelial cells are capable of giving rise to satellite cells.

Molecular identification of satellite cells

Although satellite cells are defined by their anatomical location on the muscle fiber, there existed a need to identify these cells on a molecular level. As satellite cells progress through the myogenic program, from proliferating myoblasts to terminally differentiated myofibers, they express molecular markers that are specific for each stage during differentiation. While satellite cells represent a heterogeneous population, Pax3 (Brzoska, Przewozniak et al. 2009), Pax7 (Seale, Sabourin et al. 2000), CD34

(Beauchamp, Heslop et al. 2000), c-Met (Cornelison and Wold 1997) and M-cadherin (Irintchev, Zeschnigk et al. 1994) are widely accepted and used as molecular markers for the identification of quiescent satellite cells. However, it is important to note that some of these markers are not exclusive to satellite cells and are expressed elsewhere in the body. CD34 is expressed in endothelial cells (De Angelis, Berghella et al. 1999) and Pax7 is expressed in the brain (Rodger, Ziman et al. 1999).

Pax7

Pax7 (paired box protein 7) is a well established and widely used molecular marker for the identification of quiescent and activated satellite cells (Fukada, Uezumi et al. 2007). While Pax7 is expressed by satellite cells and early proliferating myogenic precursors, its expression is subsequently downregulated at the onset of myogenic differentiation (Zammit, Relaix et al. 2006). Pax7 belongs to the family of paired box transcription factors, characterized by paired and homeodomain DNA binding motifs (Kumar, Shadrach et al. 2009). At birth, the $Pax7^{-/-}$ mouse demonstrates comparable satellite cell numbers to wild type mice; however, this population decreases during post-natal development (Relaix, Montarras et al. 2006). Not only was satellite cell proliferation perturbed in the $Pax7^{-/-}$ population, increased expression of caspase-3, indicative of apoptosis, was observed, partially explaining the loss of satellite cells in the Pax7 mutant during post-natal development (Relaix, Montarras et al. 2006). This apparent anti-apoptotic function of Pax7 cannot be compensated for by Pax3, as Pax3-expressing satellite cells from the $Pax7^{-/-}$ mouse still exhibited this striking loss of satellite cells. As $Pax7^{-/-}$ mice begin to develop, they exhibited thinner diaphragms,

reduced muscle mass and reduced fiber diameter, though fiber organization is unaffected (Seale, Sabourin et al. 2000).

The myogenic regulatory factors

The notion of a family of myogenic specific genes responsible for inducing myogenesis was established from experiments evaluating the effect of treatment of the mouse pluripotent mesenchymal cell line, C3H10T1/2, and the mouse embryonic fibroblast cell line, NIH3T3, with the demethylating agent, 5-azacytidine. The widespread and indiscriminate gene activation caused by demethylation resulted in the induction of multinucleated myotubes, among other phenotypes (Taylor and Jones 1979). Furthermore, the transfection of C3H10T1/2 cells with genomic DNA from 5-azacytidine induced myoblasts or from the mouse skeletal muscle cell line, C2C12, resulted in the conversion of C3H10T1/2 fibroblasts to myoblasts (Lassar, Paterson et al. 1986). The authors concluded that the observed frequency of myogenic conversion was representative of a single locus responsible for the commitment of non-myogenic cells into the myogenic lineage, leading to the discovery of the myogenic regulatory factors (MRFs).

The MRFs are a family of basic helix-loop-helix (bHLH) proteins necessary for the transcription of muscle specific genes (Puri and Sartorelli 2000). The members of this family include Myf5, MyoD, myogenin and MRF4 and are sequentially expressed during myogenesis (Seale, Sabourin et al. 2000). It is widely accepted that Myf5 and MyoD are expressed in proliferating myoblasts (Weintraub 1993) and are important for myogenic commitment and determination, whereas myogenin and MRF4 appear during

differentiation and are exclusively expressed in terminally differentiated cells (Megeney and Rudnicki 1995) (Fig. 1). As early markers of myogenesis, Myf5 and MyoD are responsible for regulating withdrawal from the cell cycle and inducing differentiation. Gain of function experiments have demonstrated that the expression of any member of the MRFs has the capacity to induce myogenesis in a variety of non-muscle committed cell lines (Delgado, Huang et al. 2003).

Embryonic myogenesis

In early vertebrate development, masses of paraxial mesoderm, called somites, are formed along the sides of the neural tube and will ultimately give rise to skeletal muscle (Ordahl and Le Douarin 1992). The dermomyotome is the origin of the myogenic precursor cells that will become delaminated from the epithelium of the dermomyotome at the onset of myogenesis (Kalcheim and Ben-Yair 2005). These myogenic precursor cells then migrate under the dermomyotome and differentiate to become the post-mitotic skeletal muscle of the developing embryo (Cossu, Tajbakhsh et al. 1996).

Expression of satellite cell markers Pax3 and Pax7 can be detected in the dermomyotome as well as in the myotome, with Pax3 persisting in the dermomyotome to give rise to muscle progenitor cells (Jostes, Walther et al. 1990; Bober, Franz et al. 1994). Pax3 has been demonstrated to be necessary for the migration of satellite cells from the somite to the developing limb buds (Crist, Montarras et al. 2009), as mice that are homozygous null for Pax3 fail to form limb muscle and do not survive to term (Daston, Lamar et al. 1996). Furthermore, the transplantation of lateral somite halves from a $Pax3^{-/-}$ mouse embryo into the forelimb of a chick host revealed efficient differentiation of

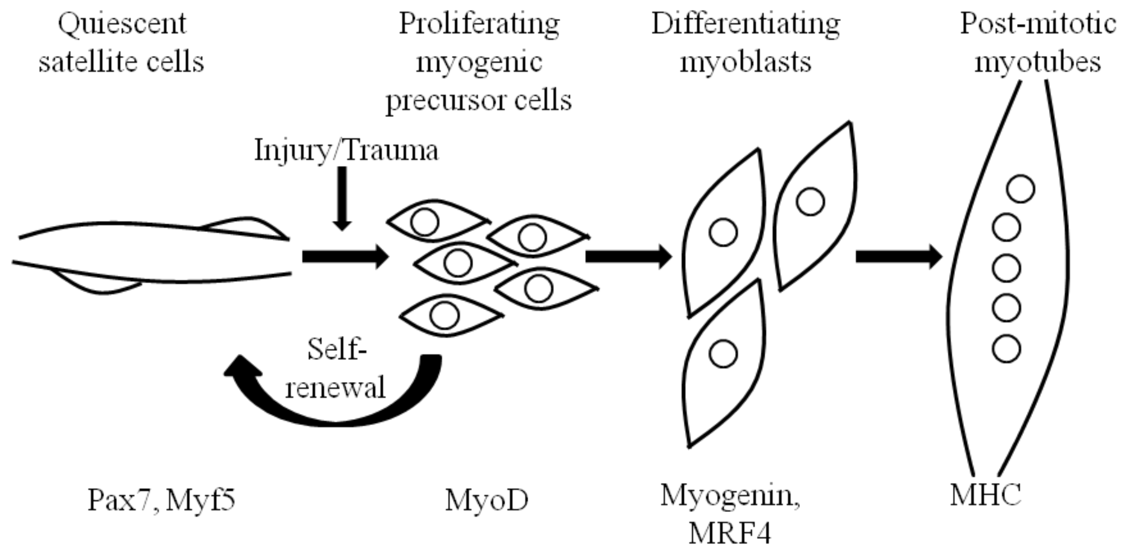


Figure 1. Myogenic regulatory factor expression during myogenesis.

Quiescent satellite cells express markers Pax7 and Myf5 and are activated upon injury or trauma to the skeletal muscle. Activated satellite cells enter the cell cycle and begin to proliferate as myogenic precursor cells (MPC) and express MyoD. A subset of cells will not proceed down the myogenic pathway and lose expression of MyoD to undergo self-renewal to re-populate the satellite cell niche. MPCs differentiate to form myoblasts which express myogenin and MRF4. The fusion of myoblasts into terminally differentiated, multi-nucleated myotubes expressing MHC is the culmination of the myogenic program, giving rise to mature myocytes capable of repairing muscle damage.

limb muscle precursors, suggesting that while Pax3 is required for migration, it is dispensable for the differentiation of limb muscle precursors (Daston, Lamar et al. 1996).

The myogenic precursor cells which migrate under the dermomyotome to give rise to the skeletal muscle of the myotome also express Myf5 and MRF4 (Buckingham 2006). Myf5 is expressed as early as 8 days post coitus (p.c.) in the dermomyotome and by 11 days p.c., Myf5 transcript levels begin to decrease and are no longer detectable by 16 days p.c. (Ott, Bober et al. 1991). In contrast, MyoD and myogenin are expressed together at about 11.5 days p.c. and persist at high levels in the limb muscles until birth (Sassoon, Lyons et al. 1989). In support of the roles of both Myf5 and MyoD in early myogenesis, mice that are deficient for both Myf5 and MyoD display a complete lack of myoblasts and differentiated skeletal muscle and these mice do not survive (Rudnicki, Schnegelsberg et al. 1993).

Post-natal myogenesis

Post-natal muscle growth and repair relies upon the satellite cells which are capable of being activated to proliferate and differentiate to give rise to new fibers (Morgan and Partridge 2003; Collins, Olsen et al. 2005). In addition to their capacity for terminal differentiation, these satellite cells also possess the ability to self-renew and a subset of cells will retain expression of satellite cell markers to re-populate the satellite cell niche (Olguin and Olwin 2004; Zammit, Relaix et al. 2006). Recent studies have suggested that the satellite cell population is composed of committed myogenic progenitors and satellite stem cells, based on the presence or absence of Myf5 expression, respectively (Kuang, Kuroda et al. 2007). Kuang et al. demonstrated the importance of

the satellite cell niche in the self-renewal and commitment of $Pax7^{+}/Myf5^{-}$ cells, which they determined to account for about 10% of the satellite cell population and represent what they termed, satellite stem cells (Kuang, Kuroda et al. 2007). Through apical-basal oriented divisions, these $Pax7^{+}/Myf5^{-}$ cells asymmetrically divide to give rise to $Pax7^{+}/Myf5^{-}$ and $Pax7^{+}/Myf5^{+}$ cells, where the newly divided daughter cell attached to the basal lamina remains $Myf5^{-}$ and contribute to the satellite stem cell population, and the other daughter cell on the apical side upregulates Myf5 expression to become a committed myogenic progenitor (Kuang, Kuroda et al. 2007). Furthermore, the daughter cells that remained $Pax7^{+}/Myf5^{-}$ after the asymmetrical division can again be activated to undergo this self-renewal and myogenic commitment process (Kuang, Kuroda et al. 2007). In addition to this pathway for self-renewal, $MyoD^{+}$ myoblasts can also exit the differentiation program though the downregulation of MyoD expression, which is estimated to occur in up to 50% of activated cells (Yoshida, Yoshida et al. 1998). These newly $MyoD^{-}$ cells will re-populate the satellite cells niche. These so-termed, reserve cells, possess characteristics similar to satellite cells and represent a mononucleated, predominantly quiescent population which are $MyoD^{-}$ and can be stimulated to proliferate and differentiate in high and low serum conditions, respectively (Yoshida, Yoshida et al. 1998). In accordance, it has been demonstrated using C2C12 myoblasts that the generation of reserve cells requires the downregulation of MyoD (Yoshida, Yoshida et al. 1998; Stuelsatz, Pouzoulet et al. 2010).

Although it is generally accepted that satellite cells represent the primary cell population responsible for post-natal growth and repair, there exists other populations of cells that demonstrate the ability to aid in the repair of skeletal muscle (Wagers and

Conboy 2005). Such populations include the muscle-resident side population (muSP) cells (Asakura, Seale et al. 2002) and interstitial $PW1^{+}/Pax7^{-}$ cells (PICs) (Mitchell, Pannerec et al. 2010). There has also been evidence that bone marrow cells can contribute to myofiber repair (Fukada, Miyagoe-Suzuki et al. 2002; LaBarge and Blau 2002), although at a much lower frequency.

Satellite cell activation

Upon injury or trauma to the muscle, normally quiescent satellite cells are activated to re-enter the cell cycle and proliferate as myoblasts which then terminally differentiate to generate new myofibers, fusing to pre-existing muscle fibers or to each other, depending on the severity of the injury (Charge and Rudnicki 2004; Kuang, Kuroda et al. 2007). An activation signal or trigger is therefore required for the transition from quiescence to proliferation. Nagata et al. demonstrated the involvement of sphingosine-1-phosphate (S1P) in the activation of quiescent satellite cells and for efficient regeneration (Nagata, Partridge et al. 2006). S1P is mitogenic and has also been shown to be involved in calcium homeostasis and angiogenesis and is produced through the phosporylation of sphingosine (Spiegel and Milstien 2003). Activation of satellite cells is also induced by mechanical stretching of the muscle fibers and this stretch promotes the satellite cell secretion of hepatocyte growth factor (HGF), which binds the c-Met receptor on satellite cells to promote their activation (Tatsumi, Liu et al. 2006; Wozniak and Anderson 2007). Tatsumi et al. further demonstrated that released HGF could freely enter the circulation and increase HGF in adjacent, unstretched muscles (Tatsumi, Liu et al. 2006). Nitric oxide, produced by nitric oxide synthase in the muscle

fiber, is another chemical signal that was demonstrated to play a role in the activation of satellite cells by triggering the release of HGF, as the inhibition of its production blocked satellite cell activation (Anderson and Pilipowicz 2002).

Physiological functions of the MRFs

The use of knockout models for each of the MRFs have helped in the elucidation of their individual roles during post-natal muscle growth and regeneration, functional redundancies and the hierarchical relationships that exists among them.

Myf5

With the generation of a Myf5$^{loxP/loxP}$ mouse line (Kassar-Duchossoy, Gayraud-Morel et al. 2004), any phenotypic effects caused by null mutations for the Myf5 gene could be studied, as previous attempts to characterize the Myf5$^{-/-}$ mouse failed due to the perinatal lethality caused by the Myf5 null alleles (Tajbakhsh, Rocancourt et al. 1996). With the Myf5$^{loxP/loxP}$ mouse, conditional mutants could be generated through breeding with a ubiquitously expressing PGK-Crem transgenic, where Cre recombinase is driven by the PGK-1 promoter and allowed for the investigation of adult mice lacking Myf5 (Lallemand, Luria et al. 1998). Although at birth Myf5$^{-/-}$ mice do not display any significant differences as compared to wild type mice, at around 7 months, Myf5$^{-/-}$ mice begin to exhibit subtle myopathy, characterized by increased fibrosis, increased fiber diameter and range of diameter, and an increase in central nucleation (Gayraud-Morel, Chretien et al. 2007). To assess the regeneration potential of Myf5$^{-/-}$ mice, injury was induced by freezing or by using cardiotoxin. These mice displayed accumulation of adipocytes at the site of regeneration, increased fibrosis, and a delay in muscle

differentiation as determined by the lack of myogenin expression, as compared to the controls (Gayraud-Morel, Chretien et al. 2007). Additionally, there were no significant differences observed in satellite cell numbers between Myf5$^{-/-}$ and wild type mice, and only when cultured on gelatin coated dishes was there a significant difference observed in proliferation rate (Gayraud-Morel, Chretien et al. 2007). Myf5$^{-/-}$ satellite cells exhibited a reduced proliferation rate, which was more pronounced at days 5-6 in culture; however, this difference in proliferation rate as compared to control cultures became negligible at later stages in culture. This reduced proliferation rate could be an explanation of the delayed skeletal muscle regeneration after injury observed in the Myf5$^{-/-}$ model (Ustanina, Carvajal et al. 2007).

MyoD

At birth, mice that are MyoD deficient do not display any abnormal phenotype and show comparable levels of Myf5, myogenin and MRF4 transcript as compared to wild type mice (Rudnicki, Braun et al. 1992). As they reach adulthood, mice that are MyoD$^{-/-}$ demonstrated severely impaired muscle regeneration after injury (Megeney, Kablar et al. 1996) and MyoD$^{-/-}$ satellite cells, although capable of being activated, do not progress normally throughout the myogenic program (Cornelison, Olwin et al. 2000). In culture, satellite cells derived from MyoD$^{-/-}$ myofibers exhibited very few multinucleated myotubes as compared to wild type satellite cells after one week in differentiation medium (Cornelison, Olwin et al. 2000). Furthermore, the differentiation frequency of MyoD$^{-/-}$ satellite cells, as defined by the expression of Myosin Heavy Chain (MHC) was determined to be eight-fold lower compared to wild type satellite cells (Cornelison, Olwin et al. 2000). Sabourin et al. demonstrated that MyoD$^{-/-}$ satellite cells continued to

proliferate, even under conditions that would normally induce terminal differentiation in wild type satellite cells (Sabourin, Girgis-Gabardo et al. 1999). This enhanced proliferative potential and failure to withdraw from the cell cycle observed in the absence of MyoD suggested that these MyoD$^{-/-}$ satellite cells display a propensity to undergo self-renewal rather than advancing through the differentiation program (Megeney et al, 1996). Furthermore, Asakura et al. demonstrated that not only did transplanted MyoD$^{-/-}$ satellite cells have higher engraftment rates but also participated in re-populating the satellite cell niche, again supporting previous results of the self-renewal properties of MyoD$^{-/-}$ satellite cells (Asakura, Hirai et al. 2007). Compared to wild type controls, MyoD$^{-/-}$ satellite cells also exhibited higher resistance to apoptosis and an upregulation in the expression of stem cell markers, Sca-1 and CD34 (Asakura, Hirai et al. 2007). These results are in accordance with the notion that satellite cells lacking MyoD represent a population of cells that possess more stem cell characteristics than wild type satellite cells. In studies evaluating the effect of forced MyoD expression, it was observed that ectopic MyoD induced growth arrest as determined by reduced colony formation (Crescenzi, Fleming et al. 1990).

Myogenin

The deletion of myogenin in the germline resulted in neonatal death (Hasty, Bradley et al. 1993), which necessitated the generation of a conditional mutant which could be controlled temporally. Knapp et al. used two different Cre recombinase expressing transgenic lines to evaluate the effect of loss of myogenin before and after embryonic muscle development. To investigate the loss of myogenin before embryonic muscle development, mice bearing the Myog^{flox} allele were mated to CMV-Cre

transgenic mice, where the Cre recombinase is under the control of the human cytomegalovirus promoter and is active in the zygote (Arango, Lovell-Badge et al. 1999). Deletion of the myogenin gene before embryonic muscle development (E17.5) resulted in a phenotype similar to the myogenin$^{-/-}$ mouse, exhibiting severe muscle deficiencies and an inability to form normal myofibers (Knapp, Davie et al. 2006). Myog^{flox} mice were mated to CAGGCre-ER^{TM} transgenic mice in which the Cre recombinase, under control of a chimeric promoter/enhancer of the cytomegalovirus immediate-early enhancer and chicken β-actin promoter/enhancer, is only activated upon tamoxifen treatment (Hayashi and McMahon 2002). Tamoxifen treatment of myogenin$^{flox/flox}$ mice at E15.5 or E17.5 resulted in viable mice that displayed normal skeletal muscle, though were 30% smaller than control littermates. Knapp et al. concluded that the deletion of myogenin after embryonic muscle development does not appear to have a detrimental effect on post-natal myogenesis, as determined by subtle differences in transcript levels of Myf5, MyoD and MRF4 as compared to control mice (Knapp, Davie et al. 2006). Furthermore, because these MRFs did not appear to be markedly upregulated in the absence of myogenin, this suggested that there is no functional redundancy between myogenin and these MRFs. In support of this, combined null mutations of myogenin with either MyoD or Myf5 demonstrated that neither of these MRFs was able to functionally compensate for the loss of myogenin and that myogenin regulates targets distinct from Myf5 and MyoD (Rawls, Morris et al. 1995).

MRF4

Double mutants lacking MRF4 and either myogenin or MyoD were generated to elucidate any functional overlaps or compensatory roles if these MRFs during

myogenesis. The myogenin/MRF4 double knockout revealed similar number of residual fibers as compared to myogenin$^{-/-}$ mice and in vitro culture of primary myoblasts isolated from myogenin$^{-/-}$/MRF4$^{-/-}$ exhibited comparable differentiation as compared to wild type mice (Rawls, Valdez et al. 1998). This suggested that either Myf5 and/or MyoD are also capable of activating the differentiation program, in the absence of myogenin and MRF4. In contrast, MyoD/MRF4 double knockouts exhibited severe skeletal muscle deficiency, despite the expression of Myf5 and myogenin, suggesting these two MRFs are unable to initiate myogenic differentiation in the absence of MyoD and MRF4 (Rawls, Valdez et al. 1998). Additionally, it suggested a functional overlap between MyoD and MRF4 during myogenesis.

In all vertebrates, MRF4 and Myf5 are linked genes which act as myogenic determination genes (Kassar-Duchossoy, Gayraud-Morel et al. 2004). Three different MRF4$^{-/-}$ models have been generated, with each exhibiting varying degrees of skeletal deformities to viability. The most severe phenotype among the three is characterized by the incomplete formation of the ribs and perinatal lethality (Braun and Arnold 1995), while the least severe phenotype exhibits minor rib defect and full viability (Zhang, Behringer et al. 1995). Interestingly, the phenotype observed with the most severe MRF4 deletion is consistent with the phenotype seen in Myf5$^{-/-}$ homozygotes (Braun and Arnold 1995). All three MRF4 null alleles demonstrated varying levels of Myf5 transcript, with the most severe phenotype having undetectable levels of Myf5 transcript and the least severe phenotype having little to no effect on Myf5 transcript expression (Braun and Arnold 1995; Zhang, Behringer et al. 1995). Yoon et al. demonstrated that these discrepancies in Myf5 expression could be attributed to differences in deleted sequences

to generate the MRF4 knock outs. Furthermore, using compound heterozygotes harbouring MRF4 and Myf5 null alleles, they showed the involvement of cis-regulatory elements within the MRF4 gene that control expression of Myf5 (Yoon, Olson et al. 1997).

Regulation of MyoD expression

MyoD has been described as the master regulator of skeletal muscle myogenesis. Myogenic conversion experiments have shown that the expression of MyoD in non-muscle cell lineages, such as primary fibroblasts, pigment cells, nerves, fat and liver is sufficient to convert these various cell types into skeletal muscle (Weintraub, Tapscott et al. 1989). Ectopic MyoD expression is able to rescue the differentiation deficiency observed in $MyoD^{-/-}$ cells (Sabourin, Girgis-Gabardo et al. 1999) as well as in C2C12 myoblasts to overcome the inhibition of differentiation imposed by Notch signaling (Nofziger, Miyamoto et al. 1999). Furthermore, ectopic expression of MyoD in myogenic cells induces cell cycle arrest, mediated by the upregulation of the cell cycle inhibitor, p21 (Crescenzi, Fleming et al. 1990). Coordinated and controlled regulation of MyoD activity and expression is essential, as all proteins belonging to the bHLH family recognize E-boxes, which occur frequently in the genome (Tapscott 2005). Strict regulation of MyoD activity is vital to ensure the proper temporal and spatial expression is induced to initiate myogenesis.

Transcriptional control

The MyoD promoter contains a core enhancer and two upstream regulatory regions (Fig. 2). A highly conserved core enhancer, found approximately 20 kb upstream

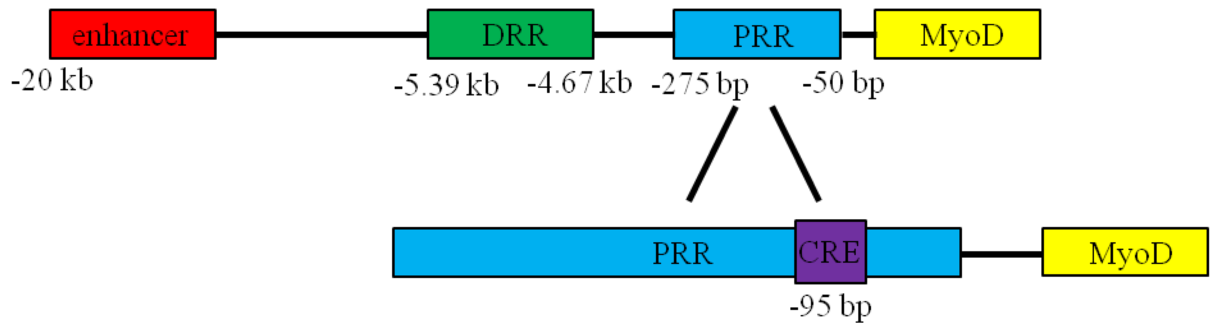


Figure 2. Schematic representation of the mouse MyoD promoter and the upstream regulatory regions.

A highly conserved core enhancer (red) is found at -20 kb upstream of MyoD (yellow) and is required for embryonic myogenesis. A second enhancer element, the distal regulatory region (DRR) (green) is located between -5.39 kb and -4.67 kb of MyoD (yellow) and is required for post-natal myogenesis. The proximal regulatory region (PRR) (blue) is found upstream of MyoD (yellow) between -275 bp and -50 bp and contains a C/EBP response element (CRE) (purple) located -95 bp upstream of MyoD (yellow).

of MyoD, is required for muscle-specific gene transcription in the somites and limb buds during embryonic myogenesis (Goldhamer, Brunk et al. 1995). A second enhancer, the distal regulatory region (DRR), is found between -5.39 kb and -4.67 kb from the transcription start site (Tapscott, Lassar et al. 1992) and is active during adult myogenesis (Chen, Ramachandran et al. 2002). The proximal regulatory region (PRR), located from -275 bp to -50 bp upstream of the transcription start site, contains various elements such as a TATA box, a CCAAT box and a GC-rich region containing a consensus specificity protein 1 (SP1) binding site (Tapscott, Lassar et al. 1992). The C/EBP response element located at -95 bp in the PRR upstream of the MyoD gene was demonstrated by our lab to be bound by C/EBPβ to inhibit MyoD expression in C2C12 myoblasts and undifferentiated primary myoblasts (F. Marchildon and C.St-Louis, unpublished). Interestingly, in mouse muscle, the PRR requires the DRR for its activity, but not so in chich muscle which, interestingly, lacks the C/EBP response element (Tapscott, Lassar et al. 1992).

C3H10T1/2 cells transfected with MyoD cDNA demonstrated increased MyoD mRNA expression and provided evidence of positive autoregulation of the MyoD gene (Thayer, Tapscott et al. 1989). Similarly, transfection with myogenin cDNA was also capable of activating endogenous MyoD expression and vice versa, suggesting a positive cross-regulation loop whereby the expression of these MRFs act synergistically to promote myogenesis (Thayer, Tapscott et al. 1989). Additionally, Zingg et al. demonstrated that the MyoD protein binds to two proximal E-boxes found within the MyoD promoter to positively autoregulate its expression (Zingg, Pedraza-Alva et al. 1994).

Post-transcriptional control

A mechanism of post-transcriptional regulation of MyoD is through nuclear factor κB (NF-κB). Previously, it was shown that tumor necrosis factor-α (TNF-α) induced NF-κB activation could repress myogenesis via the downregulation of MyoD mRNA in C2C12 myoblasts (Guttridge, Mayo et al. 2000). C2C12 cells treated with TNF-α exhibited a decrease in MyoD transcript as compared to untreated cells, despite equivalent rates of transcription, suggesting this regulation of MyoD mRNA is occurring at the post-transcriptional level (Sitcheran, Cogswell et al. 2003).

In C2C12 myoblasts and myotubes, MyoD was shown to have a half-life of approximately 30 minutes to 1 hour (Weintraub, Davis et al. 1991; Sun, Trausch-Azar et al. 2005). Langen et al. demonstrated the half-life of MyoD protein approximately doubled in the first 24 hours after cells were transferred from growth media into differentiation media. Furthermore, this increase in protein was not preceded by an increase in MyoD mRNA, suggesting this increase in MyoD protein is not governed by transcriptional control, but rather from protein stabilization. Phosphorylation by a cyclin-dependent kinase (CDK) on serine200 of MyoD targeted the protein for degradation by the ubiquitin/proteosome pathway (Song, Wang et al. 1998). MyoD stability is improved upon binding to its specific DNA response element and this protects the protein from degradation by inhibiting its conjugation to ubiquitin (Abu Hatoum, Gross-Mesilaty et al. 1998). More recently, it was shown that MyoD protein stability could also be improved upon phosphorylation of tyrosine156 by mitogen-activated protein kinase kinase 1 (MEK1) (Jo, Cho et al. 2011).

CCAAT/Enhancer Binding Proteins (C/EBPs)

CCAAT/Enhancer Binding Proteins (C/EBPs) are a family of transcription factors that regulate proliferation, differentiation, metabolism and inflammation, and can be found in a variety of cell types, such as adipocytes, hematopoietic cells and hepatocytes (Ramji and Foka 2002). All six members of this family demonstrate >90% sequence similarity in their C-terminal domains and a more divergent (<20% sequence similarity) N-terminus (Ramji and Foka 2002).

The C-terminal domain contains the highly conserved basic leucine zipper (bZIP) domain, which is responsible for mediating DNA binding and dimerization (Begay, Smink et al. 2004). The basic region assumes an α-helical structure and its affinity for DNA binding is determined by the specific sequence of basic amino acid residues (Johnson 1993). The leucine zipper motif takes on an α-helical conformation and consists of four or five leucine residues in a heptad repeat, such that a leucine residue is located at every seventh position and align on the same side of the zipper, forming a hydrophobic surface (Ramji and Foka 2002). This hydrophobic surface allows for the dimerization of two leucine zippers which form an inverted Y-shaped structure, where each arm of the Y recognizes and binds to the CCAAT box motif found in gene promoters (Ramji and Foka 2002). Despite a preference for the CCAAT box motif, C/EBP factors have been shown to bind to and activate from very divergent DNA sequences (McKnight 2001).

The N-terminal domain of the different family members is less conserved than the C-terminal domain, except for three short subregions which have been demonstrated to be the activation domains (Ramji and Foka 2002). The N-terminal domain contains conserved motifs that are involved in interaction with basal transcriptional machinery,

transcriptional co-activators and sumoylation (Nerlov and Ziff 1995; Mink, Haenig et al. 1997; Kim, Cantwell et al. 2002). Four of the six family members, C/EBPα, -β, -δ and –γ are intronless, while C/EBPε contains two exons and C/EBPζ contains four exons (Ramji and Foka 2002). All family members can form homodimers and heterodimers with each other, due to the highly conserved bZIP domain (Cappello, Zwergal et al. 2009). However, C/EBP dimerization with C/EBPζ fails to bind DNA at the CCAAT box motif due to the presence of two proline residues in the basic region of C/EBPζ (Ron and Habener 1992) and has been shown to activate gene transcription during times of cellular stress (Ubeda, Wang et al. 1996). This protein, also identified as CHOP-10 or GADD153 (Ramji and Foka 2002), therefore acts as a dominant-negative inhibitor of C/EBP activity.

CCAAT/Enhancer Binding Protein beta (C/EBPβ)

C/EBPβ is widely expressed in various tissues and has been demonstrated to play a vital role in ovarian follicle development, adipogenesis, osteogenesis, hematopoiesis and liver development (Screpanti, Romani et al. 1995; Lee, Williams et al. 1997; Sterneck, Tessarollo et al. 1997; Tang, Otto et al. 2003; Wiper-Bergeron, St-Louis et al. 2007). Due to alternate translation initiation sites, C/EBPβ mRNA can give rise to three different isoforms: the full length 38 kDa liver activator protein (LAP*), the 35 kDa LAP, which bears a truncation of the first 21 amino acids in the N-terminal domain (Kowenz-Leutz and Leutz 1999) and the 20 kDa liver inhibitory protein (LIP), bearing a deletion of the entire N-terminal domain and lacking all of the activation domains (Descombes and Schibler 1991) (Fig. 3). LAP* and LAP contain all three of the activation domains and

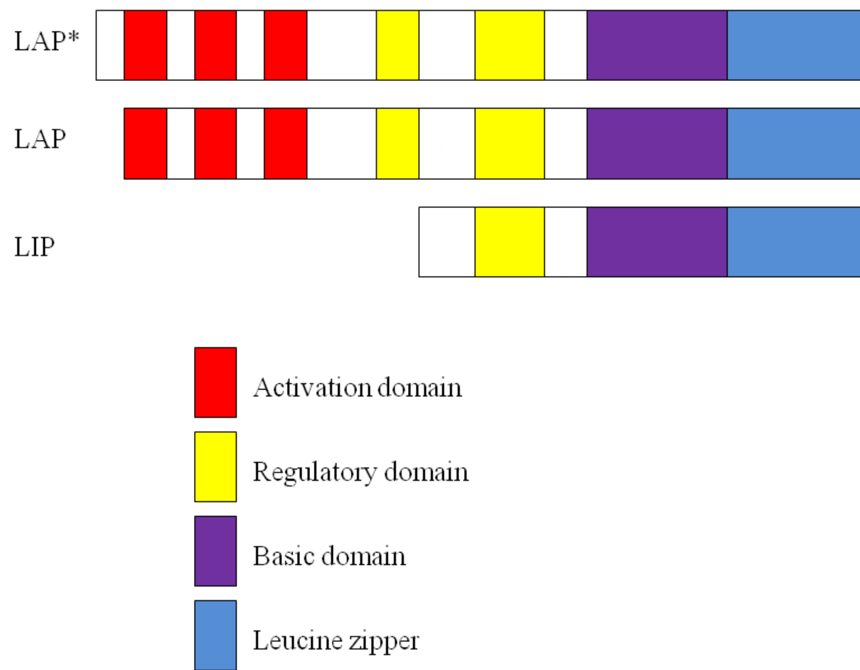


Figure 3. Schematic representation of the three C/EBPβ isoforms generated from alternate translation initiation sites.

The N-terminal transctivation domains (red) are responsible for activating transcription by the recruitment of the components of the basal transcriptional apparatus. The negative regulatory domains (yellow) inhibit the activity of C/EBPβ by suppressing its DNA binding activity and regulating its transcriptional activation.

The leucine zipper (blue) is organized so that the hydrophobic leucines are aligned on one side which can interact with the leucines on a different C/EBP protein, allowing for dimerization. The basic region (purple) mediates binding of this dimer to DNA to stimulate transcription.

The full length 38 kDa (LAP*) isoform contains a fully intact and functional C-terminal bZip domain and N-terminal transctivation domains.

The 35 kDa (LAP) isoform bears a truncation of the first 21 amino acids in the N-terminal and contains fully functional bZip and transactivation domains. These 21 amino acids have been demonstrated to interact with the SWI/SNF chromatin remodeling complex which is recruited to overcome the repressive effects of chromatin to activate gene transcription.

The 20 kDa (LIP) isoform bears a deletion of the N-terminal and does not contain any transactivation domains. With a fully functional C-terminal, LIP is still able to form heterodimers and bind DNA; however, due to the lack of any transactivation domains, it is an inhibitory protein.

are activator proteins, while LIP, though lacking activation domains, is still able to dimerize and bind DNA is a dominant negative regulator of LAP* and LAP (Luedde, Duderstadt et al. 2004). Furthermore, LAP* is the only C/EBPβ isoform that has been demonstrated to interact with the SWI/SNF chromatin-remodeling complex, which is recruited to overcome the repressive effects of chromatin to activate gene transcription (Kowenz-Leutz and Leutz 1999). The LAP and LIP isoforms are the most commonly produced proteins, though the proportion of LAP to LIP varies according to the cellular state (Descombes and Schibler 1991).

Regulation of C/EBPβ expression

The expression of C/EBPβ is regulated at several levels, including transcriptional, translational, post-translational and via protein-protein interactions (Ramji and Foka 2002).

Transcriptional control

The main known transcriptional activator of C/EBPβ expression is cAMP signaling, which results in the activation of the transcription factor, cAMP-response-element-binding protein (CREB) (Niehof, Kubicka et al. 2001). CREB binds to cAMP-response element (CRE) sequences near the TATA box of the C/EBPβ promoter (Niehof, Manns et al. 1997). Given that C/EBPβ can also bind to CRE sequences, C/EBPβ can also autoregulate its own promoter (Niehof, Streetz et al. 2001). Furthermore, during the acute phase response (APR), several inflammatory cytokines, including interleukin-6 (IL-6), have been demonstrated to induce C/EBPβ transcription through the binding of a transcription factor, STAT3, to the CRE-like sequences in the C/EBPβ promoter

(Niehof, Streetz et al. 2001). In adipogenesis, Kruppel-like factor 4 (KLF4), a member of the KLF family of transcription factors characterized by three zinc-finger proteins, has been shown to bind and activate the C/EBPβ promoter on several sites in a highly conserved upstream region (Birsoy, Chen et al. 2008).

Translational control

Regulation of C/EBPβ expression at the translational level arises from the production of the three different isoforms due to alternate initiation start sites and the ratio which the isoforms are produced. C/EBPβ is also a target of the mammalian target of rapamycin (mTOR), a signaling pathway that regulates the translational initiation machinery (Calkhoven, Muller et al. 2002). Inhibition of this signaling pathway resulted in the downregulation of the LIP isoform of C/EBPβ (Jundt, Raetzel et al. 2005). The N-terminal truncated C/EBPβ isoform has been shown to enhance epithelial cell proliferation and has been shown to be highly expressed in breast cancer (Zahnow, Cardiff et al. 2001).

Post-translational modification

Phosphorylation can act to positively or negatively regulate C/EBPβ function and is dependent upon the specific residues that are targeted for phosphorylation. TNF-α has been demonstrated to induce phosphorylation of Ser239 in the nuclear localization sequence of C/EBPβ (Williams, Angerer et al. 1997) and, mediated by a nuclear export receptor CRM1, induces the export of C/EBPβ from the nucleus into the cytoplasm (Buck, Zhang et al. 2001). Alternate mechanisms of phosphorylation-induced repression of C/EBPβ activity include inhibition by insulin (Guo, Cichy et al. 2001) and by Protein Kinase A and C (Trautwein, van der Geer et al. 1994).

The LAP isoform of C/EBPβ has been demonstrated to be an intrinsically repressed protein, due to three short regions in the N-terminal: two highly hydrophobic motifs and an auto-inhibitory domain (AID) (Lee, Miller et al. 2010). In addition, a separate hydrophobic motif was identified in the C-terminal and has been demonstrated to form a hydrophobic core with the N-terminal repressive elements to maintain C/EBPβ in the repressed state (Lee, Miller et al. 2010). This auto-inhibition of C/EBPβ DNA binding can be overcome by oncogenic Ras signaling (Lee, Shuman et al. 2010). In the presence of Ras, cells exhibited a significant increase in C/EBPβ DNA binding and Western analysis revealed phosphorylation at Ser64 (Lee, Miller et al. 2010). Additional mechanisms of phosphorylation-induced activation of C/EBPβ activity include phosphorylation by a Ras/mitogen-activated protein kinase (MAPK) (Nakajima, Kinoshita et al. 1993), cyclic AMP (cAMP) dependent kinase (Metz and Ziff 1991), protein kinase C (PKC) (Trautwein, Caelles et al. 1993), or calcium-dependent kinase (Wegner, Cao et al. 1992). Furthermore, Lee et al. demonstrated that ribosomal s6 kinase (RSK) mediated phosphorylation of Ser273 in the leucine zipper of C/EBPβ overcomes the autorepression of the DNA binding domain. In addition to this, Ser64 phosphorylation has been attributed to promoting the formation of C/EBPβ homodimers, which have been shown to be growth inhibitory (Lee, Shuman et al. 2010).

In addition to phosphorylation, C/EBPβ can also be post-translationally modified by acetylation. First evaluated in histones for its ability to activate transcription, acetylation of non-histone proteins, including transcription factors and α-tubulin, has since been implicated in functions such as modifying DNA binding, protein-protein interactions and protein stability (Kouzarides 2000). It has been demonstrated that

C/EBPβ can be acetylated at multiple lysine residues by p300, p300/CBP-associated factor (PCAF) and general control of amino acid 5 (GCN5) (Cesena, Cardinaux et al. 2007; Wiper-Bergeron, Salem et al. 2007).

Another post-translation modification is sumoylation, the covalent attachment of a small ubiquitin-related modifier (SUMO) (Shiio and Eisenman 2003). Sumoylation of C/EBPs occurs on a lysine residue within the regulatory domain motif, a conserved 5 amino acid sequence found within the inhibitory domain (Kim, Cantwell et al. 2002). Eaton and Sealy demonstrated that full length LAP* can be sumoylated with SUMO-2 and -3 while the LAP isoform cannot, suggesting that an intact N-terminus is required for sumoylation activity (Eaton and Sealy 2003). Furthermore, lysine 173 was identified as the target of sumoylation necessary for C/EBPβ repression of the cyclin D1 promoter (Eaton and Sealy 2003). Berberich-Siebelt et al. demonstrated a role for C/EBPβ in the repression of the c-myc gene and the inhibition of T-cell proliferation, which could be overcome by sumoylation (Berberich-Siebelt, Berberich et al. 2006).

Protein-protein interactions

In addition to the interactions described above, the interaction of C/EBPβ with other C/EBP members or with non-bZIP proteins, such as co-factors, can also modulate its regulation. C/EBPβ can heterodimerize with C/EBPζ, which acts to inhibit C/EBP activity, but has also been demonstrated to activate the transcription of genes involved in cellular stress (Ubeda, Wang et al. 1996). The amino terminus of C/EBPβ can interact with the E1A binding domain of the transcriptional coactivator, p300, and this recruitment of p300 by C/EBPβ promotes the translocation of the transcription

coactivator to specific gene regions to activate gene transcription (Mink, Haenig et al. 1997).

In mice after a partial hepatectomy, histone deacetylase 1 (HDAC1) has been shown to form a complex with C/EBPβ to repress activity from the C/EBPα promoter, thereby promoting liver proliferation (Wang, Salisbury et al. 2008). Furthermore, C/EBPβ is associated with a HDAC1/mSin3A complex to prevent the onset of C/EBPα expression, thereby repressing pre-adipocyte differentiation (Wiper-Bergeron, Wu et al. 2003). Wiper-Bergeron et al. also demonstrated that acetylation of C/EBPβ at K98/101/102 was sufficient to decrease its association with the HDAC1/mSin3A co-repressor complex, thereby increasing the transcription activity of C/EBPβ (Wiper-Bergeron, Wu et al. 2003).

C/EBPβ$^{-/-}$ mice

To fully evaluate the effect of the loss of C/EBPβ *in vivo*, a complete C/EBPβ$^{-/-}$ mouse was generated for study. Originally developed by Valeria Poli, the C/EBPβ$^{-/-}$ mutation was achieved through the replacement of the C-terminal portion of the gene with an MC1-Neo poly(A)+ cassette (Screpanti, Romani et al. 1995). The insertion of this replacement vector effectively inactives all C/EBPβ isoforms (Screpanti, Romani et al. 1995). C/EBPβ$^{-/-}$ mice are produced at sub-Mendelian ratios and experience high rates of perinatal death, attributed to hypoglycemia as a result of failure of the liver to release glucose (Croniger, Trus et al. 1997). In addition, mice harbouring the deletion of the C/EBPβ gene are resistant to diet-induced obesity, exhibit abnormal female reproduction

and are immunocompromised (Sterneck, Tessarollo et al. 1997; Bradley, Zhou et al. 2003; Millward, Heaney et al. 2007).

C/EBPβ in mesenchymal differentiation

Adipogenesis

Adipogenesis is initiated through a transcriptional cascade which culminates in the differentiation of pre-adipocytes into fully mature adipocytes. This cascade begins with the expression of C/EBPβ and C/EBPδ, which in turn activates the expression of PPARγ and C/EBPα, the so-termed "master regulators of adipogenesis" (Millward, Heaney et al. 2007). In vitro and in vivo, adipogenic conversion can be stimulated by the addition of glucocorticoids, insulin and insulin-like growth factor-1, which promotes the initiation of the differentiation cascade, beginning with the expression of C/EBPβ (Fox, Fankell et al. 2006). In addition to its role in the initiation of adipogenesis, C/EBPβ has also been demonstrated to be required for mitotic clonal expansion of pre-adipocytes prior to adipogenic differentiation (Tang, Otto et al. 2003).

In vivo, embryonic fibroblasts isolated from C/EBP$\beta^{-/-}$ mice exhibited less efficient adipogenic differentiation as compared to wild type embryonic fibroblasts. While C/EBP$\beta^{-/-}$ mice do have adipocytes, their differentiation is arrested at the immature adipocyte stage, characterized by low levels of lipid accumulation in the cells (Tanaka, Yoshida et al. 1997). Millward et al. demonstrated that despite consuming more calories on a high-fat diet, C/EBP$\beta^{-/-}$ mice were resistant to the accumulation of body fat as well as the accumulation of triglycerides in the liver and free fatty acids, cholesterol, blood glucose and triglycerides in the blood (Millward, Heaney et al. 2007). In addition,

C/EBPβ$^{-/-}$ mice exhibited the inability to mobilize glycogen which causes perinatal death due to hypoglycemia. Mice that do survive into adulthood display less adipose tissue accumulation, exhibit fasting hypoglycemia, a 40% reduction in hepatic glucose production and decreased plasma free fatty acids (Liu, Croniger et al. 1999).

Osteogenesis

Bone homeostasis is maintained through the activity of bone-forming and bone-resorbing cells called osteoblasts and osteoclasts, respectively (Smink, Begay et al. 2009). Osteoblastogenesis and bone formation is dependent upon the activation of the transcription factor, Runx2 (Franceschi and Xiao 2003). Mice harbouring a mutated Runx2 gene do not survive and exhibit a complete lack of any bone formation (Komori, Yagi et al. 1997). Studies evaluating the role of C/EBPβ during osteoblastogenesis have indicated positive and negative regulation pathways. Interaction of C/EBPβ with Runx2 has been shown to positively regulate the expression of a bone specific gene, osteocalcin (Gutierrez, Javed et al. 2002). Hata et al. demonstrated that the LIP isoform of C/EBPβ is induced during the osteoblast differentiation of C3H10T1/2 cells and that physical interaction between LIP and Runx2 is required for this differentiation to occur (Hata, Nishimura et al. 2005). While C/EBPβ$^{-/-}$ mice demonstrated a reduced bone volume, as determined by the number and thickness of bone trabeculae, LIP knock-in mice exhibited increased bone volume, as compared to wild type mice (Smink, Begay et al. 2009). In addition to increased bone volume, osteoblasts derived from LIP knock-in mice demonstrated enhanced proliferation and expression of osteoblast differentiation markers. In contrast, C/EBPβ has also been demonstrated to be a negative regulator of Runx2 expression, through C/EBPβ occupancy of the Runx2 P1 promoter. This repression of the

Runx2 promoter by C/EBPβ can be overcome by treatment with all-trans retinoic acid which causes the displacement of C/EBPβ and promotes Runx2 expression (Wiper-Bergeron, St-Louis et al. 2007).

C/EBPβ in inflammation

During the innate immune response, activated macrophages are recruited to the site of inflammation and induce the expression of pro-inflammatory cytokines, including interleukin-1 (IL-1), IL-6, IL-8, nitric oxide synthase and TNF-α (Baer, Williams et al. 1998; Bradley, Zhou et al. 2003). The expression of C/EBPβ is also induced by inflammatory stimuli, resulting in an increase in its transcriptional activity due to phosphorylation (Trautwein, Caelles et al. 1993; Poli 1998). In agreement with this, Bradley et al. demonstrated that high levels of endogenous C/EBPβ localized in macrophages are constitutively phosphorylated and transcription is increased upon lipopolysaccharide (LPS) stimulation (Bradley, Zhou et al. 2003). C/EBPβ binding sites have been identified in the regulatory regions of various genes involved in inflammation, including cytokines IL-6, IL-8 and TNF-α (Natsuka, Akira et al. 1992). Indeed, C/EBPβ has been demonstrated to interact with NF-κB to activate IL-6 and IL-8 gene transcription via a LPS-mediated mechanism (Matsusaka, Fujikawa et al. 1993). Moreover, Pope et al. demonstrated using Jurkat cells transfected with a mutant C/EBPβ lacking the transactivation domain that expression of this dominant negative construct was sufficient to inhibit activity from the TNF-α promoter (Pope, Leutz et al. 1994). More specifically, Uematsu et al. demonstrated that the LAP isoform of C/EBPβ is expressed in activated macrophages and is responsible for the induction of several pro-

inflammatory cytokines, including IL-12, which stimulates the production of natural killer cells, T helper type 1 cells and interferon-gamma cells (Trinchieri, Pflanz et al. 2003; Uematsu, Kaisho et al. 2007).

In vivo, C/EBPβ-deficient mice are more susceptible to infection by *Candida albicans* and *Listeria monocytogenes*, and demonstrate altered nitric oxide production from defective splenic macrophages and altered T-helper response (Screpanti, Romani et al. 1995; Uematsu, Kaisho et al. 2007). Interestingly, C/EBP$\beta^{-/-}$ mice also demonstrated increased serum levels of IL-6, which is reminiscent of individuals affected by Castleman's Disease, in which lymph nodes produce excess amounts of IL-6 (Screpanti, Romani et al. 1995). Given this apparent contradictory result, it would suggest that C/EBPβ is in fact not required for induction of IL-6 transcription. The interplay of the ratio of the different C/EBPβ isoforms as well as to other C/EBPs is most likely the main factor that determines whether C/EBPβ function is inhibitory or activating (Screpanti, Romani et al. 1995).

The regulation of C/EBPβ activity in macrophages was been evaluated using a macrophage cell line, P388D1(IL1), that expresses high levels of C/EBPβ, and IC-21, in which C/EBPβ is induced only after treatment with conditioned media from cultured P388D1(IL1) cells (Bretz, Williams et al. 1994; Baer, Williams et al. 1998). Baer et al. demonstrated the involvement of two secreted factors, TNF-α and autocrine macrophage factor (AMF), which act independently in the regulation of C/EBPβ expression, localization and activity. Whereas TNF-α was shown to stimulate C/EBPβ protein expression and this expression displayed a punctate nuclear distribution pattern, AMF

redistributed C/EBPβ from a punctate nuclear localization to a diffuse nuclear distribution (Baer, Williams et al. 1998).

Furthermore, Ruffell et al. demonstrated a muscle-extrinsic role for C/EBPβ-expressing macrophages for proper muscle repair after injury (Ruffell, Mourkioti et al. 2009). Macrophages aid in the muscle repair process by the removal of necrotic tissue and induction of anti-inflammatory genes (Ruffell, Mourkioti et al. 2009).

Experimental rationale

Taylor and Jones demonstrated the induction of several new phenotypes upon treatment of the pluripotent mesenchymal stem cell line, C3H10T1/2, with the demethylating agent, 5-azacytidine and these phenotypes were characterized as chondrocytes, adipocytes and myocytes (Taylor and Jones 1979). During early adipogenesis, C/EBPβ and C/EBPδ are induced, which in turn, induce the expression of C/EBPα and PPARγ, the master regulators of adipogenesis (Payne, Au et al. 2007). There have been several lines of evidence implicating the dual function of C/EBPβ as a transcriptional activator and repressor in various regulatory and differentiation processes. Previous work in our laboratory has demonstrated that in a mesenchymal stem cell model, C/EBPβ acts as an activator of adipogenesis and an inhibitor of osteoblastogenesis (Wiper-Bergeron, Wu et al. 2003; Wiper-Bergeron, Salem et al. 2007; Wiper-Bergeron, St-Louis et al. 2007).

We sought to evaluate the effect of ectopic C/EBPβ in 5-azacytidine treated mesenchymal stem cells. Based on previous observations from our lab as well as in the literature, we predicted that we would observe enhanced adipogenesis in the C/EBPβ-

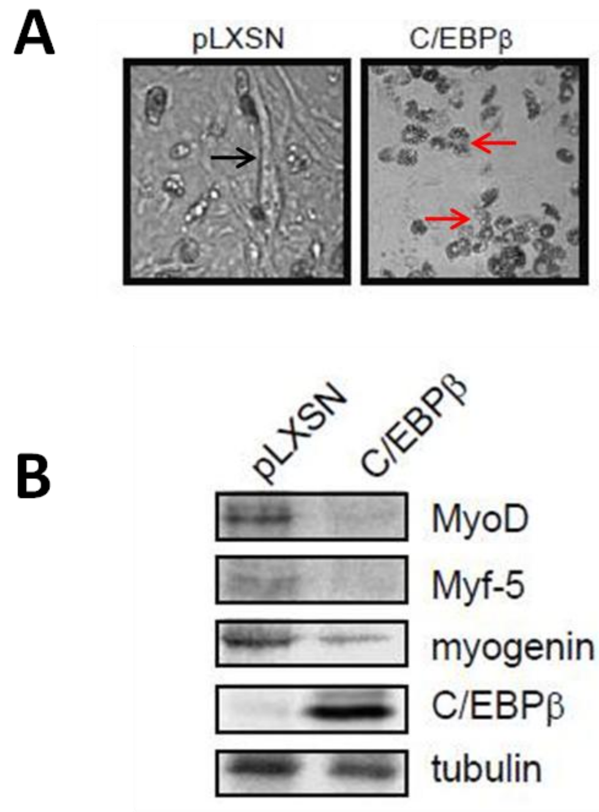


Figure 4. Ectopic C/EBPβ expression in 5-azacytidine treated mesenchymal stem cells exhibits reduced expression of myogenic factors. (C.St-Louis)

Pluripotent mesenchymal stem cells, C3H10T1/2, were retrovirally transduced to express full length C/EBPβ or with empty vector (pLXSN) and treated with 5-azacytidine (5-azaC) for 48 hours and maintained in culture for 30 days.

(A) Phase contrast images of 5-azaC treated C3H10T1/2 cells. Note the absence of fused myotubes and presence of adipocytes (red arrows) in C/EBPβ-expressing cells as compared to empty vector controls (pLXSN), which exhibited formation of myotubes (black arrow) and minimal adipogenesis.

(B) Whole cell extracts (25 μg) were resolved by SDS-PAGE and protein expression of C/EBPβ and myogenic factors, MyoD, Myf-5 and myogenin were evaluated by Western analysis. α-tubulin is used as a loading control.

expressing cultures. Our lab retrovirally transduced C3H10T1/2 cells with full length C/EBPβ or with empty vector, pLXSN, and then treated these cells with 5-azacytidine for 48 hours and maintained them in culture for 30 days. As expected, we observed an increased presence of adipocytes in the C/EBPβ-expressing cells, as compared to pLXSN control cultures, in which the adipogenic phenotype was less prominent. Interestingly, we noted in our control cultures the presence of fused myotubes, which were not observed in the C/EBPβ-expressing cultures (Fig. 4A). Further investigation by Western analysis for expression of myogenic proteins demonstrated that the C/EBPβ-expressing cells failed to upregulate the myogenic factors, MyoD, Myf5 and myogenin, in contrast to control cultures (Fig. 4B). In addition to its well established role in adipogenesis and osteoblastogenesis, our observed results suggested a potential role for C/EBPβ as a repressor of skeletal muscle differentiation.

HYPOTHESIS AND OBJECTIVES

Hypothesis

C/EBPβ is a negative regulator of skeletal muscle differentiation in C2C12 myoblasts and primary myoblasts.

Objectives

1. To evaluate the effect of ectopic C/EBPβ expression in differentiated C2C12 myoblasts and primary myoblasts.

We predicted that C/EBPβ-expressing cells will demonstrate reduced MyoD expression and expression of the late MRFs and cell cultures will exhibit reduced differentiation and fusogenic ability.

2. To characterize the muscle phenotype of the C/EBPβ$^{-/-}$ mouse.

We postulated that the absence of C/EBPβ will fail to inhibit MyoD expression, and these cells will terminally differentiate with few cells returning to quiescence after each cycle of activation. Therefore, we predicted that C/EBPβ$^{-/-}$ mice will exhibit fewer satellite cells.

MATERIALS AND METHODS

Cell Line Maintenance

C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose with L-glutamine and sodium pyruvate (Wisent Bioproducts, St-Bruno, QC, Canada) and supplemented with 1x MEM Non-essential Amino Acid solution (NEAA) (Wisent Bioproducts, St-Bruno, QC, Canada) and 10% heat inactivated fetal bovine serum (HI-FBS) (Invitrogen Canada Inc., Burlington, ON, Canada). Phoenix™ Ampho packaging cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM containing 4.5 g/L glucose with L-glutamine and sodium pyruvate and supplemented with 1x NEAA and 10% HI-FBS.

Isolation and Maintenance of Murine Primary Myoblasts

Preparation of Matrigel

BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Mississauga, ON, Canada) was diluted 1:10 in ice-cold DMEM using pre-cooled pipette tips and 1.5 mL Eppendorf tubes. To prepare Matrigel coated dishes, wells were coated with diluted Matrigel and rocked to ensure even coverage of the surface. Excess Matrigel was removed and used to coat the next dish. Dishes were left to set at room temperature for 1 hour and prior to plating, were placed in the incubator to re-hydrate the gel. To prepare Matrigel coated coverslips, glass coverslips were coated with diluted Matrigel and placed into a 6-well dish (one coverslip per well). Coverslips were left to set at room temperature for 1 hour and placed in the incubator to re-hydrate prior to cell plating.

Preparation of enzyme solution

A fresh solution of collagenase/dispase was prepared the day of isolation and comprised of DMEM (use 8 mL per mouse) with 0.2% collagenase A (Roche Applied Science, Laval, QC, Canada), 500 ul dispase (BD Biosciences, Mississauga, ON, Canada) and 0.01% of penicillin-streptomycin solution (Wisent Bioproducts, St-Bruno, QC, Canada). Solution was warmed to 37°C and filter sterilized with a 0.22 μm syringe filter (Millipore, Billerica, MA, USA) prior to use.

Isolation of murine primary myoblast cells

Mice were euthanized by cervical dislocation and hind limb muscles removed immediately and placed into a 60 mm dish containing 1x phosphate buffered saline (PBS) with 0.01% penicillin-streptomycin. Muscles were cut into small fragments and transferred to a 15 mL tube containing the collagenase/dispase enzyme solution, which was placed into a 37°C water bath for 2 hours, or until no large muscle fragments were visible. The homogenized muscle mixture was filtered through a 70 μm cell strainer (Fisher Scientific Company, Ottawa, ON, Canada) into a 50 mL tube and centrifuged at 1200 rpm for 5 minutes. Supernatant was carefully removed and the pellet was re-suspended in 10 mL of growth media and plated onto an uncoated 10 cm tissue culture dish and placed in the incubator for 1-3 hours to remove contaminating fibroblasts. The unadhered cell suspension (containing the primary myoblast cells) was then plated onto Matrigel coated dishes and placed in incubator. Media was not changed for the first 2 days to ensure complete attachment of primary myoblasts, after which media was changed daily. To induce myoblast fusion, growth media was replaced with differentiation media and changed daily until harvest.

Cell maintenance

Primary myoblasts were grown in DMEM containing 4.5 g/L glucose with L-glutamine and sodium pyruvate and supplemented with 10% HI-FBS, penicillin-streptomycin and maintained in a 37°C humidified incubator with 5% carbon dioxide.

Retroviral Infection

Transfection of Phoenix™ Ampho packaging cells

Replication incompetent pLXSN-based retroviruses (Clontech Laboratories, Inc., Mountain View, CA, USA) were generated by transfection of Phoenix™ Ampho packaging cells by calcium phosphate precipitation. Fresh media containing 25 μM of chloroquine diphosphate salt (Sigma-Aldrich, Oakville, ON, Canada) was added to 1.0 X 10^6 Phoenix cells in a 60 mm dish. In a 1.5 mL Eppendorf tube, 10 μg of the DNA was diluted in 428 μl of ddH_2O and 62 μl of $CaCl_2$ was added to the DNA/ddH_2O mixture. To this, 500 μl of 2x HBS (50 mM HEPES pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl and 1.5 mM Na_2HPO_4) was added and the contents were mixed by inverting the tube and tapping. This DNA/HBS mixture was then added to the Phoenix cells dropwise while swirling the dish for equal distribution. Cells were placed in the incubator for 10 hours after which fresh media was added. Viral supernatants were collected 48 hours post-transfection and filtered through a 0.45 μm syringe filter (Millipore, Billerica, MA, USA) and used fresh or aliquoted and stored at -80°C.

Infection of C2C12 cells

Early in the day, 10 cm dishes of 50% confluent C2C12 cells were infected with 1 mL of viral supernatant in 5 mL of DMEM containing 6 μg/μL of polybrene (Sigma-Aldrich, Oakville, ON, Canada). At the end of the day, the infection was stopped by the

addition of 4 mL of DMEM to the dishes and left overnight. Selection was started 48 hours post-infection in DMEM containing 400 µg/mL of G418 (Sigma-Aldrich, Oakville, ON, Canada) and continued for 7 days to eliminate non-transduced cells.

Differentiation of C2C12 myoblasts and primary myoblasts

To induce differentiation of C2C12 myoblasts, DMEM containing 4.5 g/L glucose with L-glutamine and sodium pyruvate and supplemented with 1x NEAA and 2% heat-inactivated horse serum (HI-HS) (Invitrogen Canada Inc., Burlington, ON, Canada) was added to 70% confluent cells. To induce differentiation of primary myoblasts, DMEM containing 4.5 g/L glucose with L-glutamine and sodium pyruvate and supplemented with 10% HI-HS was added to cells 3 days after proliferation in growth media.

Giemsa staining

Cells were washed twice with 1x PBS and fixed with ice cold methanol at room temperature for 15 minutes. Cells were then washed once with 1x PBS and stained with 10% Giemsa stain solution (Sigma-Aldrich, Oakville, ON, Canada) at room temperature for 1 hour. Cells were then washed extensively with ddH_2O to remove excess stain and left to dry at room temperature.

Western analysis and Immunodetection

Preparation of whole cell protein extracts

Cells were washed twice with 1x PBS and collected in 150 µl of IPH buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM 1,4-dithiothreitol (DTT), 1x complete protease inhibitor cocktail (Roche Applied Science, Laval, QC, Canada)). Cells were sonicated for 20 seconds at 10% duty cycle and placed on ice to

lyse for 30 minutes. Cell extracts were centrifuged at 13 000 rpm for 5 minutes at 4°C to pellet cell debris and supernatant was transferred to new tubes and stored at -20°C. Protein concentrations were determined by a standard Bradford assay reading at 595 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and membrane transfer

Protein samples (25 μg) mixed with 3x SDS loading dye were heated at 95°C for 5 minutes and centrifuged before loading. Samples were loaded on a 10-12% separating gel with a 4% stacking gel and run at 120 V until desired resolution was achieved. Immobilon-P polyvinylidene fluoride (PVDF) transfer membranes (Millipore, Billerica, MA, USA) were hydrated with methanol briefly for 30 seconds and washed twice with ddH_2O, after which they were equilibrated in 1x transfer buffer before transfer. Electrophoretic membrane transfer was carried out at room temperature for 1 hour at 100 V or at 4°C overnight at 30 V.

Immunodetection

After transfer, PVDF membranes were incubated in 0.2% blocking solution (ECL Advance™ Blocking Agent, GE Healthcare, Baie d'Urfe, QC, Canada) in 1x phosphate buffered saline with 0.05% Tween-20 (PBS-T) (BioShop Canada Inc., Burlington, ON, Canada) on a rocker at room temperature for 1 hour. After blocking, membranes were washed 3 times with 1x PBS-T and incubated overnight with primary antibody diluted in blocking solution on a rocker at 4°C. The following primary antibodies were used: C/EBPβ (C-19), MyoD (M-318), MHC (H-300), Myf5 (C-20), myogenin (M-225) (1:400, all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), α-tubulin (B-7) (1:10 000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Pax7 (1:500,

Development Studies Hybridoma Bank, Iowa City, IA, USA), cyclophilin (ab16045, 0.5 μg/mL, Abcam, Inc., Cambridge, MA, USA). The following day, membranes were washed 5 times with 1x PBS-T and incubated at room temperature for 1 hour with horseradish peroxide (HRP) conjugated secondary antibodies diluted in 1x PBS-T. The following secondary antibodies were used: sheep α-mouse IgG HRP and donkey α-rabbit IgG HRP (1:50 000, Amersham, GE Healthcare, Baie d'Urfe, QC, Canada). Membranes were washed 5 times with 1x PBS-T prior to signal detection by chemiluminescence (Amersham™ ECL Advance™ Western Blotting Detection Kit, GE Healthcare, Baie d'Urfe, QC, Canada) and images were captured using the Luminescent Image Analyzer LAS-3000 (Fujifilm Life Science).

Preparation of whole muscle protein extracts

Hind limb muscles were removed and placed into 500 μL of 1x PBS in a 1.5 mL Eppendorf tube. The muscle was cut into smaller fragments and centrifuged at 7000 rpm for 5 minutes to pellet the muscle. Supernatant was removed and 500 μL of whole cell extract buffer (20 mM HEPES, pH 7.9, 400 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1x complete protease inhibitor cocktail (Roche Applied Science, Laval, QC, Canada)) was added. Muscle fragments were homogenized and sonicated for 30 seconds at 20% duty cycle and tube was placed on ice for 30 minutes. Tube was centrifuged at 13 000 rpm for 2 minutes and supernatant was transferred to a new 1.5 mL Eppendorf tube and stored at -20°C. Protein concentrations were determined by a standard Bradford assay reading at 595 nm.

Indirect immunofluorescence

Preparation of frozen sections

Hind limb muscles were removed and immediately coated with Tissue-Tek O.C.T compound (Sakura Finetek, Torrance, CA, USA) and flash frozen using cooled isopentane. Samples were cooled in the isopentane for 1 minute and then placed into 1.5 mL cryovials which were previously cooled using liquid nitrogen. Frozen muscles were stored at -80°C. Frozen sections of the muscle samples were done by using a cooled cryostat to generate 10 µm thick sections melted onto glass microscope slides. Slides were kept at -80°C for storage.

Immunohistochemistry on frozen sections

Microscope slides were thawed to room temperature prior to fixation with 4% paraformaldehyde (PFA) for 15 minutes. Slides were washed once with 1x PBS and immediately incubated with primary antibody diluted in 1x PBS with 5% normal serum and 0.3% Triton X-100 (BioShop Canada Inc., Burlington, ON, Canada) overnight at 4°C. If 2 proteins of interest were being evaluated, both primary antibodies were diluted and a simultaneous incubation was performed. The following primary antibodies were used: laminin (1:50, ab11575, Abcam, Inc., Cambridge, MA, USA), Pax7 (1:10, Development Studies Hybridoma Bank, Iowa City, IA, USA), C/EBPβ (C-19, 1/50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The next day, slides were washed twice with 1x PBS and incubated with fluorophore-conjugated secondary antibody diluted in 1x PBS with 5% normal serum and 0.3% Triton X-100 for 1 hour at room temperature in the dark. The following secondary antibodies were used: Dylight™ 549-conjugated donkey α-mouse and Dylight™ 488-conjugated donkey α-rabbit (1:400, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Slides were washed twice with 1x PBS and incubated with 1 µg/mL of DAPI solution at room

temperature for 1 minute. Slides were washed with 1x PBS to remove excess DAPI solution and mounted with coverslips using Vectashield Mounting Medium (Vector Labs, Inc., Burlington, ON, Canada) and sealed with nail polish. Slides were stored at 4°C.

Hematoxylin and eosin staining

Microscope slides were brought up to The Morphology Unit laboratory of the Department of Pathology and Laboratory Medicine at the University of Ottawa for hematoxylin and eosin staining.

Immunocytochemistry on primary myoblast cells

Primary myoblasts were plated onto Matrigel-coated coverslips contained in 6-well dishes for immunocytochemistry. Media was aspirated and cells were washed twice with 1x PBS. Cells were fixed with ice cold methanol at room temperature for 15 minutes. Cells were washed twice with 1x PBS and incubated overnight at 4°C with primary antibody diluted in 1x PBS with 5% normal serum and 0.3% Triton X-100. The following primary antibodies were used: MHC (H-300), MYH1/2/4 (MY-32) (1:50, all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The next day, primary antibody was removed and cells were washed with 1x PBS. Incubation with fluorophore-conjugated secondary antibody, diluted in 1x PBS with 5% normal serum and 0.03% Triton X-100, was done at room temperature for 1 hour in the dark. The following secondary antibodies were used: Dylight™ 549-conjugated donkey α-mouse and Dylight™ 488-conjugated donkey α-rabbit (1:400, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cells were then washed with 1x PBS and incubated with 1 μg/mL DAPI solution at room temperature for 1 minute. Cells were washed with 1x PBS to remove excess DAPI solution and coverslips were mounted onto

glass microscope slides with Vectashield Mounting Medium (Vector Labs, Inc., Burlington, ON, Canada) and sealed with nail polish. Slides were kept at 4°C.

Analysis of C/EBPβ$^{-/-}$ mice

C/EBPβ$^{-/-}$ mice (strain *Cebpb*^{tm1Vpo}/J) were produced by breeding heterozygous female mice bearing the deletion with heterozygous male mice. Breeder mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and all animals were housed in a pathogen-free facility. Mice bearing two copies of the gene for C/EBPβ were designated as wild type for the purpose of this study. All animal handling procedures conform to the guidelines established by the University of Ottawa Animal Care Service and the Canadian Council on Animal Care.

Statistical Analysis

A student's t-test was performed to determine any significant differences between 2 sample sets for a minimum of 3 independent experiments. Statistical significance was set at $p<0.05$. Error represents the standard error of the mean.

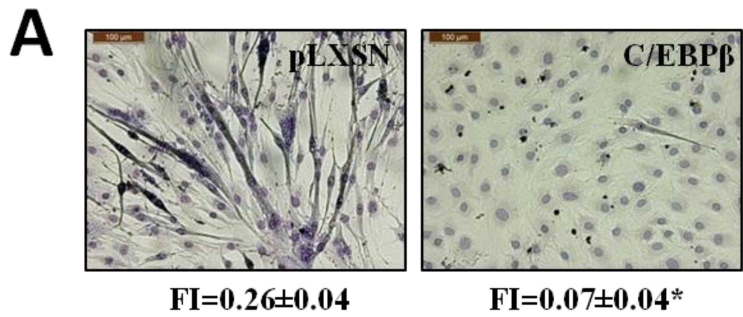
RESULTS

Ectopic expression of full length C/EBPβ inhibits expression of myogenic proteins in C2C12 cells.

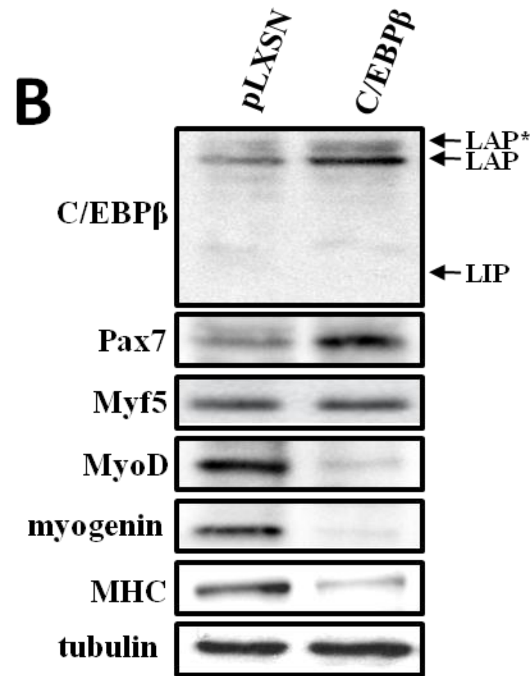
Given that ectopic expression of full length C/EBPβ in mesenchymal stem cells resulted in the inhibition of myogenesis, we sought to evaluate the role of this transcription factor in myoblast cells. To evaluate this, we first used the murine myoblast cell line, C2C12. These cells were originally derived from the serial passage of myoblasts isolated from the thigh muscle of C3H strain mice after crush injury, as described by Yaffe and Saxel (Yaffe and Saxel 1977) and are capable of forming terminally differentiated myotubes in culture.

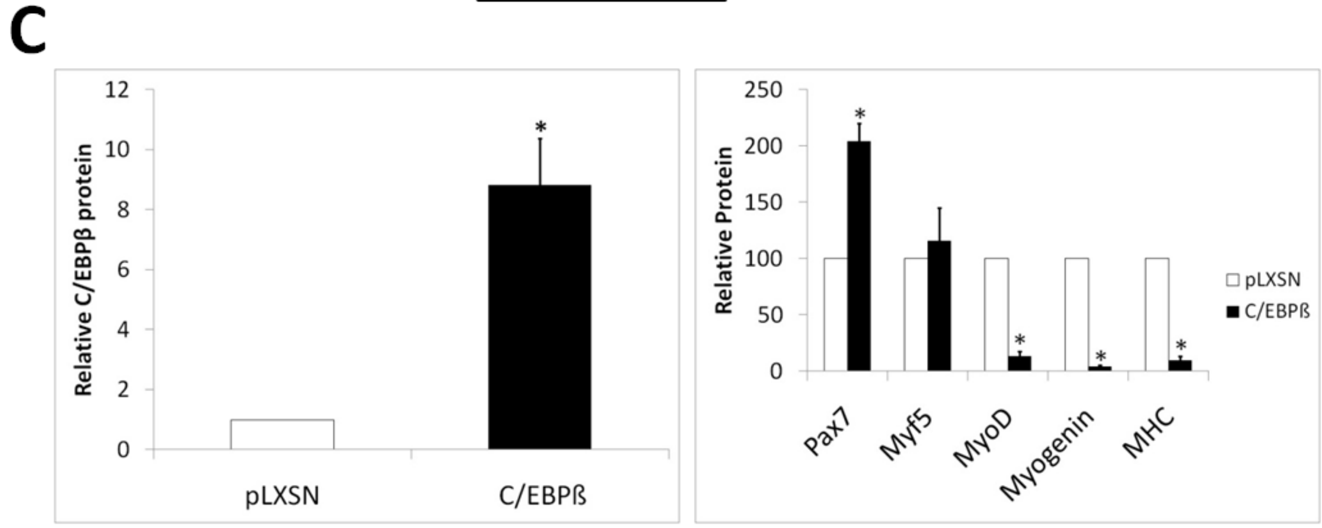
C2C12 cells were retrovirally transduced with empty vector, pLXSN, or to express full length C/EBPβ. Cells were grown to 70% confluency before induction to differentiate in low serum media for a period of 5 days. Cells were then fixed and stained with 10% Giemsa solution to visualize individual undifferentiated cells and fused myotubes (Fig. 5A). pLXSN control cultures differentiated efficiently and exhibited extensive fusion of myoblasts to multinucleated myotubes (Fig. 5A). In contrast, cells ectopically expressing full length C/EBPβ did not differentiate as efficiently and displayed only a low degree of myoblast fusion to multinucleated myotubes (Fig. 5A).

To quantify the formation of myotubes, the fusion index (FI) of each stable cell line was calculated as the number of nuclei per myotubes/total number of nuclei. Cells ectopically expressing full length C/EBPβ demonstrated a significantly reduced FI=0.07±0.04 (*$p<0.05$) compared to that of pLXSN control cultures (FI=0.26±0.04). To determine whether the observed defect in C/EBPβ-expressing cells was due to a failure of



FI=0.26±0.04 FI=0.07±0.04*





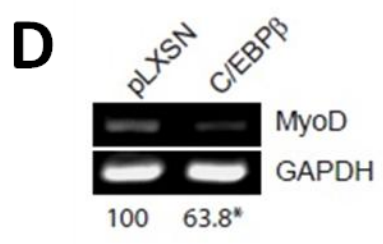


Figure 5. Ectopic C/EBPβ expression in C2C12 myoblasts decreases protein expression of myogenic factors.

(A) C2C12 myoblasts were retrovirally transduced to express full length C/EBPβ or with empty vector (pLXSN) and induced to differentiate in low serum media for 5 days. Cells were fixed with ice-cold methanol and stained with 10% Giemsa solution for visualization of differentiated myotubes and undifferentiated myoblasts. Fusion index (FI) was calculated as the number of nuclei per myocyte/total nuclei. Error represents the standard error of the mean of four independent trials (*$p<0.05$). Scale bar = 100 μm.

(B) Whole cell extracts (25 μg) from cells transduced and differentiated as in (A) were resolved by SDS-PAGE and protein expression of C/EBPβ, Pax7 and myogenic factors, Myf5, MyoD, myogenin and Myosin Heavy Chain (MHC) were evaluated by Western analysis. α-tubulin is used as a loading control.

(C) Quantification of C/EBPβ, Pax7, Myf5, MyoD, myogenin and MHC expression from (B) relative to α-tubulin expression, where pLXSN is set at 1 or 100. Error bars represent the standard error of the mean of three independent trials (*$p<0.05$).

(D) Semi-quantitative RT-PCR of MyoD mRNA expression in cells transduced and differentiated as in (A). Quantification of MyoD expression relative to GAPDH is representative of three independent trials (*$p<0.05$) (C. St-Louis).

differentiation or a failure of myocyte fusion, 5-days differentiated C/EBPβ-expressing and control cultures were analyzed for protein expression of the myogenic regulatory factors, Myf5, MyoD, myogenin and myosin heavy chain (MHC). Protein expression of C/EBPβ and Pax7 was also evaluated.

While control cultures robustly expressed Myf5, MyoD, myogenin and MHC proteins, C/EBPβ-expressing cultures demonstrated reduced levels of MyoD, myogenin and MHC, suggesting that the observed defect was indeed due to a failure of differentiation, and not of myocyte fusion (Fig. 5B). Interestingly, C/EBPβ-expressing cultures did not exhibit a reduction in Myf5 protein, suggesting the repressive action of C/EBPβ on myogenesis acts downstream of Myf5 (Fig. 5B). C/EBPβ-expressing cultures also demonstrated an increase in C/EBPβ and Pax7 protein as compared to control cultures (Fig. 5B).

Quantification of C/EBPβ protein expression revealed a significant increase in expression levels in the C/EBPβ-expressing cultures (8.8±1.6, $*p<0.05$) as compared to the pLXSN control cultures (set arbitrarily at 1) (Fig. 5C). Quantification of MyoD, myogenin and MHC protein expression demonstrated a significant decrease in the C/EBPβ-expressing cultures (13.6±3.9, 6.8±1.4 and 9.7±3.3, respectively, $*p<0.05$) as compared to control cultures (set arbitrarily at 100) (Fig. 5C). While there was a significant increase in Pax7 protein expression in C/EBPβ-expressing cultures (203.7±16.1, $*p<0.05$), there was no significant difference in Myf5 protein (115.6±29.1, $*p>0.05$) as compared to control cultures (set arbitrarily at 100) (Fig. 5C).

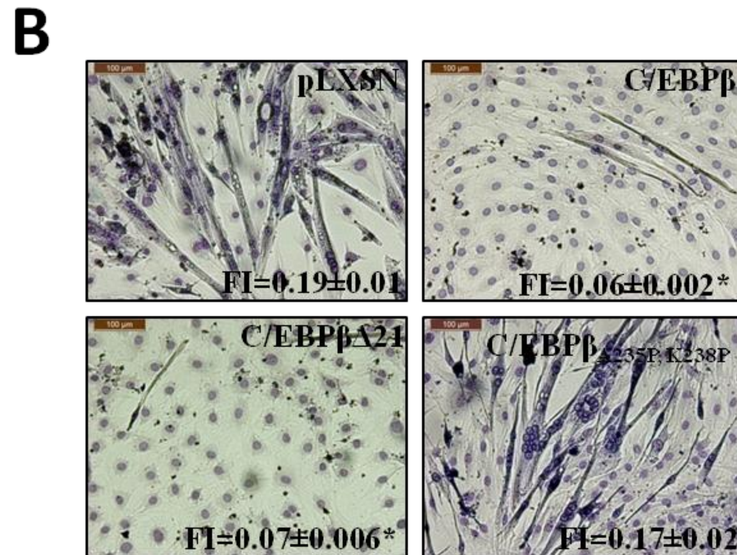
To further evaluate the level of MyoD regulation by C/EBPβ, reverse transcription polymerase chain reaction (RT-PCR) analysis evaluating the mRNA levels of MyoD in control cultures and C/EBPβ-expressing cultures was performed by Catherine St-Louis. We observed a decrease in MyoD mRNA expression in the C/EBPβ-expressing cultures (63.8, $*p<0.05$) as compared to control cultures (set arbitrarily at 100) (Fig. 5D, C. St-Louis), suggesting that C/EBPβ may act to impair MyoD expression at the level of transcription.

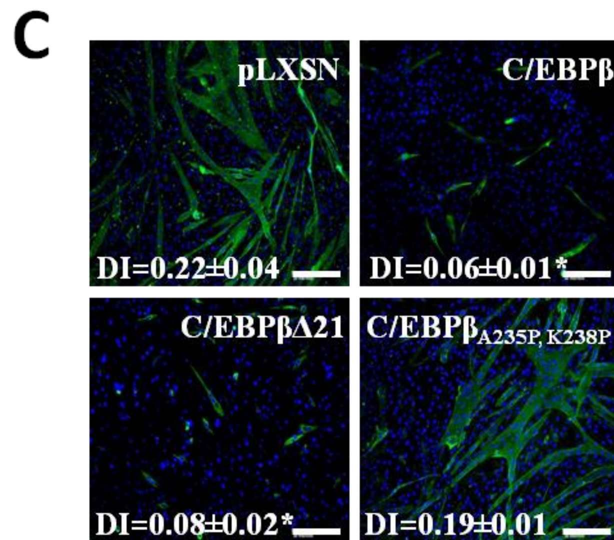
The C/EBPβ DNA binding domain is required for its inhibitory function during myogenesis.

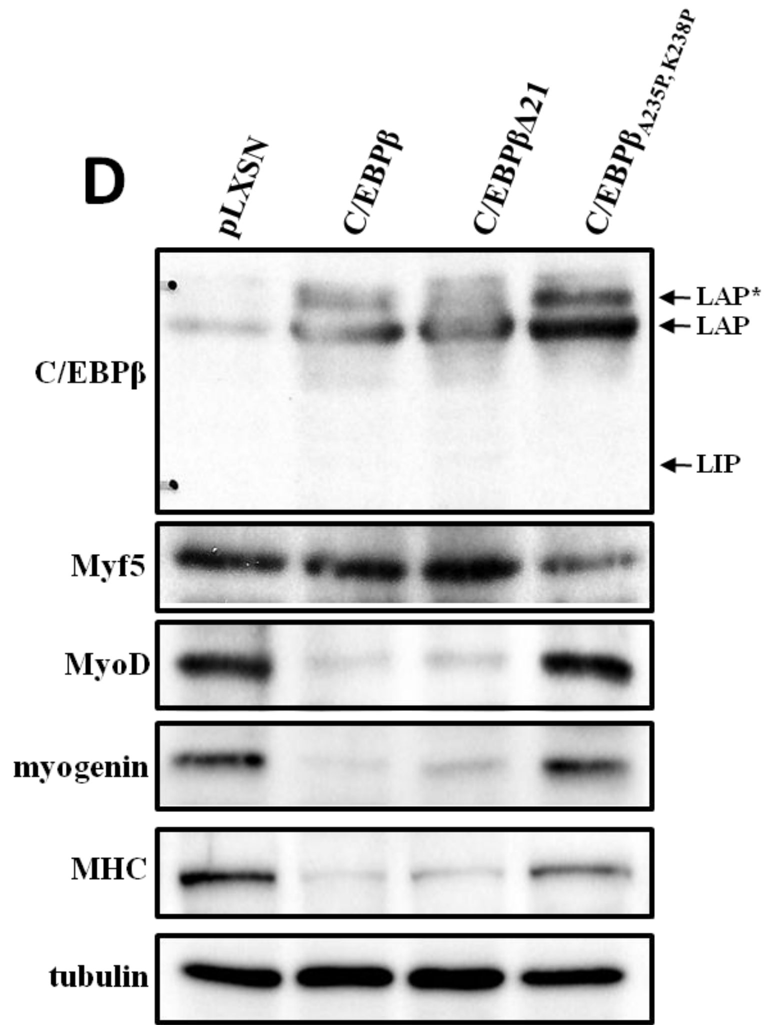
To elucidate the mechanism by which C/EBPβ inhibits myogenesis, two different C/EBPβ mutants were generated using site-directed mutagenesis of the full length C/EBPβ construct. These clones were generated and validated by C. St- Louis.

The C/EBPβΔ21 mutant bears a deletion of the first 21 amino acids in the N-terminal creating the naturally occurring LAP isoform of C/EBPβ (Fig. 6A). These 21 amino acids are known to interact with various proteins involved in chromatin remodeling including the SWI/SNF remodeling complex (Kowenz-Leutz and Leutz 1999) and the histone acetyltransferase p300 (Schwartz, Beck et al. 2003) to promote active gene transcription. Given that C/EBPβ can interact with histone deacetylase 1 (HDAC1) and the SWI/SNF complex, we postulated that recruitment of C/EBPβ to myogenic promoters could promote the silencing of chromatin, and thereby inhibit MyoD expression. The C/EBP$\beta_{A235P,\ K238P}$ mutant bears two point mutations in the C-terminal bzip domain (Fig. 6A), which while still able to form heterodimers, is unable to bind DNA.

A
activation domains
1 (22-34, 54-73, 84-93)
296
C/EBPβ
bzip
C/EBPβ
C/EBPβ
bzip
C/EBPβΔ21
C/EBPβ
bzip
C/EBPβA235P,K238P


B
pLXSN
FI=0.19±0.01
C/EBPβ
FI=0.06±0.002*
C/EBPβΔ21
FI=0.07±0.006*
FI=0.17±0.02


C
pLXSN
DI=0.22±0.04
C/EBPβ
DI=0.06±0.01*
C/EBPβΔ21
DI=0.08±0.02*
C/EBPβA235P, K238P
DI=0.19±0.01



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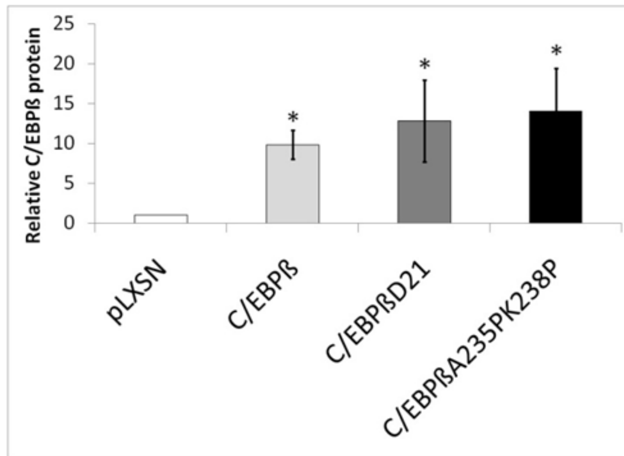


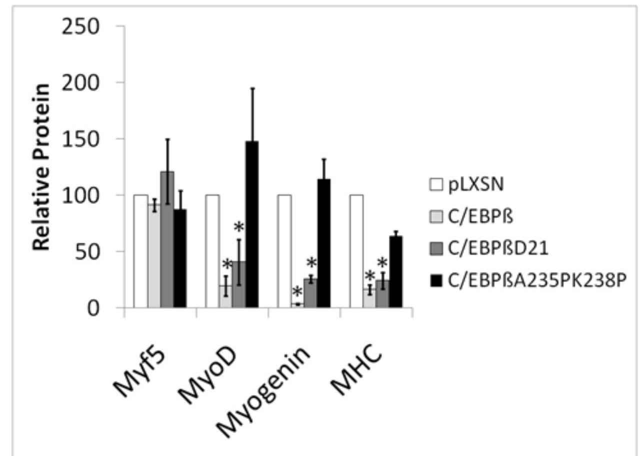


Figure 6. A functional DNA binding domain is required for the inhibitory function of C/EBPβ during myogenesis.

(A) Schematic representation of full length C/EBPβ and the two generated mutant constructs, C/EBPβΔ21 and C/EBP$\beta_{A235P,K238P}$. C/EBPβΔ21 is a naturally occurring isoform of C/EBPβ produced by the use of an alternative translation start site, which results in the deletion of the first 21 amino acids in the N-terminal of full length C/EBPβ. The N-terminal domain has been implicated to interact with chromatin remodeling proteins, such as the SWI/SNF remodeling complex and histone acetyltransferase p300 to promote gene transcription. C/EBPβA235P,K238P bears 2 point mutations in the DNA-binding region of the C-terminal bzip domain, which while still able to form heterodimers, is unable to bind DNA.

(B) C2C12 cells were retrovirally transduced to express full length C/EBPβ, C/EBPβΔ21, C/EBP$\beta_{A235P,K238P}$ or with empty vector (pLXSN). Cells were induced to differentiate in low serum media for 5 days. Cells were fixed with ice-cold methanol and stained with 10% Giemsa solution for visualization of differentiated myotubes and undifferentiated myoblasts. Fusion index (FI) was calculated as the number of nuclei per myocyte/total number of nuclei. Error represents the standard error of the mean of four independent trials (*$p<0.05$). Scale bar = 100 μm.

(C) Cells were fixed with ice-cold methanol and immunostained with antibody against MHC (green) and with DAPI (blue) for visualization of nuclei. Differentiation index (DI) was calculated as the number of MHC^+ myotubes/number of unfused cells. Error represents the standard error of the mean of three independent trials (*$p<0.05$). Scale bar = 50 μm.

(D) Whole cell extracts (25 μg) from cells transduced and differentiated as in (B) were resolved by SDS-PAGE and protein expression of C/EBPβ, Myf5, MyoD, myogenin and MHC were evaluated by Western analysis. α-tubulin is used as a loading control.

(E) Quantification of C/EBPβ, Myf5, MyoD, myogenin and MHC expression from (D) relative to α-tubulin expression, where pLXSN is set at 1 or 100. Error bars represent the standard error of the mean of three independent trials (*$p<0.05$).

Cells were retrovirally transduced to express a C/EBPβ mutant, full length C/EBPβ or with empty vector (pLXSN) and differentiated in low serum media for 5 days after which they were stained with 10% Giemsa for visualization of myotube formation (Fig. 6B). Cells ectopically expressing C/EBPβΔ21 did not differentiate efficiently and had a significantly reduced FI=0.07±0.006 (* $p<0.05$) as compared to control cultures (FI=0.19±0.01) and a fusion index comparable to cells expressing the full length C/EBPβ (FI=0.06±0.002, * $p<0.05$). In contrast, cells ectopically expressing C/EBP $\beta_{A235P,\ K238P}$ differentiated efficiently and exhibited extensive fusion of myoblasts to multinucleated myotubes, with a fusion index comparable to that of control cultures (FI=0.17±0.02 versus FI=0.19±0.01, respectively).

To further evaluate the differentiation potential of full length C/EBPβ, the two C/EBPβ mutant constructs and empty vector (pLXSN) transduced cultures, indirect immunocytochemistry was performed using an antibody against amino acids 1641-1940 of embryonic myosin heavy chain (MYH3) to visualize the formation of multinucleated myotubes and DAPI to visualize nuclei (Fig. 6C). Empty vector pLXSN and the C/EBP $\beta_{A235P,\ K238P}$ construct exhibited extensive formation of myotubes, while full length C/EBPβ and the C/EBPβΔ21 construct had only limited fusion (Fig. 6C). The differentiation index (DI) was calculated as the number of MHC^{+} cells/number of unfused cells and was used as a measure of the maturity and terminal differentiation of the cells. Cells ectopically expressing C/EBPβΔ21 demonstrated a significantly reduced DI=0.08±0.02 (* $p<0.05$) as compared to control cultures (DI=0.22±0.04) and one comparable to cells expressing full length C/EBPβ (DI=0.06±0.01, * $p<0.05$). In contrast,

cells ectopically expressing C/EBPβ$_{A235P,\ K238P}$ exhibited a differentiation index comparable to control cultures (DI=0.19±0.01 versus DI=0.22±0.04, respectively).

Differentiated cells ectopically expressing a C/EBPβ mutant, full length C/EBPβ or with empty vector (pLXSN) were subjected to Western blot analysis. Both the C/EBPβ mutants and full length C/EBPβ expressed C/EBPβ protein at a higher level than the empty vector control (Fig. 6D). Quantification of C/EBPβ protein expression demonstrated a significant increase in protein in the full length C/EBPβ, C/EBPβΔ21 and C/EBPβ$_{A235P,\ K238P}$ cultures (9.8±1.8, 12.8±5.1 and 14.1±5.3, respectively, *$p<0.05$) as compared to empty vector controls (set arbitrarily at 1) (Fig. 6E). As expected, full length C/EBPβ-expressing cultures exhibited significantly reduced MyoD, myogenin and MHC protein expression (19.6±8.8, 3.7±0.6 and 16.4±4.3, respectively, *$p<0.05$) as compared to control cultures (set arbitrarily at 100) (Fig. 6E). Interestingly, there was no significant difference in Myf5 protein expression in full length C/EBPβ, C/EBPβΔ21 and C/EBPβ$_{A235P,\ K238P}$ expressing cultures (91.3±5.4, 121±28.5 and 87.9±16.5, respectively, *$p>0.05$) as compared to control cultures (set arbitrarily at 100), again supporting the notion that C/EBPβ acts downstream of Myf5 to repress myogenesis.

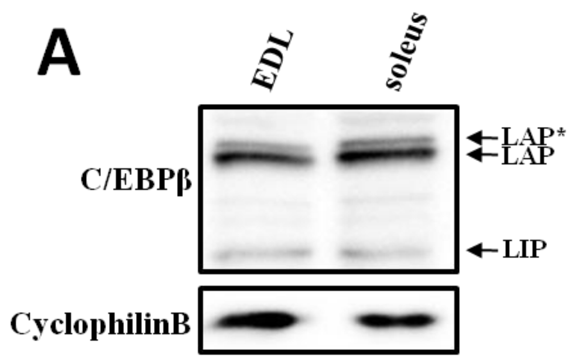
The C/EBPβ$_{A235P,\ K238P}$ mutant differentiated as efficiently as the empty vector control and demonstrated comparable levels of MyoD, myogenin and MHC protein expression, suggesting that the C-terminal DNA-binding domain of C/EBPβ is required for the inhibitory function of C/EBPβ during myoblast differentiation (Fig. 6D). Quantification of MyoD, myogenin and MHC protein expression in the C/EBPβ$_{A235P,\ K238P}$ mutant construct did not reveal significant changes in protein expression (147.8±47,

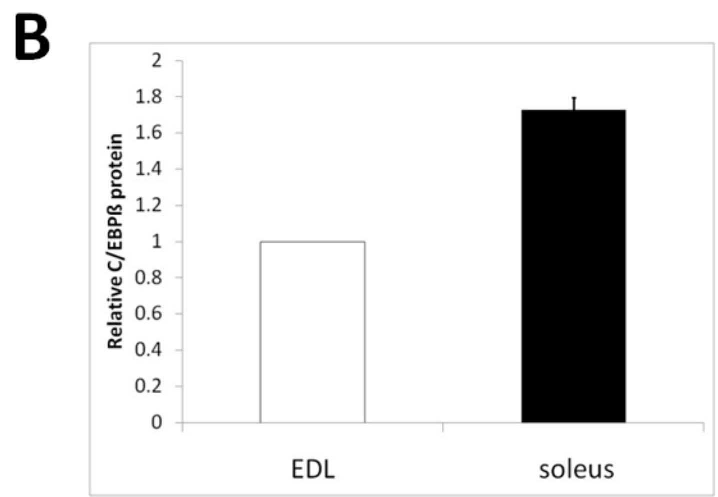
114.2±17.7 and 44.9±11.7, respectively, *p>0.05) as compared to empty vector controls (set arbitrarily at 100) (Fig. 6E).

Quantification of MyoD, myogenin and MHC protein expression in the C/EBPβΔ21 mutant construct demonstrated a significant reduction in protein expression (40.8±19.9, 26.0±3.3 and 24.3±7.4, respectively, *p<0.05) as compared to empty vector controls (set arbitrarily at 100) (Fig. 6E). These results suggested that the deletion of the first 21 amino acids in the N-terminal of C/EBPβΔ21 did not hinder its ability to effectively inhibit myoblast differentiation, implying that any co-factors which interact with the N-terminal 21 amino acids, such as the SWI/SNF complex, are dispensable for the repressive action of C/EBPβ during myogenesis. In contrast, the DNA-binding domain is required for the inhibitory function of C/EBPβ during myogenesis.

C/EBPβ is localized to the satellite cells in vivo.

To investigate the *in vivo* role of C/EBPβ in skeletal muscle, the expression pattern was first evaluated in soleus and extensor digitorum longus (EDL) muscle isolated from a 7 weeks old wild type mouse (C57BL/6). Western blot analyses revealed the presence of C/EBPβ protein in both soleus and EDL muscle (Fig. 7A). Interestingly, we observed higher, though not significantly so, C/EBPβ expression in the soleus muscle (1.7±0.07, p=0.1) as compared to the EDL muscle (set arbitrarily at 1) (Fig. 7B). We next evaluated the localization of C/EBPβ in the muscle on cross sections of the tibialis anterior (TA) muscle from a 7 weeks old wild type mouse by indirect immunohistochemistry. Double immunostaining with antibodies against C/EBPβ, Pax 7 or Pax 3 revealed the co-localization of these proteins in the satellite cells (Fig. 7C). C/EBPβ was not observed in myonuclei or myofibers in healthy muscle.


A
EDL
soleus
C/EBPβ
LAP*
LAP
LIP
CyclophilinB


B
Relative C/EBPβ protein
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
EDL
soleus

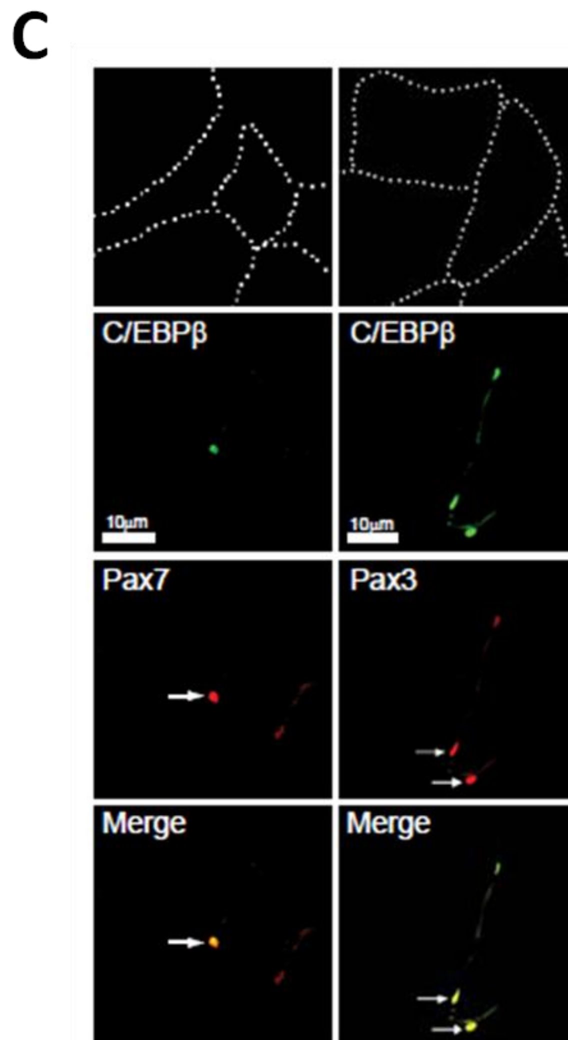

C
C/EBPβ
C/EBPβ
10μm
10μm
Pax7
Pax3
Merge
Merge

Figure 7. C/EBPβ is localized to the satellite cells.

Soleus, EDL and TA muscles were dissected from 7 weeks old C57BL/6 mice and processed for Western analysis or flash frozen for indirect immunohistochemistry.

(A) Whole cell extracts (25 μg) were resolved by SDS-PAGE and protein expression of C/EBPβ was evaluated by Western analysis. CyclophilinB is used as a loading control.

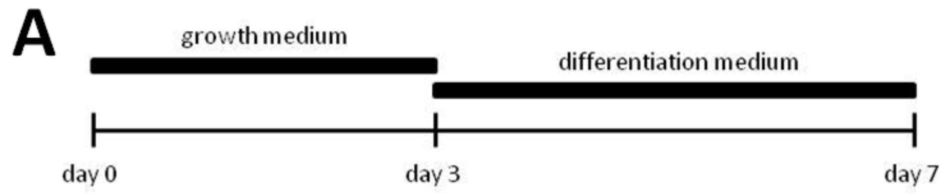
(B) Quantification of C/EBPβ expression from (A) relative to cyclophilinB expression, where protein expression in EDLmuscle is set at 1. Error bars represent the standard error of the mean of three independent trial.

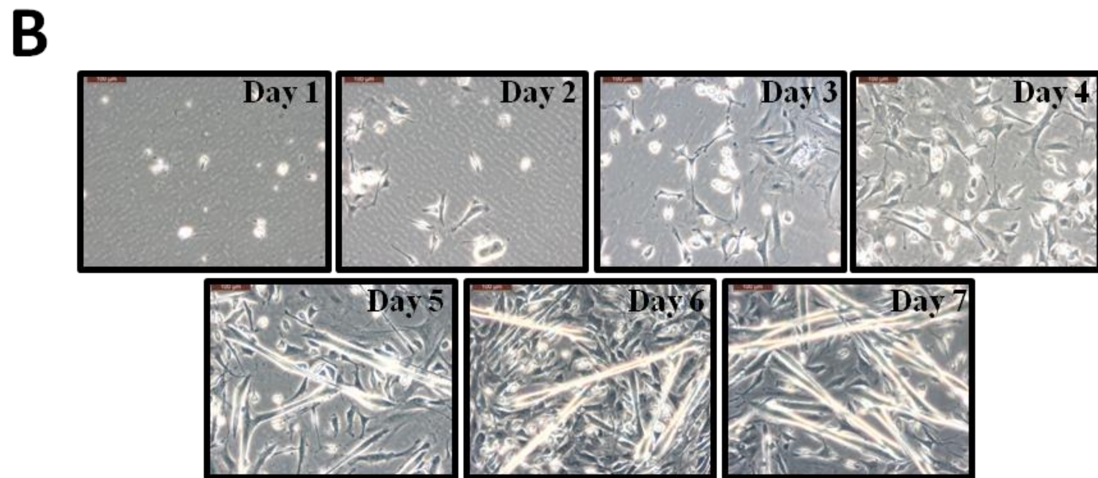
(C) Frozen cross sections of TA muscle were double immunostained with antibodies against C/EBPβ and Pax3 or Pax7 and visualized with fluorophore-conjugated secondary antibodies. White arrows indicate $Pax3^+$ and $Pax7^+$ cells. Scale bar = 10 μm.

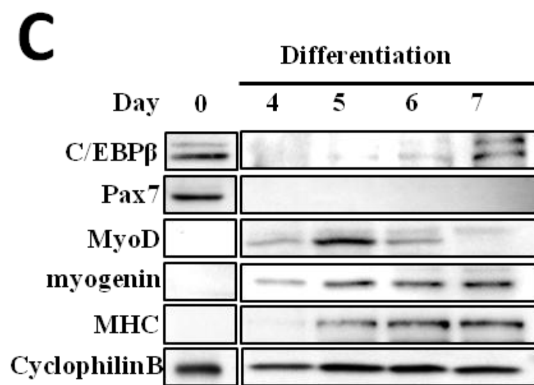
C/EBPβ protein expression is downregulated during differentiation.

We next wanted to study the regulation of C/EBPβ expression in satellite cells of wild type mice during differentiation. The satellite cells were released from the myofibers through enzymatic digestion with collagenase and dispase and pre-plating was performed to remove contaminating fibroblasts. We recognize that despite our best efforts, even after several passages of pre-plating, this method does not produce a population of pure satellite cells, though the process of pre-plating does greatly enrich this population. The day of isolation was referred to as Day 0 and the subsequent days were monitored until harvesting at Day 7. Isolated satellite cells were maintained on Matrigel coated plates, which recapitulates the extracellular environment and forms an attachment substrate for the cells. Isolated satellite cells were allowed to proliferate in growth media for 3 days before switching to low serum media to induce differentiation for an additional 4 days, prior to harvesting for protein analysis (Fig. 8A).

To elucidate the expression profiles of the key players in the myogenic program, a time course analysis for myogenic protein expression was carried out. Phase contrast images of the cultured satellite cells at each time point during the time course (except for Day 0) were taken prior to harvesting for protein to monitor proliferation and differentiation (Fig. 8B). Myoblast fusion into myotubes was observed beginning at Day 5 and several large myotubes were present at Day 7 (Fig. 8B). Western blot analyses of undifferentiated cells (Day 0) and differentiating cells (Days 4-7) demonstrated C/EBPβ expression in undifferentiated cells, following by a transient loss of this expression during differentiation and a re-emergence of C/EBPβ at Day 7 (Fig. 8C). As expected, satellite cell marker Pax7 was only observed at Day 0 in undifferentiated cells and was


A
growth medium
differentiation medium
day 0
day 3
day 7


B
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7


C
Differentiation
Day 0 4 5 6 7
C/EBPβ
Pax7
MyoD
myogenin
MHC
CyclophilinB

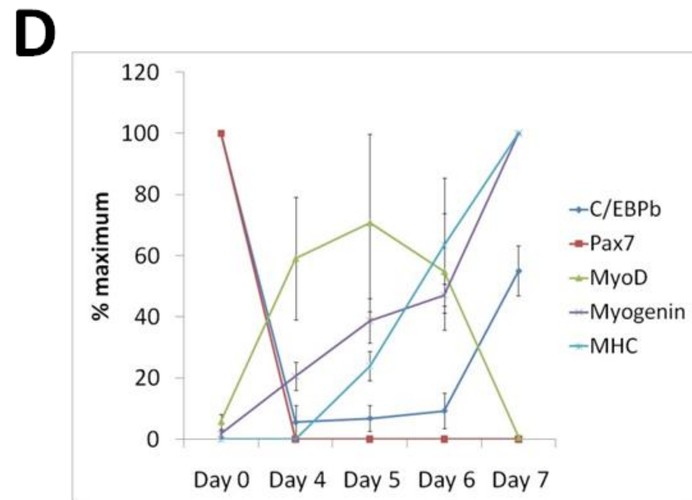

D
% maximum
120
100
80
60
40
20
0
Day 0 Day 4 Day 5 Day 6 Day 7
C/EBPb
Pax7
MyoD
Myogenin
MHC

Figure 8. C/EBPβ and myogenic factor protein expression during the differentiation of primary myoblasts in culture.

Primary myoblasts were isolated from 3 weeks old C57BL/6 mice and cultured for 7 days.

(A) Schematic representation of the experimental procedures for the culture and differentiation of isolated primary myoblasts. Cells were maintained in growth media and allowed to proliferate for 3 days prior to induction to differentiate in low serum media for an additional 4 days. Primary myoblasts were maintained on growth factor reduced Matrigel coated dishes.

(B) Phase contrast images of isolated primary myoblasts cultured and differentiated as in (A). Note the appearance of fused myotubes (black arrow) beginning at Day 5. Scale bar = 100 µm.

(C) Whole cell extracts (25 µg) from freshly isolated, undifferentiated primary myoblasts (Day 0) and differentiating myoblasts (Days 4-7) were resolved by SDS-PAGE and protein expression of C/EBPβ, Pax7, MyoD, myogenin and MHC were evaluated by Western analysis. CyclophilinB is used as a loading control.

(D) Quantification of protein expression from (C) relative to cyclophilinB expression and expressed as the % of maximal C/EBPβ, Pax7, myogenin and MHC expression during the time course analysis. Error bars represent the standard error of the mean of three independent trials.

not present in the differentiating myoblasts (Fig. 8C). MyoD was not present at Day 0 and began to be upregulated at Day 4 and disappeared at Day 7, coinciding with the re-appearance of C/EBPβ (Fig. 8C). The late myogenic markers myogenin and MHC had similar expression profiles, absent in undifferentiated cells and being induced during the later stages of differentiation (Fig. 8C). CyclophilinB was used a loading control.

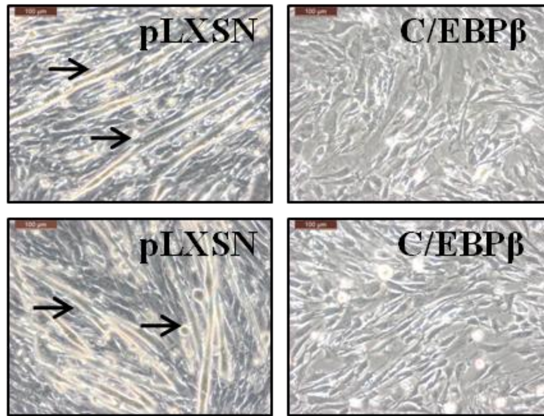
Quantification of protein expression as determined in Fig. 8C revealed an inverse correlation between C/EBPβ and MyoD (Fig. 8D). The line graph is representative of the % maximal expression for each protein of interest from undifferentiated and differentiating cells. Both C/EBPβ and Pax7 demonstrated maximal expression at Day 0 in undifferentiated cells. Furthermore, Pax7 is downregulated at Days 4-7 during differentiation (Fig. 8D). However, while C/EBPβ is downregulated at Days 4-6, it is re-expressed at Day 7. MyoD exhibits maximal expression at Day 5, while the later myogenic markers myogenin and MHC both demonstrate maximal expression at Day 7 (Fig. 8D).

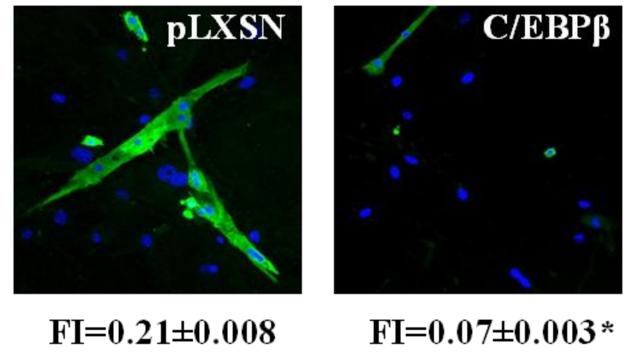
Ectopic expression of C/EBPβ in primary myoblasts inhibits expression of the myogenic factors.

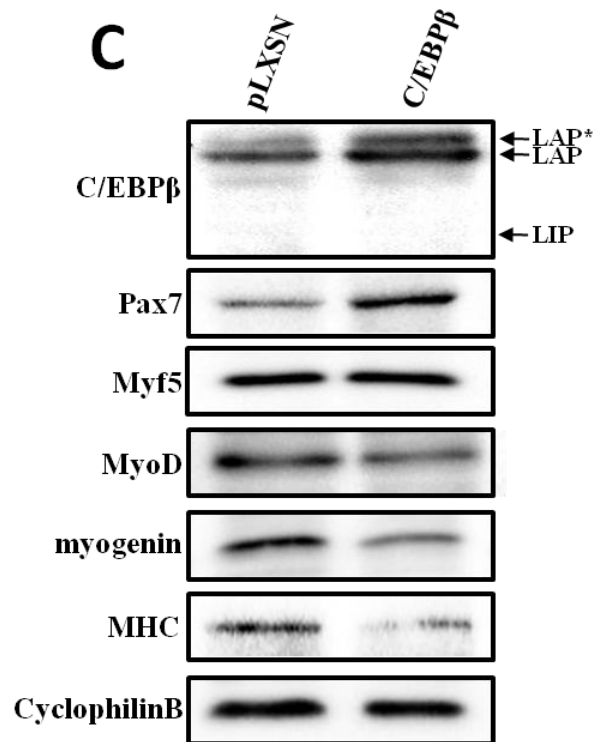
We next wanted to evaluate whether the ectopic expression of full length C/EBPβ in satellite cells would reduce their capacity to differentiate in culture and fail to express the markers defining the myogenic program. Satellite cells were isolated and upon culturing, were transduced with retrovirus to express full length C/EBPβ or with empty vector, pLXSN. The satellite cells were induced to differentiate for 4 days before harvesting for protein expression analysis.

Phase contrast images were taken prior to harvesting for protein to visualize the formation of myotubes (Fig. 9A). The empty vector control exhibited the formation of several large myotubes (representative fused myotubes are indicated with the black arrows) while the C/EBPβ-expressing satellite cells did not differentiate as efficiently (Fig. 9A). Furthermore, indirect immunocytochemistry with antibody against MHC revealed the presence of several $MHC^{+}/DAPI^{+}$ cells in the empty vector control, while in contrast, the C/EBPβ-expressing cells exhibited far fewer $MHC^{+}/DAPI^{+}$ cells that did not differentiate as efficiently as the pLXSN control cultures (Fig. 9B). Calculation of the fusion index revealed that C/EBPβ-expressing cultures demonstrated a less efficient FI=0.07±0.003 (*p<0.05) as compared to pLXSN control cultures (FI=0.21±0.008) (Fig. 9B).

Cultures were also analyzed for protein expression of the myogenic regulatory factors. Western blot analysis demonstrated an approximately two-fold increase (2.2±0.1, *p<0.05) in C/EBPβ protein expression in the cultures transduced to express full length C/EBPβ as compared to empty vector controls (set arbitrarily at 1) (Figs. 9C and 9D). Similar to C2C12 myoblasts, there was no significant difference in Myf5 protein in C/EBPβ-expressing cultures as compared to control cultures, set arbitrarily at 100 (124±19.5, *p>0.05) (Figs. 9C and 9D). While the myogenic marker protein expression (MyoD, myogenin and MHC) were all downregulated in the cells ectopically expressing C/EBPβ, the satellite cell marker, Pax7, was expressed at a higher level in these cells (Fig. 9C). Quantification of MyoD, myogenin and MHC expression demonstrated a significant reduction in protein in the C/EBPβ-expressing cells (49.1±2.9, 34.0±4.4, 32.3±2.8, respectively, *p<0.05) as compared to empty vector controls (set arbitrarily to


A
pLXSN
C/EBPβ
pLXSN
C/EBPβ


B
pLXSN
C/EBPβ
FI=0.21±0.008
FI=0.07±0.003*


C
pLXSN
C/EBPβ
LAP*
LAP
LIP
C/EBPβ
Pax7
Myf5
MyoD
myogenin
MHC
CyclophilinB

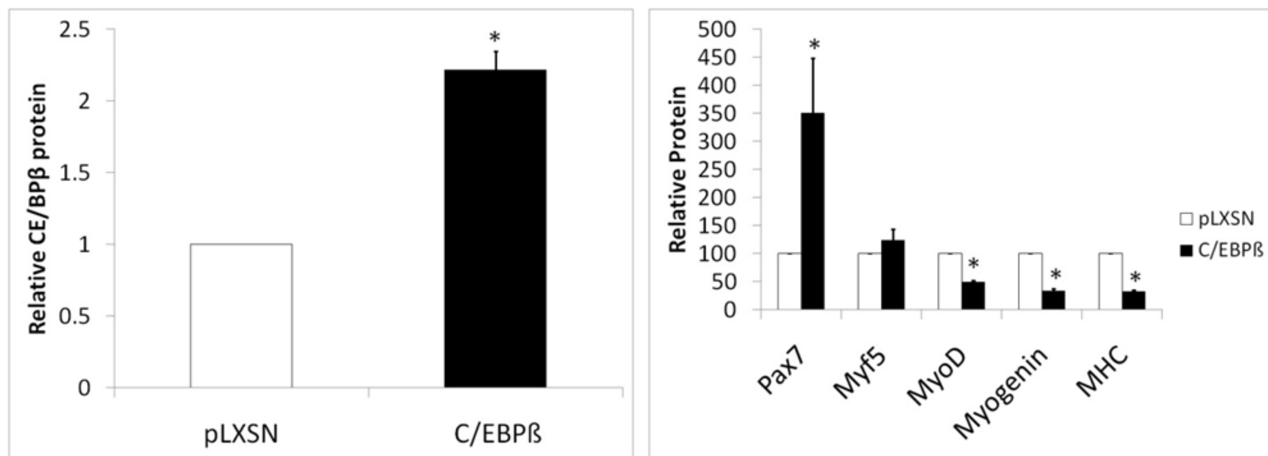

D
Relative CE/BPβ protein
pLXSN
C/EBPß
Relative Protein
Pax7
Myf5
MyoD
Myogenin
MHC
pLXSN
C/EBPß

Figure 9. Ectopic expression of C/EBPβ in primary myoblasts inhibits expression of the myogenic factors.

Primary myoblasts were isolated from 3 weeks old C57BL/6 mice and retrovirally transduced to express full length C/EBPβ or with empty vector (pLXSN). Cells were allowed to proliferate for 3 days prior to induction to differentiate in low serum media for an additional 4 days.

(A) Phase contrast images of transduced primary myoblasts. Note the presence of large, fused myotubes (black arrows) in the empty vector (pLXSN) control cultures, which are absent in the C/EBPβ-expressing cultures. Scale bar = 100 µm.

(B) Transduced primary myoblasts were fixed with ice-cold methanol and immunostained with antibody against MHC (green) and with DAPI (blue) for visualization of nuclei. Fusion index (FI) was calculated as the number of nuclei per myocyte/total number of nuclei. Error represents the standard error of the mean of three independent trials (*$p<0.05$).

(C) Whole cell extracts (25 µg) of cells transduced as described above were resolved by SDS-PAGE and protein expression of C/EBPβ, Pax7, Myf5, MyoD, myogenin and MHC were evaluated by Western analysis. CyclophilinB is used as a loading control.

(D) Quantification of protein expression from (C) relative to CyclophilinB expression, where pLXSN is set at 1 or 100. Error bars represent the standard error of the mean of three independent trials (*$p<0.05$).

100) (Fig. 9D). Quantification of Pax7 demonstrated a significant increase in protein expression (351.3±97.1, *p<0.05) as compared to control cultures (set arbitrarily at 100) (Fig. 9D). This persistent expression of Pax7 and the failure to express MyoD are consistent with a failure of quiescent satellite cells to activate and progress through the myogenic program to form myoblasts.

C/EBPβ occupies the MyoD promoter in C2C12 and primary myoblasts.

Our lab has clearly demonstrated the occupancy of the MyoD proximal promoter by C/EBPβ using chromatin immunoprecipitation (ChIP) in both C2C12 myoblasts and primary myoblasts (Figs. 10A, 10B and 10C, F. Marchildon and C. St-Louis). This occupancy correlates with decreased transcription from the promoter and is mediated, at least in part, by a C/EBP response element (CRE) located at position -95bp upstream of MyoD. The expression of C/EBPβ in unactivated satellite cells and its role in the inhibition of MyoD expression suggested that C/EBPβ may act to inhibit differentiation of these cells in the absence of appropriate stimuli for repair.

C/EBPβ$^{-/-}$ mice have smaller muscle fibers.

To evaluate the role of C/EBPβ during myogenesis in vivo, we obtained C/EBPβ$^{-/-}$ mice (Cebpb^{tm1Vpo}/J) from The Jackson Laboratory (Bar Harbor, ME, USA). Soleus and EDL muscles from 7 weeks old wild type and C/EBPβ$^{-/-}$ mice were harvested and processed for protein analysis to confirm the genotype. As expected, Western blot analysis revealed the expression of C/EBPβ protein in soleus and EDL muscle in wild type mice, but not in C/EBPβ$^{-/-}$ muscle (Fig. 11A). Soleus and EDL muscles of wild type and C/EBPβ$^{-/-}$ mice were harvested and cross sections were stained with hematoxylin and eosin for histological analysis. Visual observation with bright field microscopy did not

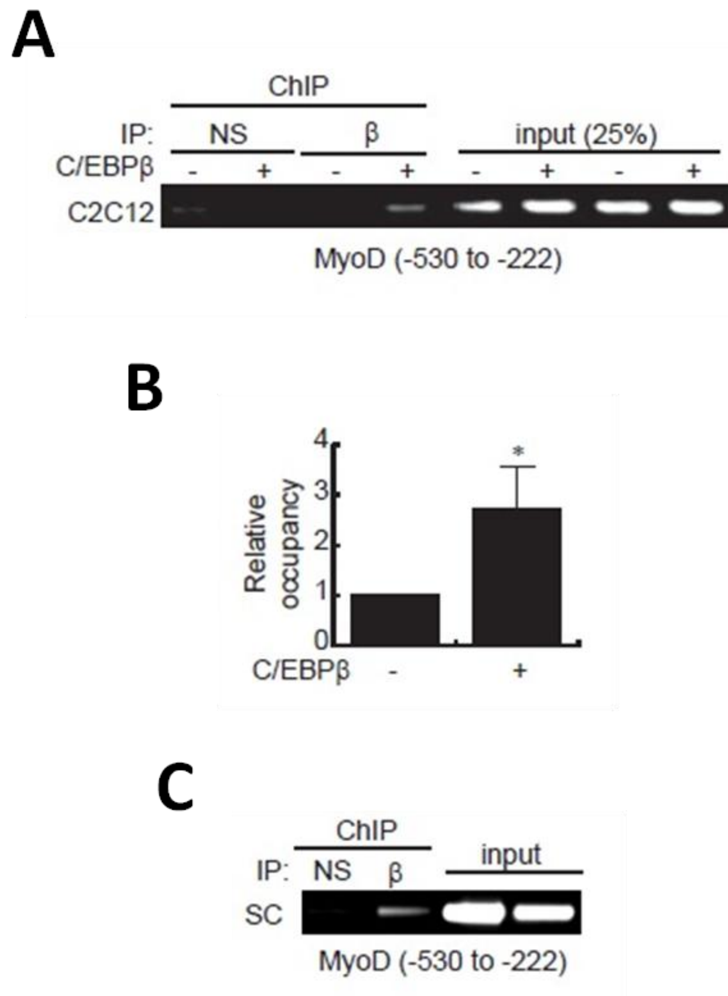
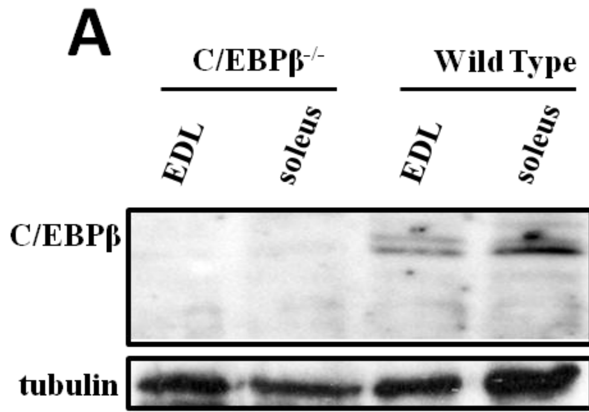


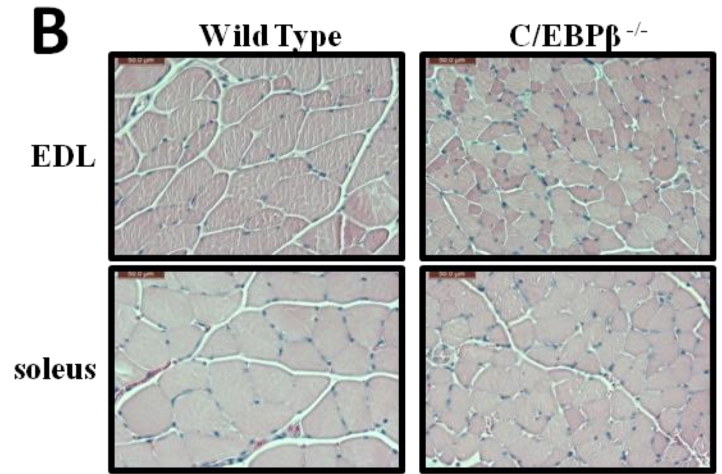
Figure 10. C/EBPβ occupies the MyoD promoter in C2C12 and primary myoblasts. (F. Marchildon, C.St-Louis)

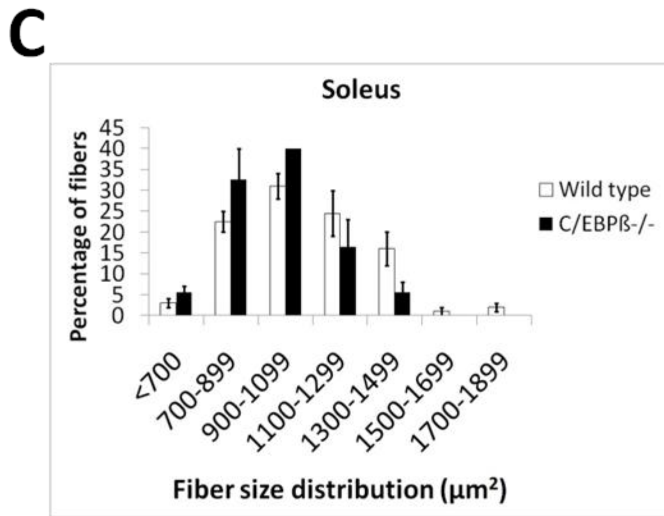
(A) C2C12 cells were retrovirally transduced to express full length C/EBPβ or with empty vector (pLXSN) and induced to differentiate for 5 days. Chromatin immunoprecipitation (ChIP) analysis of the MyoD promoter (-530 to -222) using anti-C/EBPβ antibody (β) or a type-matched non-specific antibody (NS). Inputs represent approximately 25% of the material used for immunoprecipitation.

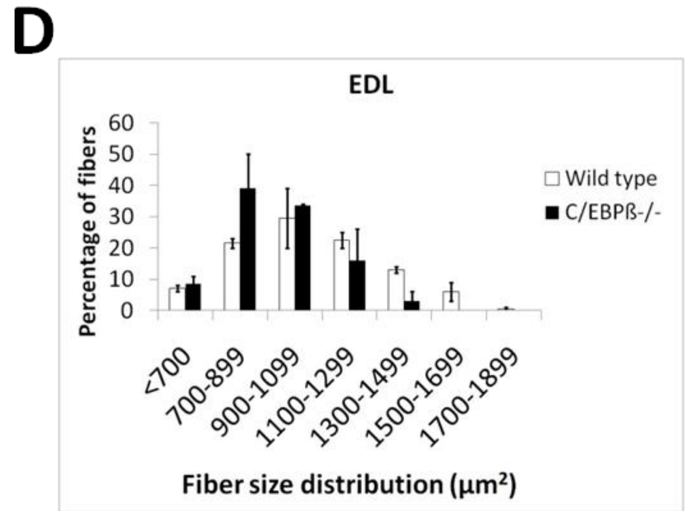
(B) Quantification of C/EBPβ occupancy on the MyoD promoter relative to input signal. Error bars represent the standard deviation of three independent trials (*$p<0.05$ as compared to pLXSN).

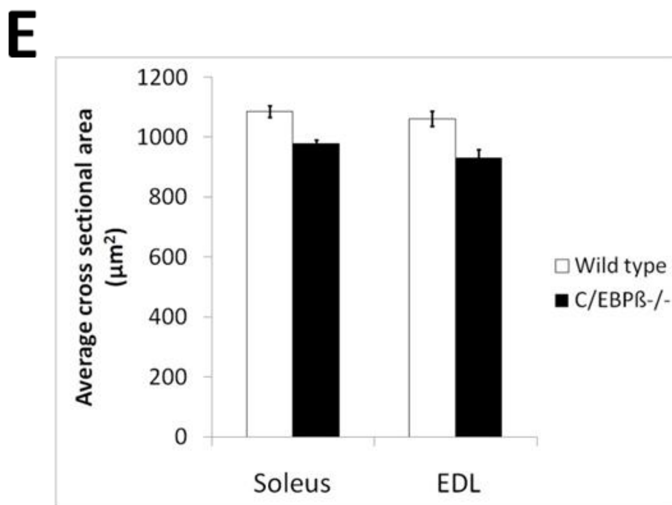
(C) Primary myoblasts were isolated from wild type hindlimb muscle and immediately processed for ChIP analysis of the MyoD promoter (-530 to -222) using anti-C/EBPβ antibody (β) or a type-matched non-specific antibody (NS). Inputs represent approximately 25% of the material used for immunoprecipitation.


A
C/EBPβ-/-
Wild Type
EDL
soleus
EDL
soleus
C/EBPβ
tubulin


B
Wild Type
C/EBPβ -/-
EDL
soleus


C
Soleus
Percentage of fibers
Wild type
C/EBPß-/-
<700
700-899
900-1099
1100-1299
1300-1499
1500-1699
1700-1899
Fiber size distribution (μm²)


D
EDL
Percentage of fibers
Wild type
C/EBPß-/-
<700
700-899
900-1099
1100-1299
1300-1499
1500-1699
1700-1899
Fiber size distribution (μm²)


E
Average cross sectional area (μm²)
Wild type
C/EBPß-/-
Soleus
EDL

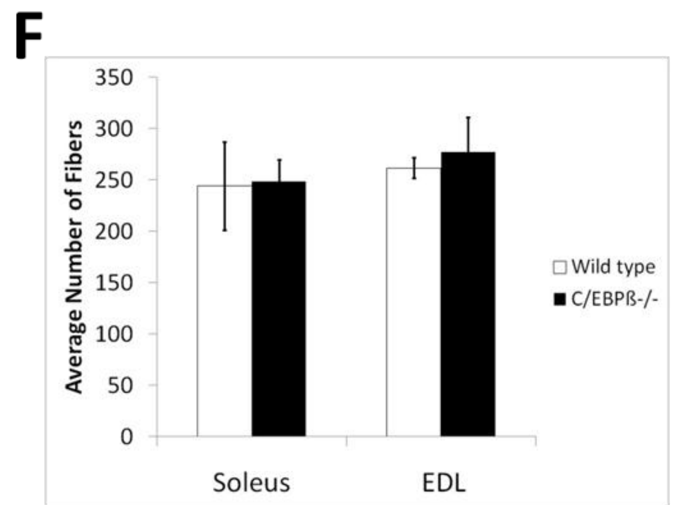

F
Average Number of Fibers
Wild type
C/EBPß-/-
Soleus
EDL

Figure 11. C/EBPβ$^{-/-}$ mice have smaller sized fibers.

Soleus and EDL muscles were excised from 7 weeks old wild type and C/EBPβ$^{-/-}$ mice and processed for Western analysis or for histological analysis.

(A) Whole cell extracts (25 μg) were resolved by SDS-PAGE and protein expression of C/EBPβ was evaluated by Western analysis. α-tubulin is used as a loading control.

(B) Bright field images of hematoxylin and eosin stained EDL and soleus muscle cross sections from wild type and C/EBPβ$^{-/-}$ mice. N=2 for each genotype. Scale bar = 50 μm.

(C) Cross sectional area analysis of fiber size from (B) and plotted as a fiber size distribution from wild type and C/EBPβ$^{-/-}$ soleus muscle. Error bars represent the standard error of the mean of two independent trials, where n=2 for each genotype.

(D) Cross sectional area analysis of fiber size from (B) and plotted as a fiber size distribution from wild type and C/EBPβ$^{-/-}$ EDL muscle. Error bars represent the standard error of the mean of two independent trials, where n=2 for each genotype.

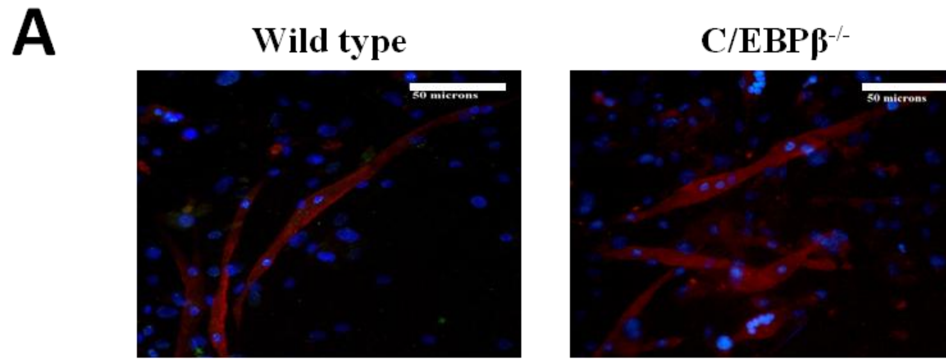
(E) Average cross sectional area analysis of fiber size from (B). Error bars represent the standard error of the mean of two independent trials, where n=2 for each genotype.

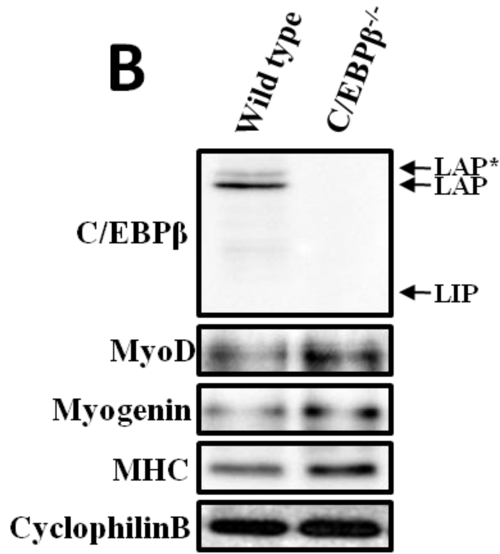
(F) Average number of fibers from (B). Error bars represent the standard error of the mean of two independent trials, where n=2 for each genotype.

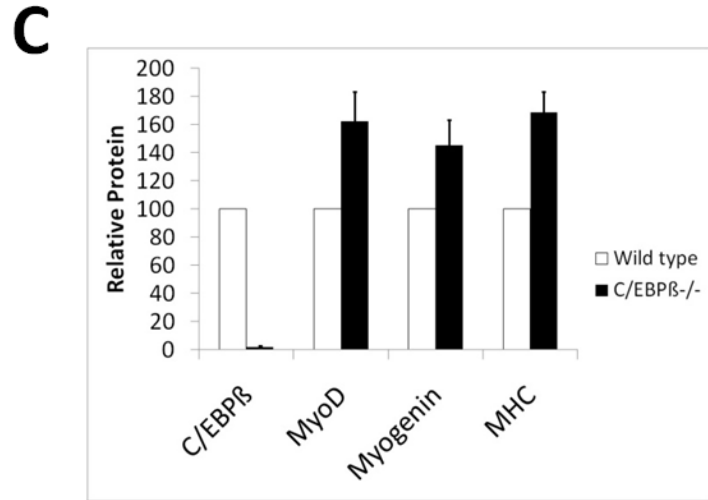
reveal any gross histological differences between wild type and C/EBPβ$^{-/-}$ mice, for both soleus and EDL muscle, though the muscle fibers appeared smaller in the null model (Fig. 11B). Fiber cross sectional area was calculated and their frequency plotted as a distribution of fiber sizes (Figs. 11C and 11D). In soleus muscle, C/EBPβ$^{-/-}$ mice demonstrated a greater proportion of smaller sized muscle fibers as compared to wild type mice (Fig. 11C). A similar trend was observed when the cross sectional area of EDL muscle was measured, with C/EBPβ$^{-/-}$ mice exhibiting a shift towards smaller fibers (Fig. 11D). Calculation of the average cross sectional area of soleus and EDL muscle demonstrated that wild type mice had slighter larger sized fibers as compared to C/EBPβ$^{-/-}$ mice, though this was not a significant result (Fig. 11E). Furthermore, wild type and C/EBPβ$^{-/-}$ mice demonstrated comparable number of fibers in both soleus and EDL muscles (Fig. 11F).

C/EBPβ$^{-/-}$ primary myoblasts differentiate comparable to wild type primary myoblasts in culture.

To evaluate the role and importance of C/EBPβ expression during the differentiation of primary myoblasts, we isolated primary myoblasts from wild type and C/EBPβ$^{-/-}$ mice and maintained them in culture. Indirect immunocytochemistry for MHC expression in satellite cells differentiated for 21 days demonstrated that satellite cells isolated from C/EBPβ$^{-/-}$ mice differentiated in culture as efficiently as wild type satellite cells (Fig. 12A). Protein analysis of C/EBPβ demonstrated both the LAP* and LAP isoforms, but not LIP, present in wild type satellite cells differentiated for 3 days in low serum media (Fig. 12B). Protein analysis of myogenic marker expression (MyoD, myogenin and MHC) from 3-days differentiated cells demonstrated comparable


A
Wild type
C/EBPβ-/-
50 microns
50 microns


B
Wild type
C/EBPβ-/-
LAP*
LAP
LIP
C/EBPβ
MyoD
Myogenin
MHC
CyclophilinB


C
Relative Protein
200
180
160
140
120
100
80
60
40
20
0
C/EBPß
MyoD
Myogenin
MHC
Wild type
C/EBPß-/-

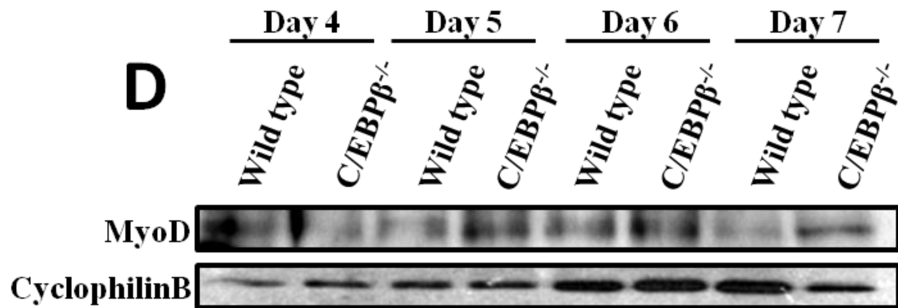

D
Day 4
Day 5
Day 6
Day 7
Wild type
C/EBPβ-/-
MyoD
CyclophilinB

Figure 12. C/EBPβ $^{-/-}$ satellite cells differentiate comparable to wild type satellite cells in culture.

Primary myoblasts were isolated from 7 weeks old wild type and C/EBPβ$^{-/-}$ littermates and maintained in culture.

(A) Wild type and C/EBPβ$^{-/-}$ satellite cells were differentiated for 21 days in low serum media and immunostained with antibody against MHC (red) and with DAPI (blue) for visualization of nuclei. Scale bar = 50 µm.

(B) Whole cell extracts (25 µg) of 3-days differentiated satellite cells (6 days post-isolation) from wild type and C/EBPβ$^{-/-}$ mice were resolved by SDS-PAGE and protein expression of C/EBPβ, MyoD, myogenin and MHC were evaluated by Western analysis. CyclophilinB was used as a loading control.

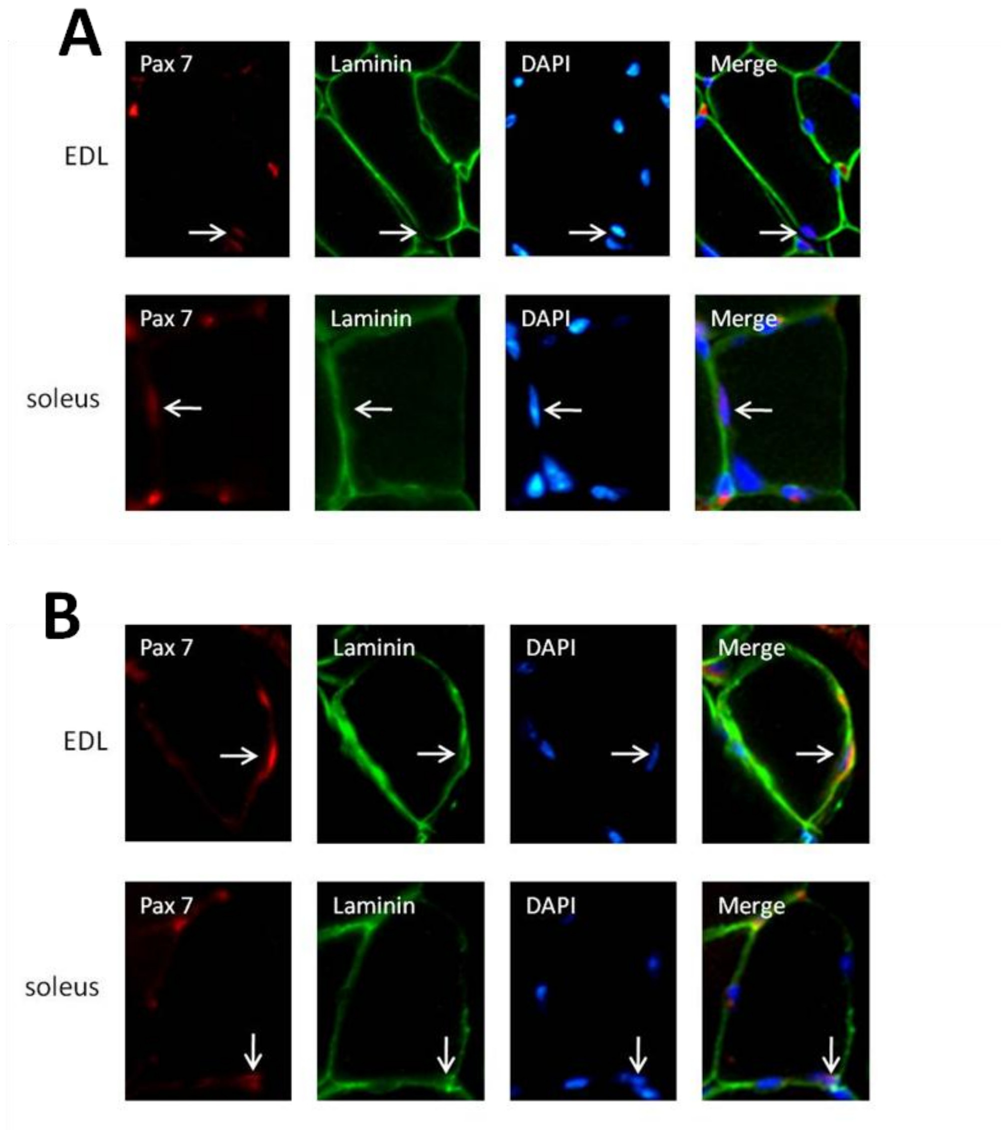
(C) Quantification of C/EBPβ, MyoD, myogenin and MHC from (B) relative to CyclophilinB, where protein expression from wild type is set at 100. Error bars represent the standard error of the mean of three independent trials.

(D) Whole cell extracts (25 µg) of satellite cells isolated from wild type and C/EBPβ$^{-/-}$ mice and differentiated for 1 to 3 days (4-7 days post-isolation) were resolved by SDS-PAGE and protein expression of MyoD was evaluated by Western analysis. CyclophilinB was used as a loading control.

expression between wild type and C/EBPβ$^{-/-}$ mice (Figs. 12B and 12C). Further analysis of MyoD expression revealed that despite the loss of C/EBPβ, MyoD was not precociously expressed in C/EBPβ$^{-/-}$ mice (Fig. 12D). Time course analysis revealed an induction of MyoD protein expression at similar time points between wild type and C/EBPβ$^{-/-}$ myoblasts (Fig. 12D). However, while MyoD expression disappeared at Day 7 in wild type cultures, similar to what we observed in the time course analysis and coinciding with the re-appearance of C/EBPβ protein (Fig. 8C), this expression persisted in C/EBPβ$^{-/-}$ cultures (Fig. 12D). These results suggest that the inhibition of MyoD expression by C/EBPβ is separate from the activation of MyoD expression *in vivo*, likely requiring additional factors.

C/EBPβ$^{-/-}$ mice have a smaller satellite cell population.

To assess the satellite cell population size, we performed indirect immunohistochemistry on cross sections of soleus and EDL muscles harvested from wild type and C/EBPβ$^{-/-}$ mice. Cross sections were double immunostained with antibodies against laminin, which highlights the basal lamina, and against Pax7, to identify satellite cells. DAPI was used for the visualization of nuclei. Satellite cells scored for quantification were identified as Pax7^{+}/DAPI^{+} and residing below the basal lamina (Figs. 13A and 13B). White arrows indicate Pax7^{+}/DAPI^{+} cells). Quantification of the number of satellite cells (expressed as a percentage of the total number of nuclei) showed that C/EBPβ$^{-/-}$ mice had reduced numbers of Pax7^{+} cells as compared to wild type mice, in both soleus and EDL muscles (Fig. 13C).


A
Pax 7
Laminin
DAPI
Merge
EDL
soleus
B
EDL
soleus

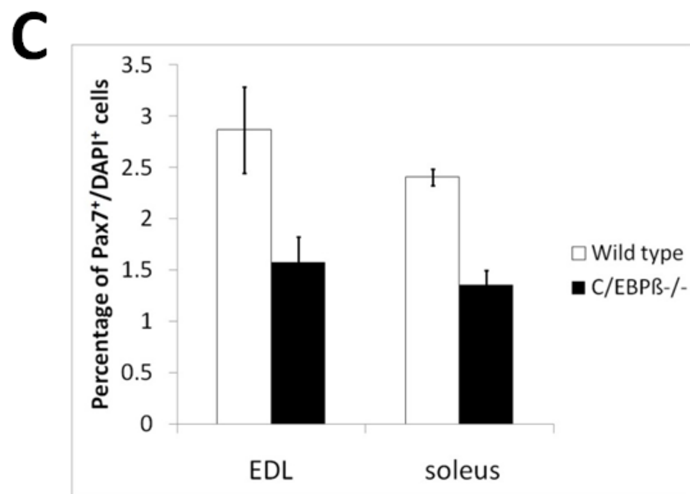

C
Percentage of Pax7+/DAPI+ cells
3.5
3
2.5
2
1.5
1
0.5
0
EDL
soleus
Wild type
C/EBPß-/-

Figure 13. C/EBPβ$^{-/-}$ mice have reduced numbers of Pax7^{+} satellite cells.

Soleus and EDL muscles were excised from 7 weeks old wild type and C/EBPβ$^{-/-}$ mice and flash frozen for indirect immunohistochemistry. Frozen cross sections were double immunostained with antibodies against Pax7 (red), laminin (green) and with DAPI (blue) for visualization of nuclei.

(A) Photomicrographs of satellite cell localization in wild type EDL and soleus muscles. Pax7^{+} cells were identified by their sub-laminal position in the muscle. White arrows indicate Pax7^{+} and DAPI^{+} cells, residing beneath the basal lamina.

(B) Photomicrographs of satellite cell localization in C/EBPβ$^{-/-}$ EDL and soleus muscles. Pax7^{+} cells were identified by their sub-laminal position in the muscle. White arrows indicate Pax7^{+} and DAPI^{+} cells, residing beneath the basal lamina.

(C) Quantification of Pax7^{+} cells from (A) and (B). Error bars represent the standard error of the mean, where n=2 for each genotype.

DISCUSSION

The progressive loss of muscle observed with age (sarcopenia) and the dramatic muscle wasting seen in certain cancers (cachexia) can be attributed, at least in part, to a failure of the skeletal muscle stem cells, satellite cells, to initiate the myogenic differentiation program. Furthermore, sepsis and spaceflight are also both associated with muscle wasting. In all conditions, there is a shift in the equilibrium between protein synthesis and protein catabolism, leading to muscle atrophy. The debilitating loss of skeletal muscle associated with the aforementioned conditions can lead to muscle weakness and loss of strength, and can result in more serious consequences, such as increased risk of injury and reduced chances of survival for cancer patients. In all of these models of muscle wasting, C/EBPβ expression has been demonstrated to be upregulated (Penner, Gang et al. 2002; Giresi, Stevenson et al. 2005; Allen, Bandstra et al. 2009).

This thesis suggests that the direct consequence of upregulated C/EBPβ expression in wasting skeletal muscle would be the inhibition of skeletal myogenesis. In C2C12 myoblasts and primary myoblasts, the ectopic expression of C/EBPβ can downregulate the expression of the myogenic regulatory factors and MHC, suggesting an impairment of myoblast differentiation and fusogenic ability. With the use of C/EBPβ mutant constructs, the data revealed the importance of the DNA binding domain for the repressive action of C/EBPβ, acting at the level of the MyoD promoter to prevent myogenesis. The results demonstrate an inverse correlation between MyoD and C/EBPβ protein expression. In addition to its role in inhibiting skeletal myogenesis, the data propose a potential involvement of C/EBPβ in the maintenance of the undifferentiated state of the satellite cells.

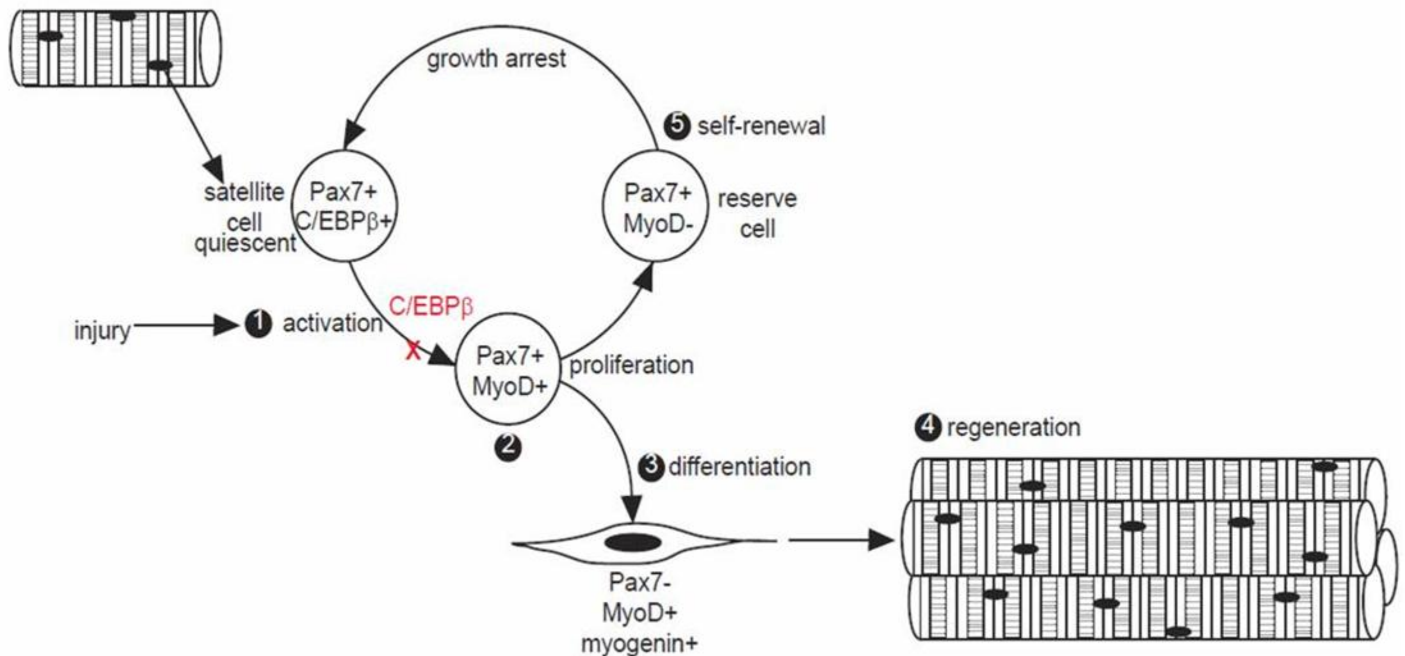


Figure 14. C/EBPβ is a negative regulator of myogenesis.

Following injury or trauma to the muscle fiber, quiescent satellite cells ($Pax7^{+}$/C/$EBP\beta^{+}$/$MyoD^{-}$) are activated **(1)** to initiate the myogenic program. Early satellite cell progenitors maintain Pax7 expression, while the downregulation of C/EBPβ expression correlates with the expression of MyoD. Cells re-enter the cell cycle as proliferating myoblasts **(2)**. The majority of these $Pax7^{+}$/$MyoD^{+}$ cells will lose expression of $Pax7^{+}$ and begin to express in a coordinated fashion, the late markers of differentiation, including myogenin **(3)**. These cells will become mature myocytes and fuse together or with existing myofibers to repair the injured muscle **(4)** Some proliferating myoblasts do not progress down the myogenic pathway and maintain expression of Pax7 while downregulating expression of MyoD. They re-express C/EBPβ and return to the quiescent state as reserve cells, capable of being re-activated to differentiate **(5)**.

We propose a model in which C/EBPβ is a negative inhibitor of skeletal muscle differentiation (Fig. 14). Upon injury or trauma to the skeletal muscle, the quiescent satellite cells ($Pax7^+$/C/EBPβ^+/$MyoD^-$) are activated to initiate the myogenic program (Fig. 14-1). Cells will lose expression of C/EBPβ and begin to proliferate as $Pax7^+$/$MyoD^+$ myoblasts (Fig. 14-2). Cells downregulate Pax7 expression and begin to express in a coordinated fashion the late markers of myogenesis (Fig. 14-3). These cells differentiate into mature myocytes which fuse together or to pre-existing muscle fibers thereby repairing the injured muscle (Fig. 14-4). A sub-population of $Pax7^+$/$MyoD^+$ myoblasts do not progress down the differentiation pathway but rather lose expression of MyoD, re-express C/EBPβ and return to quiescence as reserve cells, which still retain myogenic potential and can be re-activated to differentiate (Fig. 14-5).

Our results also generated many important questions, namely (1) How does C/EBPβ act to inhibit MyoD expression?, (2) Does regulation of MyoD by C/EBPβ expression promote self-renewal of the satellite cells niche?, and (3) Does C/EBPβ expression regulate Pax7 expression to maintain the undifferentiated state?

(1) C/EBPβ is a transcriptional repressor of MyoD activity and expression

Characterized as the master regulator of myogenesis, MyoD is expressed exclusively in committed myogenic cells (Weintraub, Davis et al. 1991). When C/EBPβ was ectopically expressed in C2C12 myoblasts and primary myoblasts, there was no effect on Myf5 protein expression, in contrast to previous observations in C3H10T1/2 mesenchymal stem cells in which Myf5 protein was downregulated. Since we did not observe a downregulation of Myf5 protein in C/EBPβ-expressing C2C12 myoblasts and

primary myoblasts, this suggested that C/EBPβ did not have an effect on commitment to the myogenic lineage, but rather affected the later stages of myogenesis. In accordance, we observed a significant decrease in MyoD protein expression, suggesting that C/EBPβ negatively regulates myogenesis at the level of MyoD. Indeed, chromatin immunoprecipitation (ChIP) analysis performed in our lab demonstrated the occupancy of the MyoD promoter by C/EBPβ in both C2C12 myoblasts transduced to overexpress C/EBPβ and undifferentiated primary myoblasts, resulting in a decrease in transcriptional activity from the MyoD promoter. This repression of transcriptional activity from MyoD is consistent with the downregulation of the late markers of myogenesis, including myogenin and MHC.

Expression of two different C/EBPβ mutants revealed the requirement of the DNA binding domain for the inhibitory function of C/EBPβ during myogenesis. The C/EBPβ mutant featuring a truncation of the first 21 amino acids in the N-terminus, mimicking the naturally occurring LAP isoform, exhibited impaired MyoD expression comparable to full length C/EBPβ. In contrast, the C/EBPβ mutant bearing two point mutations in the DNA binding domain was just as efficient as control cultures in activating MyoD expression and initiating myogenesis. The N-terminal 21 amino acids of C/EBPβ have been demonstrated to recruit the SWI/SNF chromatin remodeling complex (Kowenz-Leutz and Leutz 1999) and the histone acetyltransferease p300 (Schwartz, Beck et al. 2003) to activate gene transcription. Our observations suggest that these 21 amino acids and any interacting co-factors are dispensable for the repressive action of C/EBPβ on MyoD. Rather, these results further support a possible mechanism by which C/EBPβ

acts to inhibit MyoD expression, through binding directly to the MyoD promoter and repressing its transcriptional activity.

Based on these observations, we would expect that in the absence of C/EBPβ, there would be no brake on MyoD regulation, and thus in C/EBPβ$^{-/-}$ myoblasts, we might predict precocious expression of MyoD. However, our analysis of cultured C/EBPβ$^{-/-}$ myoblasts did not demonstrate precocious MyoD expression as compared to wild type myoblasts. Rather, we observed an induction of MyoD expression at similar time points in C/EBPβ$^{-/-}$ and wild type myoblasts. However, while wild type cultures exhibited a downregulation of MyoD expression during differentiation, correlating with a transient reappearance of C/EBPβ at Day 7 post isolation, C/EBPβ$^{-/-}$ cultures exhibited persistent MyoD expression which continued to rise. Since we did not observe precocious MyoD expression in the C/EBPβ$^{-/-}$ cultures, this suggested that additional regulators of MyoD are required for the initiation of expression, as the loss of C/EBPβ was not sufficient to induce early MyoD expression. Indeed, FoxO3 and Pax3/7 were demonstrated to simultaneously occupy the MyoD promoter in C2C12 myoblasts and primary myoblasts to positively regulate transcriptional activity from the MyoD promoter (Hu, Geles et al. 2008).

The family of inhibitor of DNA binding (Id) proteins have also been shown to inhibit myogenesis by sequestering E proteins and forming non-functional heterodimers unable to bind DNA (Olson 1990). At the onset of myogenesis, Id is downregulated and liberates the E proteins to heterodimerize with MyoD to activate gene transcription. Interestingly, Id2 is a direct target of C/EBPβ during lobuloalveolar development of the mammary gland (Karaya, Mori et al. 2005) and thus may also interfere with MyoD

activity in addition to inhibition at the level of transcription. It would be of interest to speculate whether the ectopic expression of C/EBPβ in C2C12 myoblasts and primary myoblasts causes an upregulation of Id and further represses skeletal myogenesis.

C/EBPβ mediated repression of transcriptional targets.

Our work suggests that C/EBPβ inhibits transcription through direct binding to the MyoD promoter, though the inhibitory mechanism is as of yet unknown. There is plenty of evidence linking inhibitory C/EBPβ action with interaction with co-repressor proteins. C/EBPβ can form an inhibitory complex with HDAC1 to repress activity from the C/EBPα promoter to stimulate liver proliferation (Wang, Salisbury et al. 2008) and with HDAC1/mSin3A to repress differentiation of pre-adipocytes (Wiper-Bergeron, Wu et al. 2003). During microbial infection, C/EBPβ can interact with the p50 subunit of NF-κB to form a complex repressing transcription of the microRNA, let-7i, and resulting in an increase in pathogen recognition and activation of the innate immune response conferred by toll-like receptor 4 (O'Hara, Splinter et al. 2010). Furthermore, C/EBPβ and Oct-1 can simultaneously bind to adjacent sites in the enhancer of the gonadotrophin-releasing hormone gene, thereby repressing its expression through a nitric oxide mediated pathway (Belsham and Mellon 2000). C/EBPβ has also been demonstrated to play a role in the regulation of immunoglobulin proteins, more specifically, the repression of IgM transcript and protein by the LIP isoform (Hatada, Chen-Kiang et al. 2000). In human foreskin keratinocytes, C/EBPβ can repress expression of the human papillomavirus type 11 (HPV11) gene (Wang, Liu et al. 1996); however, recent work has demonstrated that C/EBPβ can switch from a repressor to an activator of HPV11 gene expression through the mutation of a consensus binding site in the upstream regulatory region of HPV11

(Ralph, Liu et al. 2006). Pancreatic β-cells subjected to high glucose concentrations exhibited decreased insulin levels and elevated C/EBPβ levels, suggesting a possible role for C/EBPβ in the regulation of insulin gene transcription (Lu, Seufert et al. 1997). Despite a high affinity binding site in the rat insulin 1 promoter, C/EBPβ represses transcription from the rat insulin 1 gene through an interaction with E47 and preventing the dimerization and binding of E47 to the E-boxes in the rat insulin 1 promoter (Lu, Seufert et al. 1997), suggesting that C/EBPβ could potentially interfere with MRF function as well as MyoD expression.

(2) Self-renewal pathway of satellite cells

For continual muscle growth and regeneration throughout an organism's life, satellite cells must possess the capacity for self-renewal to replenish satellite cells that have progressed along the myogenic pathway to become terminally differentiated myofibers. There appears to be several mechanisms by which satellite cells achieve self-renewal. First, asymmetrical division of $Pax7^{+}/Myf5^{-}$ satellite stem cells gives rise to both myogenic daughter cells and those that remain undifferentiated. Once these myogenic daughter cells enter the differentiation pathway and express MyoD, nearly half will downregulate MyoD expression and return to quiescence (reserve cells),contributing once again to the self-renewal of satellite cells (Yoshida, Yoshida et al. 1998). Examination of the biochemical markers of myogenesis in C/EBPβ$^{-/-}$ myoblasts in differentiation conditions revealed that while MyoD levels were downregulated in wild type cells, this downregulation did not occur in C/EBPβ$^{-/-}$ cells. This result suggests that C/EBPβ's role in satellite cells may not be to prevent precocious MyoD expression per se, but rather to

promote the downregulation of MyoD in activated cells. While the experiments contained in this thesis did not directly address this possibility, we predict that in the absence of C/EBPβ, the formation of reserve cells through downregulation of MyoD expression would be perturbed, resulting in both robust myogenesis and a reduction in the satellite cells pool over time. This is in accordance with our observations of fewer $Pax7^{+}$ satellite cells in the $C/EBP\beta^{-/-}$ mouse.

$C/EBP\beta^{-/-}$ model

In addition to the reduced number of $Pax7^{+}$ satellite cells, we observed that $C/EBP\beta^{-/-}$ mice have a greater proportion of fibers with a smaller cross sectional area, although the average cross sectional area and the number of fibers is comparable to wild type littermates. These observations are in support with our prediction that over time, the loss of C/EBPβ will result in progressively fewer $Pax7^{+}$ satellite cells capable of differentiating to form new muscle, and thus have an effect on post-natal growth and repair. However, $C/EBP\beta^{-/-}$ animals have a complex phenotype including but not limited to metabolic dysregulation, immune dysfunction and reduced adipose tissue. Since $C/EBP\beta^{-/-}$ mice exhibit fasting hypoglycemia as a result of impaired liver function to increase glucose production (Liu, Croniger et al. 1999), we might predict that these mice are less active than their wild type littermates and do not utilize their muscles in the same capacity, another limitation in properly evaluating skeletal muscle growth and repair. In addition, $C/EBP\beta^{-/-}$ mice also demonstrate decreased levels of plasma fatty free acids and enhanced insulin signaling in skeletal muscle, both of which contribute to the increased insulin sensitivity of $C/EBP\beta^{-/-}$ mice (Wang, Shao et al. 2000). Smaller sized fibers in the $C/EBP\beta^{-/-}$ mouse could be explained by (1) a role for C/EBPβ in the normal growth of

muscle post-natally; (2) the requirement of C/EBPβ expression for myoblast proliferation; or (3) metabolic defects in the C/EBPβ$^{-/-}$ animal.

The complete C/EBPβ$^{-/-}$ model is thus, not an ideal animal model to investigate the role of C/EBPβ expression in satellite cells. Indeed, in addition to the metabolic defects, it has recently been shown that during muscle regeneration after injury, C/EBPβ-expressing macrophages are necessary for the removal of necrotic tissue and for the induction of anti-inflammatory genes, leading to proper muscle repair (Ruffell, Mourkioti et al. 2009).

A more appropriate model for studying the loss of C/EBPβ in satellite cells and its effect on post-natal growth and repair would be to employ Cre/lox P recombination technology to generate a conditional knockout mouse. For example, expression of tamoxifen-activated Cre recombinase driven by the Pax7 promoter in floxed C/EBPβ mice produces a temporal and spatial knock out of C/EBPβ expression in the Pax7^{+} satellite cells. Limitations with using an inducible Cre recombinase include reaching high levels of excision of the targeted gene. Since Pax7 is a transcription factor and not abundantly expressed in the skeletal muscle, there may be difficulties in reaching high excision rates to inactivate C/EBPβ. Another possibility would be to use a Cre recombinase that is under the control of the endogenous Myf5 locus as we have demonstrated that C/EBPβ exerts its effect downstream of Myf5 expression. With this, we are still able to achieve tissue specific inactivation of C/EBPβ in skeletal muscle as Myf5 is expressed in both quiescent satellite cells and proliferating myoblasts.

(3) C/EBPβ expression maintains the undifferentiated state of satellite cells

Pax7 and C/EBPβ express similar expression patterns during myogenesis, both present in quiescent satellite cells and downregulated during differentiation. Indeed, ectopic Pax7 expression in satellite cells was demonstrated to downregulate MyoD and promote exit from the cell cycle, returning to a state of quiescence (Olguin and Olwin 2004). Likewise, the constitutive expression of C/EBPβ prevented MyoD expression and initiation of the myogenic program, as observed in C2C12 myoblasts and primary myoblasts, acting instead to maintain the undifferentiated state. Accordingly, in C2C12 myoblasts and primary myoblasts retrovirally transduced to overexpress C/EBPβ, we see a significant increase in Pax7 protein expression as compared to our control cultures, suggesting C/EBPβ might be actively acting to maintain the undifferentiated state of satellite cells.

In addition to the maintenance of the undifferentiated state, Pax7 has been implicated in the specification of satellite cells, as muscle-derived stem cells from Pax7$^{-/-}$ mice were highly capable of forming hematopoietic colonies (Seale, Sabourin et al. 2000). Furthermore, Pax7$^{-/-}$ mice exhibited impaired myogenesis (Seale, Sabourin et al. 2000) and while they have comparable satellite cell numbers at birth, they demonstrated a dramatic loss of satellite cells during post-natal growth (Relaix, Montarras et al. 2006). Similarly, C/EBPβ$^{-/-}$ mice demonstrated a nearly 50% reduction in Pax7^{+} satellite cells compared to wild type mice. However, C/EBPβ$^{-/-}$ mice did not exhibit any gross muscle abnormalities and C/EBPβ$^{-/-}$ primary myoblasts differentiated as efficiently in culture, suggesting that C/EBPβ does not have a role in satellite cell specification.

Interestingly, when Pax7 is inactivated in satellite cells after post-natal day 21 (P21), there was no detrimental effect on muscle regeneration in response to injury and the cells were also able to re-occupy the satellite cell niche, demonstrating the capacity for self-renewal, suggesting that another factor is able to compensate for Pax7 functions (Lepper, Conway et al. 2009). Since the data presented herein suggests that C/EBPβ is capable of maintaining the undifferentiated state, it is interesting to speculate that C/EBPβ is this factor in the post-natal organism. Exploiting this ability of C/EBPβ to maintain the undifferentiated state in the adult can lead to the generation of therapies targeted at keeping C/EBPβ levels in satellite cells elevated. Myoblast transplantation is a potential therapeutic approach that can be used for treatment of such myopathies as Duchenne muscular dystrophy (Palmieri, Tremblay et al. 2010). An important limitation of myoblast transplantation is the activation of satellite cells immediately post-isolation, thus initiating the myogenic program and limiting their effectiveness in host muscle. The development of treatments preventing the downregulation of C/EBPβ to keep the undifferentiated state of satellite cells, thus improving engraftment rates and satellite cell niche repopulation, are of value and importance.

Biological implications of C/EBPβ regulation of MyoD

Loss of skeletal muscle and muscle wasting is observed in cancer (Zhou, Wang et al. 2010), sepsis (Penner, Gang et al. 2002), spaceflight (Allen, Bandstra et al. 2009) and with age (Giresi, Stevenson et al. 2005). The significant loss of muscle associated with these conditions renders the afflicted individuals with increased risk of injury due to frailty and weakness and for cancer patients, reduced chances of survival.

In sarcopenia, Giresi et al. have demonstrated upregulated C/EBPβ transcript levels in aged human skeletal muscle (Giresi, Stevenson et al. 2005). Preliminary results from our lab demonstrated an upregulation in C/EBPβ protein in soleus and EDL muscles of 32 weeks old mice as compared to 7 weeks old mice.

Furthermore, our lab has demonstrated upregulated expression of C/EBPβ protein in mice suffering from cancer cachexia, and this correlated with a decrease in MyoD protein expression. C/EBPβ expression can be induced by inflammatory cytokines, such as IL-6, and its transcription is increased upon lipopolysaccharide stimulation (Trautwein, Caelles et al. 1993; Bradley, Zhou et al. 2003). Several inflammatory cytokines, such as TNF-α and IL-6, have been shown to be responsible for initiating cachexia, as mice injected with repeated rounds of cytokines exhibited evidence of cachexia (Matthys and Billiau 1997). Given that certain cancers are associated with systemic inflammation this can induce elevated C/EBPβ levels in muscle satellite cells.

Cachexia is also observed in sepsis, which is characterized by an upregulation of C/EBPβ expression and activity (Penner, Gang et al. 2002). This upregulation in expression and activity is glucocorticoid dependent, as treatment with a glucocorticoid receptor antagonist was sufficient to inhibit this sepsis-induced increase in C/EBPβ (Penner, Gang et al. 2002).

Furthermore, after 12 days of spaceflight, C/EBPβ mRNA levels were upregulated in the gastrocnemius muscle of mice subjected to spaceflight (Allen, Bandstra et al. 2009). Spaceflight has been associated with having detrimental effects on

skeletal muscle and even short duration spaceflights can result in significant muscle atrophy (LeBlanc, Rowe et al. 1995).

Conditions associated with a loss of skeletal muscle, such as sarcopenia, cancer cachexia, sepsis and spaceflight all demonstrated elevated levels of C/EBPβ which, as we proposed in this thesis, would have a negative impact on skeletal muscle growth and repair. Based on the observations in this thesis, we propose a model of C/EBPβ inhibition of myogenesis, whereby elevated levels of C/EBPβ in the satellite cells negatively regulate expression of MyoD, thus repressing muscle differentiation. We postulate that elevated C/EBPβ contributes to and exacerbates the wasting phenotype by preventing efficient muscle growth and repair in the conditions described. It is clear that the impact of impaired muscle growth and repair can have damaging and debilitating consequences for individuals suffering from conditions associated with significant muscle wasting. Therapies targeted at downregulating C/EBPβ or preventing C/EBPβ activity at the MyoD promoter would conceivably help to re-establish the initiation of myogenic differentiation required for proper muscle growth and repair.

FUTURE DIRECTIONS

Based on our results, we propose that C/EBPβ levels must first be downregulated for differentiation to occur. In muscle wasting conditions, we suggest that signals which trigger the downregulation of C/EBPβ are impaired or absent or are overcome by signals which maintain C/EBPβ expression, thus keeping C/EBPβ levels elevated and preventing efficient muscle regeneration. In healthy muscle, satellite cells are capable of

downregulating C/EBPβ and activating MyoD expression in the occurrence of injury to initiate differentiation and repair. An important topic that is raised by this thesis is the question of what triggers C/EBPβ downregulation in the satellite cells of injured muscle. As C/EBPβ expression is transcriptionally activated through cAMP signaling by CREB (Niehof, Manns et al. 1997), we might suggest that there are alterations in cAMP signaling to effectively downregulate C/EBPβ for muscle differentiation. Indeed, elevated cAMP signaling has been shown to inhibit skeletal muscle differentiation through activity of PKA (Li, Heller-Harrison et al. 1992). In contrast, there is evidence that cAMP signaling actually promotes skeletal muscle myogenesis. The administration of a phosphodiesterase 4 (PDE4) inhibitor into mice displaying muscle atrophy was sufficient to further inhibit muscle loss by preventing the degradation of intracellular cAMP levels (Hinkle, Dolan et al. 2005). In addition, the treatment of septic mice with PDE resulted in elevated cAMP levels and reduced skeletal muscle proteolysis (Lira, Graca et al. 2007). A suggested mechanism for this cAMP-mediated inhibition of skeletal muscle atrophy is through the decrease of atrogin-1 expression and inhibition of the ubiquitin-proteosome system, thus preventing muscle protein catabolism (Goncalves, Lira et al. 2009). Elevated cAMP signaling would increase C/EBPβ expression, which may be occurring in the infiltrating macrophages during muscle regeneration. C/EBPβ-expressing macrophages are necessary for efficient muscle regeneration after injury by removing necrotic tissue and inducing the expression of anti-inflammatory genes (Russell and Tisdale 2010), thus protecting the muscle from further proteolysis.

Although we propose a role for C/EBPβ in the self-renewal process of satellite cells to generate reserve cells, this remains a hypothesis until we are able to determine

differences in self-renewal capabilities between the wild type and C/EBPβ$^{-/-}$ mice. One method to determine this would be to isolate single muscle fibers from C/EBPβ$^{-/-}$ and wild type littermates and maintain them in culture, as described by Rosenblatt et al. (Rosenblatt, Lunt et al. 1995). This technique results in the detachment of satellite cells from the muscle fiber which demonstrate the ability to proliferate and can be induced to differentiate to form a large, multinucleated network. Rosenblatt et al. demonstrated that these cells that migrate from single fibers are all myogenic, as determined by desmin staining, in contrast to the enzymatic digestion of muscle which releases not only satellite cells but also non-myogenic cells, such as fibroblasts. Thus, to evaluate the role of C/EBPβ in self-renewal, we can culture single fibers from wild type and C/EBPβ$^{-/-}$ mice and monitor the progression of detached satellite cells during differentiation and the subsequent generation of a population of reserve cells. Using indirect immunocytochemistry, we can use antibodies against Pax7 and MyoD to distinguish between the two populations of cells. Based on our observations, we would predict that C/EBPβ$^{-/-}$ satellite cells will differentiate as efficiently as the wild type satellite cells; however, these cells will fail to downregulate MyoD and fail to undergo the self-renewal process, leading to reduced numbers of Pax7^{+}/MyoD^{-} reserve cells with every cycle of activation. We would therefore predict to observe fewer Pax7^{+}/MyoD^{-} cells in our C/EBPβ$^{-/-}$ cultures as compared to our wild type cultures, which we predict are capable of undergoing this self-renewal process to generate Pax7^{+}/MyoD^{-} reserve cells.

It would also be of interest to determine if C/EBPβ plays a role in regulating Pax7 expression and activity. Analysis of the regulatory region of human Pax7 revealed binding sites for such transcription factors as MyoD and CREB. C/EBPβ can bind to

CREB response elements and in addition, a CCAAT box motif in reverse orientation was identified upstream of the Pax7 promoter (Syagailo, Okladnova et al. 2002), suggesting that C/EBPβ may directly regulate Pax7 expression. We demonstrated that ectopic C/EBPβ expression in primary myoblasts resulted in upregulated Pax7 protein, characteristic of a less committed myogenic phenotype. C/EBPβ and Pax7 demonstrate similar expression patterns in regards to myogenesis, so it would be interesting to speculate that downregulation of C/EBPβ expression in activated satellite cells results in the loss of Pax7 expression and the expression of MyoD.

C/EBPβ has been previously described to be necessary for the differentiation of pre-adipocytes in vitro (Wiper-Bergeron, Wu et al. 2003) and for the formation of fat in the post-natal organism, where it is expressed in post-natal adipose tissue (Tanaka, Yoshida et al. 1997; Lee, Hausman et al. 1998). In 5-azacytidine treated mesenchymal stem cells, the ectopic expression of C/EBPβ promoted the adipogenic phenotype while inhibiting the formation of myotubes (Fig. 4A). When we overexpressed C/EBPβ in C2C12 myoblasts and primary myoblasts, we observed inhibition of myogenesis without induction towards the adipogenic lineage. C2C12 myoblasts and primary myoblasts are committed to the myogenic lineage and accordingly, we observed no effect on Myf5 expression in C/EBPβ-expressing cultures, suggesting C/EBPβ does not influence commitment to the myogenic lineage (Fig. 4B). Interestingly, it has been shown that muscle satellite cells also possess the ability to differentiate into osteocytes or adipocytes given the appropriate signals (Asakura, Komaki et al. 2001). In addition, satellite cells were demonstrated to convert to a fibrogenic lineage in response to signals from the systemic environment of aged mice, resulting in increased fibrosis (Brack, Conboy et al.

2007). In conditions where C/EBPβ levels are elevated, such as in sarcopenia, this could prevent the differentiation of satellite cells into myocytes and permit their conversion to an adipogenic and/or fibrogenic lineage, both of which are observed in sarcopenic muscle (Brack, Conboy et al. 2007).

In vivo muscle injury and repair experiments would be of importance to elucidate the physiological relevance of C/EBPβ during myogenesis. In culture, we have described comparable differentiation potential of C/EBPβ$^{-/-}$ myoblasts. However, *in vivo*, we would predict that in response to muscle injury, C/EBPβ$^{-/-}$ mice would exhibit impaired regeneration based on the reduced numbers of Pax7^{+} satellite cells available for activation and repair.

To determine the role of C/EBPβ in post-natal myogenesis, we can use the Cre/lox P transgenic mice to temporally inactivate C/EBPβ before and after P21. Since our observations in the juvenile C/EBPβ$^{-/-}$ mouse revealed reduced Pax7^{+} cell numbers, consistent with our hypothesis of C/EBPβ in the self-renewal of satellite cells, it would be of interest to quantify the Pax7^{+} population at birth. We would predict that at birth, similar to Pax7$^{-/-}$ mice (Relaix, Montarras et al. 2006), C/EBPβ$^{-/-}$ mice would exhibit normal numbers of satellite cells but that this population would progressively decrease during post-natal development.

CONCLUDING REMARKS

This thesis provides evidence for an inhibitory function of C/EBPβ on MyoD regulation during myogenesis. This repression is mediated, at least in part, by the DNA

binding domain of C/EBPβ interacting with the MyoD promoter. At the onset of differentiation, C/EBPβ expression must first be downregulated for the expression of the myogenic regulatory factors and the proper initiation and progression of myogenesis. In vivo observations revealed smaller fibers and a reduced $Pax7^+$ satellite cell population in the absence of C/EBPβ. Loss of C/EBPβ is not sufficient to promote precocious MyoD expression, suggesting that additional regulators must also be involved for the transcriptional activation of MyoD. Furthermore, we propose that C/EBPβ may act to maintain the undifferentiated state of the satellite cells, based upon upregulated Pax7 protein in C/EBPβ-expressing cultures. C/EBPβ expression is upregulated in muscle wasting conditions such as sarcopenia, cancer cachexia, sepsis and spaceflight and we propose a novel mechanism in which C/EBPβ is a negative regulator of skeletal muscle differentiation.

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