Regulation of Satellite Cell Homeostasis by C/EBPβ: Therapeutic Perspectives

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ABSTRACT

Regeneration of adult skeletal muscle relies upon a population of quiescent myogenic progenitor cells, called satellite cells (SCs). Upon injury, SCs activate, proliferate, differentiate and fuse to make new myofibers or to repair damaged ones. SCs can also self-renew to repopulate the SC niche. The balance between differentiation and self-renewal is critical to maintain muscle homeostasis and changes in this equilibrium can lead to chronic muscle degeneration. For example, Duchenne's muscular dystrophy (DMD) is characterized by rounds of muscle degeneration and regeneration leading to increased muscle wasting. One approach to treat DMD is transplantation of SCs. For this treatment to be viable, transplanted cells must contribute to repairing injured muscle and repopulating the SC niche. Here, we show that the transcription factor CCAAT/Enhancer Binding Protein beta (C/EBPβ) regulates SC function. C/EBPβ is down-regulated during differentiation and persistent expression of C/EBPβ inhibits differentiation and expression of the myogenic regulatory factors MyoD and Myogenin. C/EBPβ also promotes Pax7 expression by directly binding to and regulating *Pax7* transcription. Using genetic tools to conditionally excise C/EBPβ expression in SCs, we found that C/EBPβ-null SCs lose quiescence and precociously differentiate at the expense of self-renewal. After a single injury, C/EBPβ-deficient SCs failed to self-renew, resulting in impaired muscle repair after a second injury. C/EBPβ-induced quiescence also requires upregulation of caveolin-1. Furthermore, pharmacological manipulation of C/EBPβ expression with the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), increased the number of cells available for transplantation into dystrophic muscle and enhanced the expression of stem cell markers in a C/EBPβ-dependent fashion. IBMX

treatment improved cell survival and migration, engraftment into the SC niche and repair of dystrophic muscle. Together, these results demonstrate that C/EBPβ is an important regulator of SC function and that pharmacological manipulation of C/EBPβ improves culture conditions for the expansion and selection of SCs available for cell therapy for the treatment of muscular dystrophies.

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LIST OF ABBREVIATIONS

ALCL	Anaplastic Large Cell Lymphoma
$BaCl_2$	Barium Chloride
bFGF	basic Fibroblast Growth Factor
BrdU	Bromodeoxyuridine
bZIP	Basic Leucine Zipper
cAMP	cyclic Adenosine Monophosphate
Cav-1	Caveolin-1
C/EBP	CCAAT/Enhancer Binding Protein
C/EBPβ	CCAAT/Enhancer Binding Protein Beta
CEE	Chick Embryo Extract
cGMP	cyclic Guanosine Monophosphate
ChIP	Chromatin Immunoprecipitation
cKO	Conditional Knock-Out
CRE	cAMP Response Element
CREB	cAMP Response Element-binding Protein
CTX	Cardiotoxin
DGC	Dystrophin-Glycoprotein Complex
DI	Differentiation Index
DM	Differentiation Media
DMD	Duchenne's Muscular Dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DPI	Days Post Injury

DTR	Diphtheria Toxin Receptor
EDL	Extensor Digitorum Longus
ECM	Extracellular Cellular Matrix
eIF-4E	Elongation Translation Initiation Factor 4E
eMyHC	Embryonic Myosin Heavy Chain
ERK	Extracellular Signal-Regulated Kinase
FBS	Fetal Bovine Serum
FI	Fusion Index
GFP	Green Fluorescent Protein
GH	Growth Hormone
GM	Growth Media
HDAC1	Histone Deacetylase 1
HGF	Hepatocyte Growth Factor
HS	Horse Serum
HSPG	Heparan Sulfate Proteoglycan
HuR	Human Antigen R
IBMX	3-Isobutyl-1-Methylxanthine
IHC	Immunohistochemistry
IL-1β	Interleukin-1 Beta
KLF4	Kruppel-Like Factor 4
LAP	Liver-enriched Activator Protein
LIP	Liver-enriched Inhibitory Protein
Mdm2	Mouse Double Minute 2 homolog

MEF	Mouse Embryonic Fibroblasts
miR	microRNA
MPC	Myogenic Precursor Cell
MRF	Myogenic Regulatory Factor
MRF4	Myogenic Regulatory Factor 4
MS	Mass Spectrometry
MSC	Mesenchymal Stem Cell
mTOR	Mammalian Target of Rapamycin
Myf5	Myogenic factor 5
MyHC	Myosin Heavy Chain
MyoD	Myoblast determination protein
MyoG	Myogenin
mRNA	messenger Ribonucleic Acid
NICD	Notch Intracellular Domain
NF-κβ	Nuclear Factor-kappa Beta
p21	Postnatal day 21
p56	Postnatal day 56
Pax7	Paired-box 7
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PDEi	Phosphodiesterase inhibitor
PFA	Paraformaldehyde
PIAS1	Protein Inhibitor of Activated STAT 1

PKA	Protein Kinase A
PTM	post-translational modifications
RBP	RNA-binding protein
RNA-Seq	RNA-Sequencing
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
SC	Satellite Cell
SDF-1	Stromal Cell-Derived Factor-1
SEM	Standard Error Mean
Ser	Serine
SFM	Serum Free Media
SP	Side Population
Sol	Soleus
TA	Tibialis anterior
TBP	TATA-Binding Protein
TGFβ	Transforming Growth Factor Beta
Thr	Threonine
WT	Wild-Type

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CHAPTER ONE: INTRODUCTION

1.1. Developmental Myogenesis and Specification of Satellite Cells

Skeletal muscle is one of the most dynamic tissues of the mammalian body and accounts for approximately 40% of total body weight (Frontera & Ochala 2015). It is made up of an arrangement of multinucleated muscle fibers and associated connective tissue. The main function of adult skeletal muscle is to convert chemical energy into mechanical force for voluntary movement, to maintain posture and to allow breathing (Frontera & Ochala 2015). Adult skeletal muscle relies on a mechanism that maintains tissue homeostasis during regeneration of terminally differentiated myofibers. This mechanism is the activation, proliferation and differentiation of resident muscle stem cells called satellite cells (Mauro 1961; Bischoff 1975; Collins et al. 2005).

The majority of skeletal muscles in vertebrates originate from segmental mesodermal structures called the somites (Buckingham & Vincent 2009). The somites differentiate along the dorsal-ventral axis to give rise to the dorsally located dermomyotome, which gives rise to a number of tissues including skeletal muscle of trunk and limbs (Buckingham & Vincent 2009). Myogenic progenitors of the limb muscle separate from the hypaxial dermomyotome, migrate to the limb field where they proliferate, differentiate and fuse to generate multinucleated myotubes which eventually become mature muscle fibers (Buckingham et al. 2003).

Satellite cells share the embryonic origins as the muscle they are localized in. Using quail-chick chimaeras and genetic tracing in chick and mice, studies have shown that limb and trunk muscle satellite cells originate from the dermomyotome (Gros et al. 2005; Schienda et al. 2006; Tajbakhsh 2009). Progenitor cells expressing the paired box transcription factor Pax7 are first seen loosely associated with differentiating muscle

fibers late in fetal development, at embryonic day 15.5 (E15.5), in the mouse and become satellite cells by taking their position under a basal lamina and outside the sarcolemma of the muscle fiber (Kassar-duchossoy et al. 2005; Relaix et al. 2005; Mauro 1961; Lepper & Fan 2010). The number of satellite cells decrease significantly after birth from 30% of sublaminar nuclei to less than 5% in adult (P56, postnatal day 56) (White et al. 2010). In the adult these cells are maintained in a quiescent state to elicit repair and maintain homeostasis of adult muscle (Schultz et al. 1978; Pallafacchina et al. 2010).

1.2. Adult Myogenesis Depends on Satellite Cells

In intact muscle, quiescent satellite cells make up approximately 2-5% of myonuclei and are characterized by their expression of Pax7 (Cornelison & Wold 1997; Seale et al. 2000; Fukada et al. 2007; Kuang & Rudnicki 2008; Gnocchi et al. 2009). Upon muscle injury, physiological or pathological, these cells re-enter the cell cycle and start to proliferate (Zammit et al. 2004). Proliferating satellite cells are commonly referred to as myogenic precursor cells (MPCs) or myoblasts and are characterized by expression of the myogenic regulatory factors (MRFs), MyoD and Myf5 (Cooper et al. 1999; Cornelison & Wold 1997; Füchtbauer & Westphal 1992; Grounds et al. 1992; Smith et al. 1994; Yablonka-Reuveni & Rivera 1994; Murphy & Kardon 2011). Subsequently, myoblasts downregulate Pax7, irreversibly withdraw from the cell cycle, upregulate the expression of the MRFs, myogenin and MRF4, and commit to terminal differentiation to become myocytes (Hasty et al. 1993; Chen & Goldhamer 1999; Zammit et al. 2004; Tedesco et al. 2010). Following cell cycle exit, myocytes undergo cell-to-cell fusion to repair damaged muscle or to form new multinucleated fibers (Schmalbruch & Hellhammer

1976; Robertson et al. 1990; Papadimitriou et al. 1990; Robertson et al. 1993). In contrast to myoblasts that undergo differentiation and fusion, some myoblasts can self-renew and re-enter quiescence. These myoblasts maintain Pax7 expression, downregulate MyoD expression, and return to mitotic quiescence (Collins et al. 2005; Zammit et al. 2004; Zammit et al. 2006) (Figure 1).

Self-renewal is a crucial process for maintaining the satellite cell pool required for sustainable muscle regeneration (Collins et al. 2005; Montarras, Morgan, Collins, Frederic Relaix, et al. 2005; Sacco et al. 2008). Several studies have shown that satellite cells can generate both differentiated myofibers and myogenic precursors when transplanted into mouse muscle (Gross & Morgan 1999; Heslop et al. 2001; Montarras, Morgan, Collins, Frederic Relaix, et al. 2005). For example, transplantation of a single satellite cell or a single muscle fiber contributed to the repair of hundreds of new muscle fibers (Collins et al. 2005; Sacco et al. 2008). Furthermore, when GFP-positive Pax3 expressing cells were isolated from Pax3-GFP^{+} mice and grafted into the muscle of dystrophic nude mice, these cells were not only able to contribute to fiber repair, but some also persisted in the satellite cell niche and expressed Pax7 (Montarras, Morgan, Collins, Frederic Relaix, et al. 2005).

The importance of satellite cells in regeneration was further demonstrated in a number of studies in which satellite cells were ablated using Cre-lox recombination technology (Lepper et al. 2011; McCarthy et al. 2011; Murphy et al. 2011; Sambasivan et al. 2011). Satellite cells were eliminated using mice with an inducible Pax7^{CreERT2} allele crossed with Rosa$^{DTR/+}$ mice to activate a Diptheria toxin sequence (Lepper et al. 2011; McCarthy et al. 2011; Murphy et al. 2011) or administration of the Diptheria toxin into

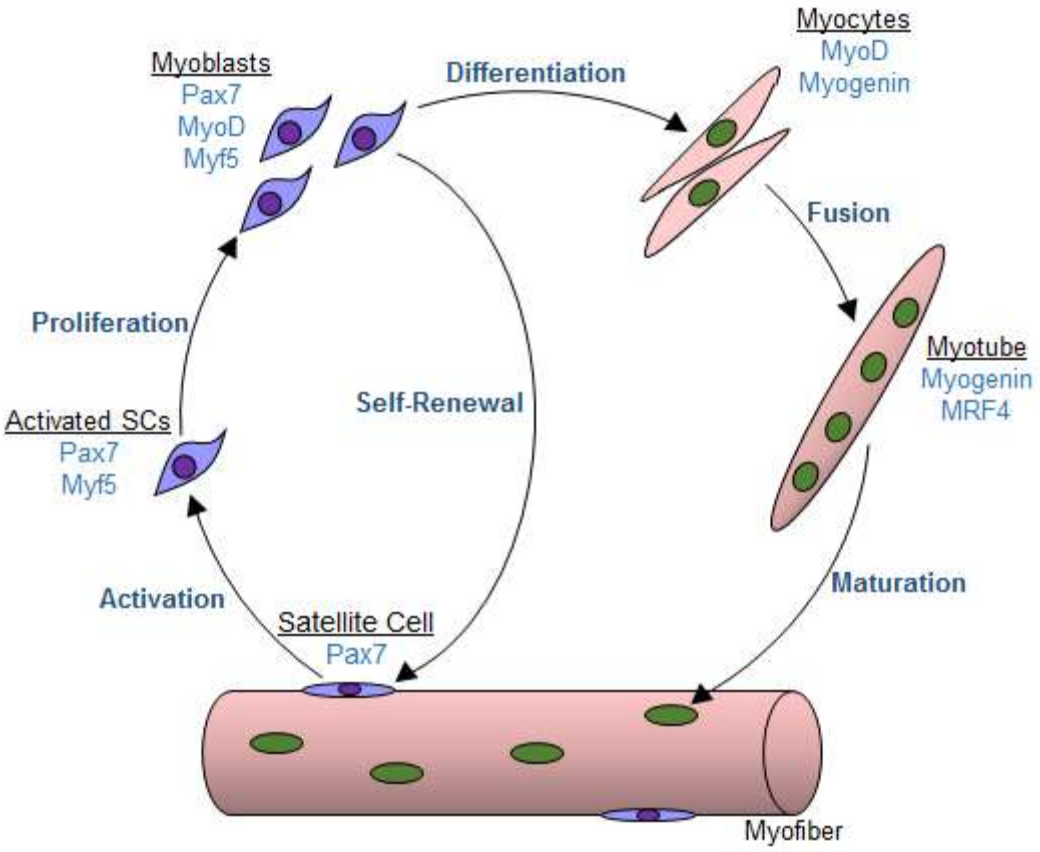

Figure 1. Lineage Progression during Adult Myogenesis. Postnatal muscle regeneration relies on quiescent satellite cells (SCs) characterized by Pax7 expression. Upon muscle injury, satellite cells activate and upregulate Myf5 protein expression. Activated satellite cells quickly upregulate MyoD and proliferate as myoblasts. Subsequently, myoblasts upregulate myogenin and differentiate into myocytes which upregulate MRF4 and form myotubes. In addition to differentiation, satellite cells can also self-renew by retaining Pax7 expression and downregulating MyoD expression after activation.

$Pax7^{DTR/+}$ mice in which a Pax7 allele encodes for the Dipteheria toxin receptor (Sambasivan et al. 2011). In each study, ablation of $Pax7^{+}$ satellite cells led to a failure to regenerate after injury accompanied by an increase in fibrosis (Murphy et al. 2011) as well as fatty tissue accumulation (Sambasivan et al. 2011). Furthermore, non-satellite cell types were inadequate for muscle regeneration in these mice (Sambasivan et al. 2011; Tedesco et al. 2010) and was rescued only by transplantation of $Pax7^{+}$ cells (Sambasivan et al. 2011). Thus, Pax7-expressing satellite cells are essential for muscle regeneration and other potential myogenic cells that do not express Pax7 cannot compensate for loss of these cells.

1.2.1. Requirement of Pax7 for Satellite Cell Function

The Pax transcription factors play an important role in organogenesis during embryonic development and are characterized by a conserved paired domain that confers sequence-specific DNA binding. Some of the family members, including Pax7, may contain full or partial homeodomains that also interact with DNA (Lang et al. 2007; Buckingham & Relaix 2015).

At birth, Pax7-null mice appear normal but display perinatal muscle growth retardation and die prematurely around 2 to 3 weeks after birth (Mansouri et al, 1996; Kuang et al., 2006; Seale et al., 2000). A small number of Pax7-null mice (5-10%) survive to adulthood and exhibit reduced satellite cell numbers (Kuang et al. 2006; Oustanina et al. 2004). When isolated, the few remaining satellite cells displayed reduced proliferation and differentiation in culture (Kuang et al. 2006; Oustanina et al. 2004). Muscle weakness and smaller muscle fibers containing fewer myonuclei have also been

reported in Pax7 mutant mice (Kuang et al. 2006; Frédéric Relaix et al. 2006; Seale et al. 2000); however, one study reported that Pax7 mutant mice did not display a significant reduction in the number or size of myotubes in postnatal muscle (Oustanina et al. 2004). These differences are mostly likely due to the difference in the genetic background of the mutant mice (Buckingham & Relaix 2015).

The role of Pax7 in adult satellite cells has been examined using conditional mutations of the gene in $Pax7^{CreERT2/flox}$ mice and $Pax7^{CE/loxP\text{-}Gu}$ mice (Lepper et al. 2009; von Maltzahn et al. 2013; Günther et al. 2013). The first study done by Lepper et al. using the $Pax7^{CreERT2/flox}$ mouse, in which Pax7 was specifically excised in satellite cells when injected with tamoxifen, reported that Pax7 function is critical only before postnatal day 21 (P21) and is dispensable in adult regenerative myogenesis (Lepper et al. 2009). Surprisingly, these results are in contrast to those obtained from $Pax7^{-/-}$ mice which suggested that Pax7 plays a central role in satellite cell function (Kuang et al. 2006; Oustanina et al. 2004; Frédéric Relaix et al. 2006). Contrary to the Lepper findings, two more recent studies demonstrated that Pax7 is absolutely required for normal function of satellite cells during regeneration of adult skeletal muscle (von Maltzahn et al. 2013; Günther et al. 2013).

Using the same inducible Pax7-Cre allele, von Maltzahn et al. showed a severe regeneration defect one week after cardiotoxin injury in mice administered with tamoxifen between P40 and P44 (von Maltzahn et al. 2013). Unlike the previous study (Lepper et al. 2009), mice subjected to intraperitoneal injections of tamoxifen were also maintained on food containing tamoxifen throughout the experiment to prevent the re-growth of satellite cells that escaped excision of Pax7 (von Maltzahn et al. 2013; Brack

2014). Furthermore, the loss of Pax7 resulted in a reduced number of satellite cells in both uninjured and injured muscle demonstrating that Pax7-deficient satellite cells cannot self-renew (von Maltzahn et al. 2013). The role of Pax7 in satellite cell function was further assessed using another conditional Pax7 mutant mouse in which the first three exons of *Pax7* are deleted to prevent the generation of a truncated Pax7 protein that may contain some biological activity (Günther et al. 2013). Similar to the results from the Rudnicki lab (von Maltzahn et al. 2013), Pax7 expression in satellite cells was essential for efficient repair and satellite cell self-renewal during regenerative myogenesis.

Furthermore, forced expression of Pax7 in myoblasts correlates with the notion that Pax7 regulates satellite cell expansion and self-renewal (Olguin & Olwin 2004; Zammit et al. 2006; Olguin et al. 2007; McFarlane et al. 2008). When overexpressed in myoblasts or myofiber-associated satellite cells, Pax7 delayed myogenin induction and differentiation (Olguin & Olwin 2004; Zammit et al. 2006; Olguin et al. 2007; McFarlane et al. 2008). Pax7 was also shown to repress myogenesis independently of its transcriptional activity, regulating MyoD activity and MyoD protein stability (Olguin et al. 2007).

1.3. Function of Myogenic Regulatory Factors in Myogenesis

Adult skeletal myogenesis is a well-orchestrated process regulated by a number of transcription factors known as the myogenic regulatory factors (MRFs). MRFs are a family of basic helix loop helix (bHLH) transcriptional regulators that are responsible for regulating the transcription of muscle specific genes (Weintraub et al. 1989). The members of this family include Myf5, MyoD, myogenin and MRF4 which are

sequentially expressed during myogenesis. Gene targeting experiments have provided much insight into the functions of the MRFs and has revealed the existence of a hierarchical relationship among these transcription factors. For example, MyoD and Myf5 act as primary MRFs and are required for the conversion of pre-myogenic cells into skeletal myoblasts (Chen & Goldhamer 1999), whereas, myogenin and MRF4 are secondary MRFs that are required for normal myoblast differentiation and myocyte fusion into myotubes (Chen & Goldhamer 1999; Hasty et al. 1993).

1.3.1. Myf5 and MyoD Act as Primary MRFs During Myogenesis

Since Myf5 is the first MRF expressed during embryonic development, it was surprising to see the normal skeletal muscle phenotype of the Myf5 knockout mouse (Ott et al. 1991; Braun et al. 1992). Myf5-null mice died immediately after birth due to the absence of a major part of the ribs (Braun et al. 1992). However, histological analysis revealed no abnormalities in skeletal muscle and analysis of MRF transcripts revealed normal levels of *Myod1, myogenin* and *Mrf4*, suggesting that Myf5 is dispensable for skeletal muscle development (Braun et al. 1992). Another study using a Myf5 null allele in which LacZ was inserted into the *Myf5* locus, revealed that Myf5-null muscle progenitors remain multipotent and differentiate into other cell types depending on their local environment (Tajbakhsh et al. 1996). Thus, Myf5 is required for cells to respond correctly to positional cues and to adopt their myogenic fate (Tajbakhsh et al. 1996).

To examine the role of Myf5 during regenerative myogenesis, a $Myf5^{loxP}$ mouse was created that escaped the perinatal mortality of the Myf5-null mouse described previously (Braun et al. 1992; Tajbakhsh et al. 1996; Kassar-Duchossoy et al. 2004; Gayraud-Morel

et al. 2007). In young mice, muscle from Myf5-null mice did not differ from that of wild-type mice; however, at 7 months of age, Myf5-null muscle showed increased fibrosis, a sign of chronic degeneration and regeneration (Gayraud-Morel et al. 2007). This phenotype was exacerbated after acute injury as demonstrated by delayed differentiation and increased accumulation of fatty and fibrotic tissue (Gayraud-Morel et al. 2007). Furthermore, Myf5-null satellite cells showed delayed proliferation when cultured in vitro, but no appreciable differences were seen in differentiation (Gayraud-Morel et al. 2007; Ustanina et al. 2007). However, in another study, Myf5-null myoblasts were shown to undergo precocious differentiation (Montarras et al. 2000). Thus, Myf5 appears to regulate regenerative myogenesis by enabling transient expansion of myoblasts.

In contrast to Myf5-null mice, MyoD-null mice are viable and fertile; however, like Myf5-null mice, these mice show no morphological abnormalities in their skeletal muscle and no changes in mRNA expression of muscle specific genes (Rudnicki et al. 1992). Interestingly, *Myf5* mRNA levels are elevated in postnatal MyoD-null muscle, suggesting that MyoD is dispensable for skeletal muscle development due to some degree of functional redundancy between MyoD and Myf5 (Rudnicki et al. 1992; Braun et al. 1992; Megeney & Rudnicki 1995). To address this hypothesis, mice deficient in both Myf5 and MyoD were generated (Rudnicki et al. 1993). Indeed, Myf5:MyoD double mutant mice are born alive, but they die soon after birth and show a complete absence of skeletal muscle and myoblasts (Rudnicki et al. 1993).

In adult myogenesis, MyoD expression is rapidly upregulated in activated satellite cells, suggesting that MyoD may play a role in postnatal growth and repair of skeletal muscle (Smith et al. 1994; Zammit et al. 2004; Yablonka-Reuveni & Rivera 1994;

Cooper et al. 1999). The function of MyoD in adult skeletal muscle was examined by crossing MyoD-null mice (Rudnicki et al. 1992) with *mdx* mice carrying a loss-of-function mutation in the *dystrophin* gene (Bulfield et al. 1984). These mice showed a marked increase in myopathy characterized by reduced muscle mass, and smaller muscle fiber cross-sectional area leading to premature death (Megeney et al. 1996). Furthermore, injured muscle of MyoD-null mice failed to regenerate efficiently demonstrating that MyoD plays a role in satellite cell biology (Megeney et al. 1996). Interestingly, MyoD-null muscle had increased number of satellite cells, but showed a reduced propensity to proliferate and differentiate despite the expression of Myf5 in these cells (Megeney et al. 1996; Cornelison et al. 2000; Sabourin et al. 1999; Yablonka-Reuveni et al. 1999). Thus, satellite cells deficient in MyoD favour self-renewal over progression through the myogenic program.

1.3.2. Myogenin and MRF4 Act as Secondary MRFs During Myogenesis

Unlike the Myf5-null and MyoD-null mice, myogenin-null mice are born immobile and die immediately after birth (Hasty et al. 1993; Nabeshima et al. 1993). These mice show a severe reduction in skeletal muscle tissue with reduced fiber density and increased number of unfused myoblasts (Hasty et al. 1993; Nabeshima et al. 1993). Furthermore, analysis of MRF mRNA expression revealed no change in *Myod* levels, but a decrease in *Mrf4* expression (Hasty et al. 1993; Venuti et al. 1995). Thus, myogenin is essential for the development of functional skeletal muscle and other members of the MRFs cannot compensate for this defect. Interestingly, myogenin is not sufficient to

generate skeletal muscle as Myf5:MyoD double mutants with compromised MRF4 expression are completely devoid of skeletal muscle (Kassar-Duchossoy et al. 2004).

To examine the role of myogenin in postnatal myogenesis, mice with floxed alleles of myogenin were bred to mice expressing Cre recombinase to avoid the perinatal death observed in myogenin-null mice (Knapp et al. 2006; Hasty et al. 1993; Nabeshima et al. 1993). In sharp contrast to deletion of myogenin before embryonic muscle development, the deletion of myogenin after embryonic muscle development did not result in changes to postnatal growth or function of skeletal muscle (Knapp et al. 2006; Meadows et al. 2008). Myoblasts isolated from myogenin-null mice showed no changes in their ability to differentiate suggesting that other factors may be compensating for the loss of myogenin (Meadows et al. 2008). Indeed, the expression of *Myf5, Myod* and *Mrf4* mRNA was upregulated in myogenin-null myoblasts (Meadows et al. 2008). Furthermore, myogenin was found to be dispensable for muscle regeneration as demonstrated by the phenotype of mdx:myogenin-deleted mice (Meadows et al. 2011). Thus, myogenin has distinct roles during embryonic skeletal muscle development and postnatal life.

MRF4 is transiently expressed in the somite between embryonic day 9 (E9) and 11.5 (E11.5) and reappears in differentiated muscle fibers around E16 (Hinterberger et al. 1991; Bober et al. 1991; Summerbell et al. 2002). Three *Mrf4*-null mice have been generated by deleting part of the MRF4 protein coding sequences; however, each mutation differed in their phenotypes due to the part of the sequence deleted and its proximity to the 5' end of *Myf5* (Braun & Arnold 1995; Patapoutian et al. 1995; Zhang et al. 1995; Olson et al. 1996). The only *Mrf4* mutant allele for which homozygotes survived to adulthood was produced by the Olson lab (Zhang et al. 1995). Skeletal

muscle from these mice appeared normal, but exhibited an increase in *myogenin* expression, suggesting a possible compensatory mechanism for loss of *Mrf4* (Zhang et al. 1995).

A more recent study used a conditional cell ablation and tracing strategy to further examine the role of MRF4 in muscle development (Haldar et al. 2008). These mice were immobile and died soon after birth and were born with a complete lack of differentiated myofibers. However, robust expression of *Myf5*, *Myod* and *myogenin* suggests that the presence of Mrf4 expressing cells is not required for early myogenesis (Haldar et al. 2008).

1.4. Regulation of Satellite Cell Fate Decisions

Studies have suggested significant heterogeneity within the satellite cell population (Beauchamp et al. 2000; Collins et al. 2005; Kuang et al. 2007; Sherwood et al. 2004). However, it is not known whether this heterogeneity has any biological significance regulating, for example, the satellite cell's intrinsic ability to self-renew. After re-entry into the cell cycle, the cell fate of satellite cells must be determined, specifically whether they self-renew or generate committed myogenic progenitors. The balance between these cell fates can be achieved through asymmetric division and signals from the niche.

1.4.1. Symmetric and Asymmetric Divisions

Analysis of myogenic regulatory factor, *Myf5* mRNA expression in proliferating satellite cells revealed asymmetric expression of this factor in newly divided daughter cells (Kuang et al. 2007). Using a Myf5-Cre/ROSA26-YFP animal model, Kuang and

colleagues found that 10% of Pax7 cells have never expressed Myf5 (Kuang et al. 2007). The $Pax7^{+}/Myf5^{-}$ population are satellite stem cells, whereas $Pax7^{+}/Myf5^{+}$ SCs are more committed satellite cells. Both populations can divide symmetrically to produce two identical daughter cells; however, $Pax7^{+}/Myf5^{-}$ cells can also divide asymmetrically to produce one $Pax7^{+}/Myf5^{-}$ daughter cell and one $Pax7^{+}/Myf5^{+}$ daughter cell. It has been suggested that symmetric expansion of satellite stem cells ($Pax7^{+}/Myf5^{-}$) represents the primary mechanism of self-renewal under certain conditions (Kuang et al. 2007).

MyoD and Myogenin have also been shown to be asymmetrically distributed between daughter cells (Liu et al. 2012; Troy et al. 2012; Yennek et al. 2014). For example, Pax7 and MyoD co-staining revealed MyoD was asymmetrically distributed giving rise to one $Pax7^{+}MyoD^{+}$ (committed) daughter cell and one $Pax7^{+}MyoD^{-}$ (self-renewing) daughter cell (Liu et al. 2012; Troy et al. 2012). Similarly, myogenin was asymmetrically distributed giving rise to one $Pax7^{+}MyoG^{-}$ and one $Pax7^{+}MyoG^{+}$ daughter cell (Yennek et al. 2014).

Recent literature suggests that Notch signaling is crucial for satellite cells to self-renew and return to quiescence (Bjornson et al. 2012; Fukada et al. 2011; Mourikis et al. 2012; Wen et al. 2012); therefore, it is not surprising that the Notch antagonist Numb was shown to be asymmetrically distributed into committed $myogenin^{+}$ daughter cells (Conboy & Rando 2002). Furthermore, during asymmetric division, the Notch3 receptor is enriched in $Myf5^{-}$ daughter satellite cells whereas the Notch ligand, Delta1, is enriched in $Myf5^{+}$ daughter satellite cells (Kuang et al. 2007).

1.4.2. The Satellite Cell Niche

The stem cell niche is the local microenvironment that provides essential cues for the maintenance of stem cell identity and regulation of stem cell function. Specifically, the satellite cell niche is composed of the underlying myofiber and the basal lamina which consists mainly of laminin, collagen and proteoglycans (Mauro 1961; Fuchs et al. 2004). During muscle regeneration, the signals sent by the myofiber and extracellular matrix (ECM) regulate orientation of cell division (planar versus apicobasal) which is important in determining daughter cell fate (Kuang et al. 2007; Siegel et al. 2011).

A number of proteins located in the satellite cell niche have been shown to affect cell fate decisions in satellite cells. For example, loss of collagen VI impaired *in vivo* muscle regeneration and reduced the self-renewal ability of satellite cells (Urciuolo et al. 2013). Additionally, the transmembrane heparan sulfate proteoglycans (HSPGs), syndecan-3 and syndecan-4, specifically mark satellite cells and are important in satellite cell function (Cornelison et al. 2001; Cornelison et al. 2004). Syndecan-3 and -4 are expressed in satellite cells and can interact with growth factors and ECM proteins such as Notch receptors and fibronectin, respectively, to promote satellite cell quiescence and self-renewal (Pisconti et al. 2010; Bentzinger et al. 2013).

1.5. Duchenne's Muscular Dystrophy

Duchenne's Muscular Dystrophy (DMD) is the most common muscular dystrophy with reported incidences ranging from 1:3802 to 1:6291 male births (Flanigan 2014; Mendell et al. 2012). Most boys with DMD present symptoms between 3 and 5 years of age including gross motor delay, abnormal gait, and difficulty getting up after falling

(Yiu & Kornberg 2015). Muscle weakness is first seen in the upper leg and arms and is followed by progressive muscle strength deterioration until loss of movement (Yiu & Kornberg 2015). Patient death occurs in their mid-20s, frequently due to severe cardiopulmonary complications (Flanigan 2014).

DMD is caused by spontaneous mutations or inherited nonsense point mutations in the *dystrophin* (*DMD*) gene resulting in an absence of protein (Kunkel et al. 1986; Hoffman et al. 1987; Monaco et al. 1988; Burghes et al. 1987). Indeed, the *DMD* gene contains 79 exons which makes it susceptible to *de novo* mutations. Dystrophin, a cytoskeletal protein, is a major component of the dystrophin-glycoprotein complex (DGC) which acts as a mechanical link between the cytoplasm and ECM (Petrof 2002; Gao & McNally 2015). This complex is responsible for maintaining cellular integrity, and mediating cytoplasmic signalling and muscle function (Petrof 2002; Gao & McNally 2015; Rando 2001). Loss of dystrophin results in cycles of muscle fiber degeneration and regeneration resulting in satellite cell exhaustion and increased muscle wasting (Blake et al. 2002).

1.5.1. Muscle Stem Cell Therapy for DMD

Current therapies for DMD involve the use of corticosteroids, such as Prednisone and Deflazacort, which merely delay symptoms for a short amount of time (Angelini 2007; Angelini & Peterle 2012). Corticosteroids have anti-inflammatory properties, but the side effects of these drugs, such as bone weakness, often outweigh the benefits (Angelini 2007). Transplantation of myogenic precursor cells (MPCs) offer a promising approach to restore dystrophin expression and reduce muscle pathology in DMD patients

(Sienkiewicz et al. 2015); however, isolation of MPCs and their introduction into muscle tissue typically results in poor engraftment and death of transplanted cells providing little repair of the diseased or injured muscle (Price et al. 2007; Wilschut et al. 2012).

Given that satellite cells are the major contributors to regenerative myogenesis, they have been extensively studied for use of stem cell transplantation (Sienkiewicz et al. 2015). Clinical trials were performed in the early 1990s using satellite cells or myoblasts in combination with immunosuppression, but these trials were met with disappointing results (Miller et al. 1997; Mendell et al. 1995). Unfortunately, the small number of MPCs available requires that they be expanded in culture which significantly reduces their stem cell identity as well as their myogenic potential (Montarras, Morgan, Collins, Frederic Relaix, et al. 2005), thus efforts are being made to improve culture conditions for the expansion of MPCs to enhance the efficiency of their engraftment (Schaaf et al. 2012).

Indeed, a simple combination of myogenic cell media with matrigel-coated culture plates was shown to improve expansion of Pax3- and Pax7-expressing MPCs (Wang et al. 2014). Activation of Notch signalling during *ex vivo* expansion was shown to inhibit differentiation of MPCs and maintain donor cell engraftment potential (Parker et al. 2012). A short treatment with Wnt7a improved MPC engraftment and tissue dispersal upon transplantation leading to improved muscle function (Bentzinger et al. 2014). Forskolin treatment was also shown to enhance proliferation of mouse satellite cells in culture and maintain their ability to engraft muscle *in vivo* (Xu et al. 2013). Furthermore, MPCs expanded in the presence of a combination of four pro-inflammatory cytokines, IL-1α, IL-13, TNF-α, and IFN-γ, could contribute to muscle repair and replenish the host

satellite cell niche after transplantation (X. Fu et al. 2015). Therefore, the establishment of an *in vitro* system to expand MPCs while maintaining their "stemness" is a promising strategy to treat DMD.

1.5.2. The mdx Mouse as an Animal Model of DMD

The most commonly used animal model for DMD research is the mdx mouse. The mdx mouse (C57BL/10ScSn-Dmdmdx/J mice) is a genetic and biochemical model of DMD, which lacks dystrophin due to a non-sense point mutation (C-to-T) (Bulfield et al. 1984). The mdx mutation occurred spontaneously resulting in a premature stop codon in exon 23 (Sicinski et al. 2016; Ryder-Cook et al. 1988). Although this model has no detectable dystrophin protein, sporadic revertant myofibers expressing dystrophin can appear (Yokota et al. 2006).

The loss of dystrophin underlies the progressive muscle pathology (increased myofiber necrosis and elevated blood creatine kinase) which begins around 3 weeks of age (Bulfield et al. 1984; Dangain & Vrbova 1984; DiMario et al. 1991). Muscle necrosis, indicated by immune cell infiltration, peaks at around 25-26 days with massive muscle fiber degeneration and regeneration by day 28 (DiMario et al. 1991). An accumulation of fibrotic tissue in dystrophic muscle can be observed in mice aged 10 to 13 weeks with extensive fibrous tissue in older mice (16 to 20 months of age) (Christian Pastoret & Sebille 1995; C. Pastoret & Sebille 1995). Despite being dystrophin-deficient, the lifespan of mdx mice is only reduced by approximately 25%, whereas the lifespan of DMD patients is reduced by approximately 75% (Chamberlain et al. 2007).

1.6. CCAAT/Enhancer Binding Proteins

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that regulate cellular growth and differentiation, metabolism, liver regeneration and inflammation (Ramji & Foka 2002). The expression of C/EBPs has been shown in numerous cell types including hepatocytes, adipocytes and hematopoietic cells. There are six members of this family (C/EBPα – C/EBPδ) that demonstrate >90% sequence similarity in their C-terminal domains and a more divergent (<20% sequence similarity) N-terminus (Ramji & Foka 2002).

The C-terminal domain contains the bZIP domain, a domain that consists of a basic-amino-acid-rich DNA-binding region followed by a dimerization motif termed the leucine zipper (Agre et al. 1989; Landschulz et al. 1989; Tsukada et al. 2011). The leucine zipper consists of a heptad repeat of four or five leucine residues that assume a coiled coil α-helical configuration (Agre et al. 1989; Landschulz et al. 1989). C/EBP dimerization, with other C/EBP family members or the NF-κB and Fos/Jun family, is a prerequisite for DNA binding (Landschulz et al. 1989). DNA binding is mediated by the basic region which also forms an α-helical structure (Landschulz et al. 1989). The ideal binding site for C/EBP transcription factors is the dyad symmetrical repeat, RTTGCGYAAY, where R is A or G, and Y is C or T (Osada et al. 1996). The N-termini of C/EBP proteins are divergent except for three short subregions that are conserved in most members and represent the activation domains that interact with the transcription apparatus and stimulate transcription (Williams et al. 1995; Williamson et al. 1998; Tang & Koeffler 2001; Ramji & Foka 2002). One of the family members, C/EBPγ lacks an

activation domain and thus represses gene transcription by forming inactive heterodimers with other members (Cooper et al. 1995).

1.6.1. C/EBPβ is a Regulator of Mesenchymal Stem Cell Fate

C/EBPβ was first characterized as a mediator of IL-6 signalling in the immune system (Poli et al. 1990) and since then its expression has been shown in hepatocytes, adipocytes, keratinocytes, osteoclasts and chondrocytic cells (Ramji & Foka 2002; Smink & Leutz 2012). This member of the C/EBP family of transcription factors is encoded by an intronless gene, yet it can be found in three N-terminally truncated isoforms due to alternate translational sites (Figure 2) (Calkhoven et al. 2000; Wethmar et al. 2010). The three isoforms include the full length liver activator protein (LAP*) (38kDa), LAP (35kDa) which bears a truncation of the first 21 amino acids and the liver inhibitory protein (LIP) (20kDa) which lacks all the activation domains (Descombes & Schibler 1991). LAP* and LAP contain both the activation and bZIP domains, whereas LIP only contains the bZIP domain, thus, making it a dominant negative inhibitor of C/EBP function by forming inactive heterodimers with other members of this family (Descombes & Schibler 1991).

The C/EBPβ$^{-/-}$ mouse was generated by replacement of the C-terminal portion of *Cebpb*, coding for the leucine zipper and part of the basic domain, with an MC1-Neo poly(A)$^{+}$ cassette (Screpanti et al. 1995) and are produced at sub-Mendelian ratios (Croniger et al. 1997). Half of the C/EBPβ knockout mice have normal glucose homeostasis (phenotype A), whereas the other half die perinatally, attributed to

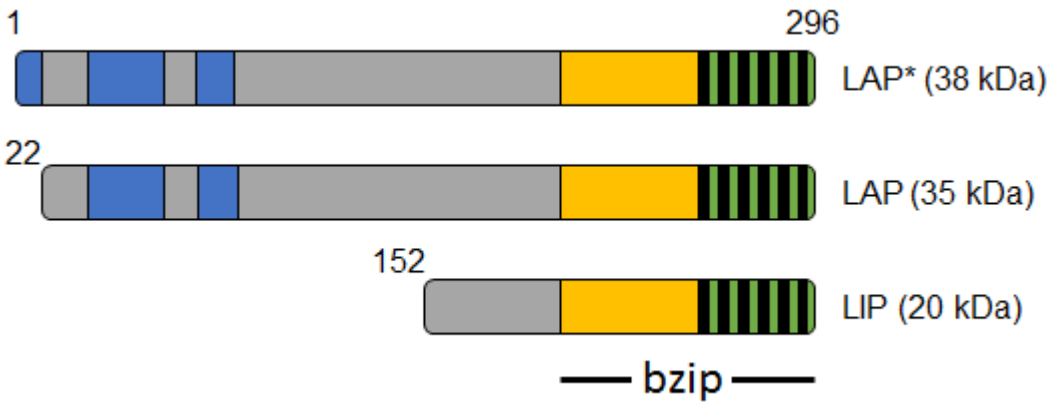

Figure 2. Schematic Model of C/EBPβ Isoforms. The bZIP domain, located at the C-terminus contains a leucine zipper (green with black vertical lines depicting the leucine residues) responsible for homodimerization and a basic region (yellow) which binds DNA. The activation domains (blue) are present in the full length C/EBPβ (LAP*) protein isoform which is 296 amino acids in length. The LAP isoform bears a truncation of the first 21 amino acids whereas the LIP isoform lacks the first 151 amino acids and all the activation domains making it a negative regulator of C/EBP function.

hypoglycemia due to liver failure (phenotype B) (Croniger et al. 1997; Greenbaum et al. 1998). In addition, these mice are resistant to diet-induced obesity, exhibit abnormal female reproduction, and suffer from immunodeficiency (Millward et al. 2007; Sterneck et al. 1997; Bradley et al. 2003). C/EBPβ has also emerged as a transcriptional regulator in at least three of the mesenchymal stem cell lineages: adipocytes, osteoblasts and chondrocytes (Smink & Leutz 2012) (Figure 3).

Several members of the C/EBP family participate in adipogenesis. The expression of C/EBPβ and C/EBPδ is induced early in adipocyte differentiation prior to the expression of PPARγ and C/EBPα, the key drivers of terminal adipocyte differentiation (Darlington et al. 1998). C/EBPβ is pro-adipogenic, as absence of the C/EBPβ gene in mice results in reduced adipocyte differentiation potential of mouse embryonic fibroblasts (MEFs), whereas induction of C/EBPβ in fibroblasts, together with hormones that activate PPARγ, can promote adipocyte differentiation (Cao et al. 1991; Yeh et al. 1995; Tanaka et al. 1997).

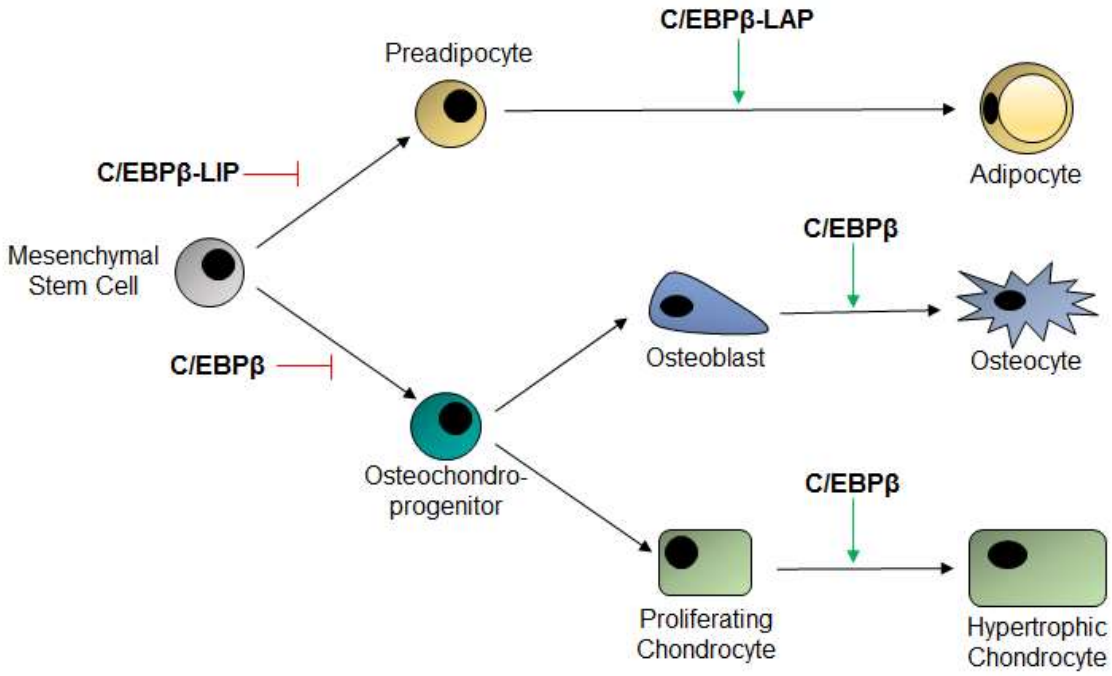

Figure 3. C/EBPβ Regulates Mesenchymal Stem Cell Fate. Mesenchymal stem cells (MSC) can differentiate into adipocytes, osteocytes and chondrocytes via intermediate progenitors. Osteocytes and chondrocytes share a common osteochondroprogenitor. Activating isoforms of C/EBPβ are pro-adipogenic and are required for adipocyte differentiation. In contrast, LIP inhibits adipocyte differentiation and induces MSC differentiation towards the osteoblast lineage. C/EBPβ (LIP and LAP isoforms) has a dual role in the differentiation of osteocytes and chondrocytes. For example, prior to commitment, C/EBPβ inhibits differentiation of MSCs into osteoblasts and proliferating chondrocytes; however, after commitment, C/EBPβ enhances differentiation of osteoblasts and proliferating chondrocytes to osteocytes and hypertrophic chondrocytes, respectively.

Osteoblasts are bone forming cells that eventually differentiate into osteocytes which act as a mechanical sensor to adjust bone formation. Osteoblast differentiation requires Runx2 and Osterix (Komori 2002; Nakashima et al. 2002). C/EBPβ appears to have a dual role in osteoblast differentiation at the cellular level. For example, loss of C/EBPβ in mice results in decreased bone mass and C/EBPβ-deficient osteoblast displayed impaired differentiation suggesting C/EBPβ stimulates osteoblast differentiation (Smink et al. 2009; Zanotti et al. 2009; Tominaga et al. 2008). C/EBPβ was also shown to induce the expression of Runx2 (Gutierrez et al. 2002; Tominaga et al. 2008). However, before lineage commitment, C/EBPβ may function as an inhibitor of osteoblast differentiation (Wiper-Bergeron, St-Louis, et al. 2007; Dingwall et al. 2011). Indeed, ectopic expression of C/EBPβ in C3H10T1/2 mesenchymal stem cells resulted in a reduction of Runx2 expression and a decrease in osteogenic potential (Wiper-Bergeron, St-Louis, et al. 2007; Dingwall et al. 2011).

Finally, the expression of C/EBPβ has also been shown in the growth plate, specifically in late proliferative and early hypertrophic chondrocytes (Tominaga et al. 2008; Hirata et al. 2009). From C/EBPβ$^{-/-}$ mouse models, C/EBPβ appears to be pro-chondrocytic (Tominaga et al. 2008; Hirata et al. 2009). Indeed, C/EBPβ$^{-/-}$ mice display growth retardation and C/EBPβ-deficient chondrocytes had enhanced proliferation and suppressed hypertrophic differentiation (Tominaga et al. 2008; Hirata et al. 2009). However, similar to C/EBPβ's dual role in osteoblast differentiation, studies performed in monopotential undifferentiated chondrogenic cells revealed that C/EBPβ is a negative regulator of cartilage genes by competing with Sox9, the central transcription factor in chondrocytes (Okazaki et al. 2002; Akiyama et al. 2002).

1.6.2. Phosphodiesterase Inhibitors as Regulators of C/EBPβ Expression

Phosphodiesterases are a class of enzymes that are responsible for controlling the cellular concentration of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by cleaving their phosphodiester bond to yield 5'-cyclic nucleotides (5' AMP or 5'-GMP, respectively) (Bender & Beavo 2006; Omori & Kotera 2007). Inhibitors of phosphodiesterases (PDEi) are a class of drugs that are widely used for various pharmacological properties including anti-inflammatory, vasodilator and antithrombotic functions (Tilley 2011; Nieder et al. 2005; Rendell & Bamisedun 1992; Rahimi et al. 2010). A class of PDEis termed methyl-xanthine non-selective PDE inhibitors has long been used for pharmacological research (Boswell-Smith et al. 2006). This class includes isobutylmethylxanthine (IBMX) and the approved drugs theophylline and pentoxifylline (Essayan 2001). Theophylline is used to treat asthma, whereas, pentoxifylline is used to improve circulation in diabetes and reduce fibrosis following radiation therapy for breast cancer (Tilley 2011; Nieder et al. 2005; Rendell & Bamisedun 1992).

IBMX is known to be well tolerated by many cell types including adipocytes (Yeh et al. 1995; Goethe et al. 2007). Furthermore, IBMX has been used to transiently stimulate C/EBPβ expression in pre-adipocytes during early adipogenesis for many years (Cao et al. 1991). IBMX increases cellular cAMP which leads to activation of protein kinase A (PKA) and the phosphorylation of cAMP response element-binding protein (CREB) (Goethe et al. 2007; Monika Niehof et al. 1997; J. W. Zhang et al. 2004; Lechner et al. 2013; Cao et al. 1991). Phosphorylated CREB binds to cAMP response elements (CRE)

in the *Cebpb* promoter, thus stimulating the expression of C/EBPβ (Monika Niehof et al. 1997) (Figure 4).

1.7. Rationale and Hypothesis

Although C/EBPβ has been shown to be a major regulator of mesenchymal stem cell fate and the differentiation of adipocytes, osteoblasts and chondrocytes, little is known about its function in the myocyte branch of mesenchymal stem cell differentiation. Indeed, C/EBPβ is upregulated in muscle wasting such as sarcopenia and has been associated with upregulation of atrogin-1 expression in muscle fiber in cancer cachexia (Giresi et al. 2005; Zhang et al. 2011). Furthermore, *Cebpb* was found to be among the most highly expressed genes in adult quiescent satellite cells identified by genome-wide expression analysis (Fukada et al. 2007).

Thus, I hypothesized that ***C/EBPβ inhibits satellite cell differentiation and pharmacological treatments that increase C/EBPβ expression in myoblasts will improve myoblast transplantation protocols***. For example, treatment with IBMX can reversibly induce C/EBPβ expression by enhancing cAMP-signalling in adipocytes (Goethe et al. 2007).

The ***specific aims*** of this study were: **(1)** to examine the role of C/EBPβ in self-renewal, proliferation and differentiation of satellite cells; **(2)** to examine the effect of IBMX treatment on the proliferation, self-renewal and differentiation of primary myoblasts and satellite cell cultures; and **(3)** to assess muscle repair and satellite cell niche reconstitution in mdx mice following transplantation of IBMX- or vehicle-treated primary myoblasts.

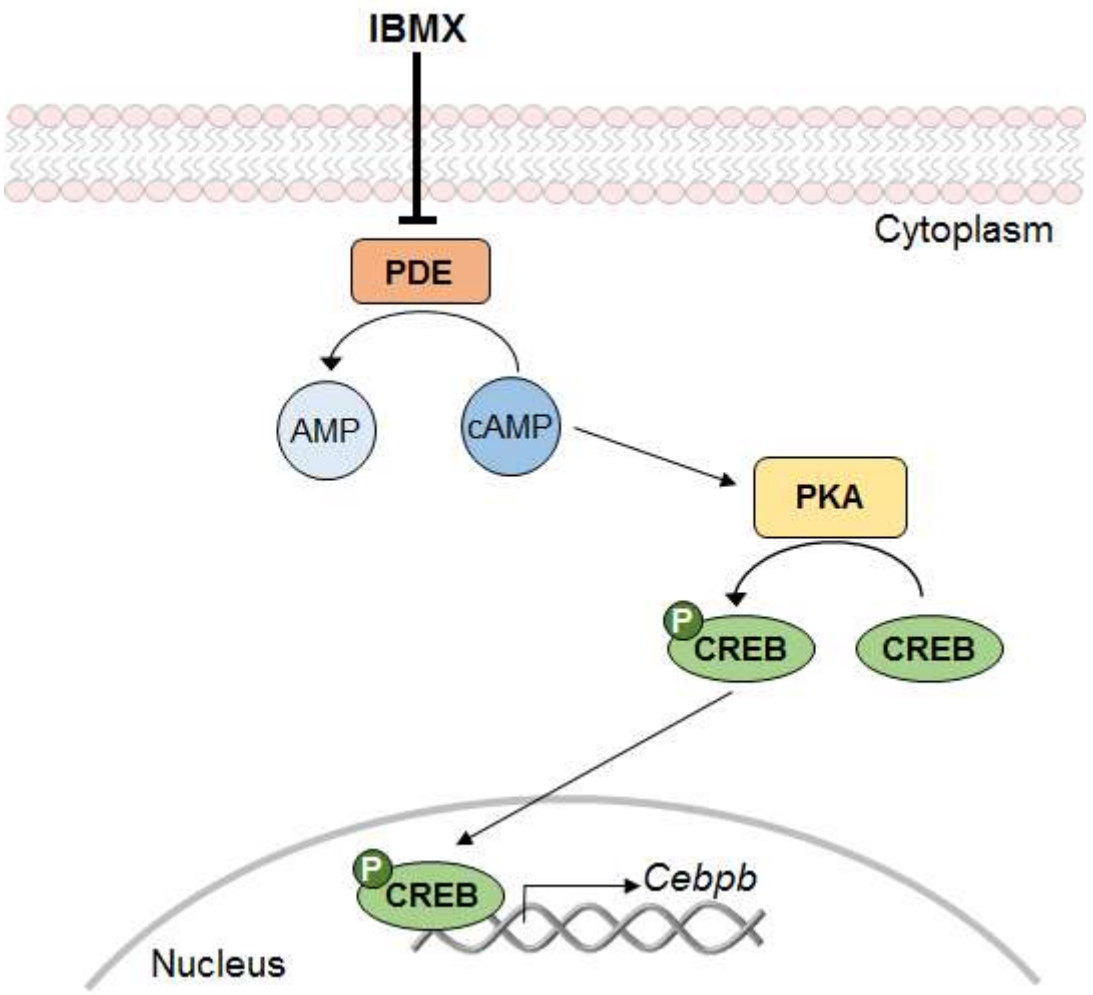

Figure 4. IBMX Stimulates *Cebpb* Gene Expression in Adipocytes. Phosphodiesterases (PDE) catalyze the hydrolysis of cyclic adenosine monophosphate (cAMP) to 5'AMP. Isobutylmethylxanthine (IBMX) inhibits PDEs and thus increases intracellular cAMP levels which leads to activation of Protein Kinase A (PKA). PKA phosphorylates the cAMP response element-binding protein (CREB) which translocates into the nucleus and binds to the cAMP response element in the Cebpb promoter, thus stimulating expression of C/EBPβ.

CHAPTER TWO: CCAAT/ENHANCER BINDING PROTEIN BETA IS EXPRESSED IN SATELLITE CELLS AND CONTROLS MYOGENESIS.

CCAAT/Enhancer Binding Protein Beta is Expressed in Satellite Cells and Controls Myogenesis.

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Running Head: C/EBPβ is an inhibitor of myogenesis.

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Author contributions:

François Marchildon: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript

Neena Lala: Collection and assembly of data, data analysis and interpretation, final approval of manuscript (Figure 7, Figure 8, Figure S2)

Grace Li: Collections and assembly of data, data analysis and interpretation, final approval of manuscript

Catherine St-Louis: Administrative support, collection and assembly of data, final approval of manuscript

Daniel Lamothe: Collection and assembly of data, data analysis and interpretation, final approval of manuscript

Charles Keller: Provision of study materials, manuscript writing, final approval of manuscript

Nadine Wiper-Bergeron: Conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript

Abstact

Upon injury, muscle SCs become activated and produce skeletal muscle precursors that engage in myogenesis. We demonstrate that the transcription factor CCAAT/Enhancer Binding Protein beta (C/EBPβ) is expressed in the SCs of healthy muscle. C/EBPβ expression is regulated during myogenesis such that C/EBPβ is rapidly and massively downregulated upon induction to differentiate. Further, persistent expression of C/EBPβ in myoblasts potently inhibits differentiation at least in part through the inhibition of MyoD protein function and stability. As a consequence, myogenic factor expression, myosin heavy chain expression and fusogenic activity was reduced in C/EBPβ-overexpressing cells. Using knockout models, we demonstrate that loss of *Cebpb* expression in SCs results in precocious differentiation of myoblasts in growth conditions and greater cell fusion upon differentiation. *In vivo*, loss of *Cebpb* expression in SCs resulted in larger muscle fiber cross-sectional area and improved repair after muscle injury. Our results support the notion that C/EBPβ inhibits myogenic differentiation and that its levels must be reduced to allow for activation of MyoD target genes and the progression of differentiation.

Introduction

Muscle SCs (SC) are thought to be the primary source of regenerative capacity for skeletal muscle and are found between the sarcolemma and the basement membrane of mature muscle fibers (Mauro 1961). These cells can be activated to both proliferate and differentiate in response to external stimuli, most importantly muscle injury and exercise, and are defined by their protein marker expression, namely $CD34^+$, $\alpha7\beta1$ $integrin^+$, $Pax3^+$ and $Pax7^+$ (Beauchamp et al. 2000; Chargé & Rudnicki 2004; Schultz et al. 1985). As SCs become activated, they progressively lose expression of Pax7 and express in a coordinated fashion the myogenic basic helix-loop-helix factors MyoD, myogenin and MRF4 that are responsible for the induction of myocyte-specific genes (Wang & Rudnicki 2012).

Significant functional redundancy exists among the myogenic factors. While loss of either *Myod1* or *Myf5* is without effect on muscle development, deletion of both genes results in a complete lack of skeletal muscle and myoblasts, suggesting that MyoD and Myf5 can functionally compensate for one another (Rudnicki et al. 1992; Rudnicki et al. 1993). However, MyoD does play an important role in the maintenance of muscle mass in the adult as following injury, $Myod1^{-/-}$ muscle is unable to repair (Megeney et al. 1996; Wang & Rudnicki 2012). Indeed $Myod1^{-/-}$ mice crossed into a dystrophin-deficient background die prematurely due to the exacerbation of muscle wasting (Megeney et al. 1996). When $Myod1^{-/-}$ myoblast gene expression profiles were determined and compared to that of wild-type (WT) controls, it was noted that $Myod1^{-/-}$ cells, while expressing higher levels of Myf5, were deficient in other myogenic markers such as desmin and MRF4, and expressed the stem cell markers Sca-1 and CD34 (Asakura et al. 2007;

Rudnicki et al. 1992). Despite low expression levels of myogenin and myosin heavy chain in *Myod1*$^{-/-}$ myoblasts, these cells failed to differentiate efficiently and displayed a dramatic reduction in fusion (Cornelison et al. 2000; Sabourin et al. 1999).

Notwithstanding its important role in myogenesis, little is known about the factors that regulate *Myod1* expression. Since inhibition of MyoD expression and/or function can be linked to the inhibition of adult myogenesis, regulatory pathways that control MyoD expression are of great interest. NF-kB can regulate *Myod1* mRNA expression through increased turnover (Guttridge et al. 2000; Li & Reid 2000), while the E3 ubiquitin ligase MAFbx/atrogin-1 has also been associated with increased degradation of MyoD protein and the development of muscle wasting (Lagirand-cantaloube et al. 2009; Tintignac et al. 2005). Overexpression of paired domain homeobox transcription factor Pax7 has also been shown to reduce MyoD protein levels and to block MyoD function independent of its mRNA expression, resulting in decreased myogenin expression and inhibited myogenesis (Olguin et al. 2007).

Appearing first in the dermomyotome, Pax7 expression becomes localized to mononucleated cells of the trunk and limb muscles between E12.5 and E16.5 (Buckingham et al. 2003). While Pax7$^{-/-}$ animals have normal fetal myogenesis, their post-natal myogenesis is severely compromised (Oustanina et al. 2004; Seale et al. 2000). As such, Pax7 is thought to participate in the maintenance of the satellite cell undifferentiated state by blocking differentiation and promoting self-renewal (McKinnell et al. 2008; Wang & Rudnicki 2012). Conditional depletion of Pax7-expressing cells abrogates muscle regeneration following injury (Sambasivan et al. 2011), though loss of

Pax7 expression after postnatal day 21 is without effect on muscle repair, suggesting that Pax7 is dispensable in older animals (Lepper et al. 2009).

C/EBPs form a family of bzip transcription factors of which C/EBPβ is involved in many regulatory and differentiation processes as both an activator and a repressor. For example, it is required for liver regeneration, acts as a potent commitment factor for adipocyte differentiation, and regulates the acute phase response of the immune system (Buck & Chojkier 2003; Friedman 2007; Grimm & Rosen 2003; Rosen et al. 2000; Sebastian & Johnson 2006). While C/EBPβ is expressed in muscle, it does not appear to be required for embryonic myogenesis as loss of *Cebpb* in mice results in no overt defects in muscle histology, though increased muscle insulin sensitivity was observed (Wang et al. 2000). C/EBPβ-expressing infiltrating macrophages have been shown to be necessary for efficient repair of an acute muscle injury, while loss of *Cebpb* expression in the muscle fiber itself was without impact on repair (Ruffell et al. 2009). C/EBPβ expression in myonuclei has been associated with the upregulation of atrogin-1 expression in the muscle fiber in cancer cachexia (Zhang et al. 2011).

Our own work has demonstrated that C/EBPβ is a major regulator of mesenchymal stem cell fate where it acts as an activator of adipogenesis and a repressor of osteoblastogenesis (Wiper-Bergeron, Salem, et al. 2007; Wiper-Bergeron, St-Louis, et al. 2007; Wiper-Bergeron et al. 2003). Since C/EBPβ's role in muscle stem cell function remains unknown, we sought to determine if C/EBPβ participated in adult myogenesis.

Materials and methods

Constructs. The C/EBPβ expression vector and retroviral expression vector have been described previously (Wiper-Bergeron et al. 2003). The Pax7-luc reporter construct was kindly provided by Dr. L. Shen (Lang et al. 2009). The -2kb myogenin-luc reporter construct was a gift from Dr. Alexandre Blais (Liu et al. 2010).

Retroviral infection and cellular differentiation. Replication incompetent pLXSN-based retroviruses (Clontech) were generated in Phoenix Ampho packaging cells (ATCC) as described (Wiper-Bergeron et al. 2003). Following infection, cells were selected in media containing 400 ug ml^{-1} G418 for 7 days prior to differentiation to ensure expression in all cells. To stimulate skeletal muscle differentiation, 70% confluent C2C12 cells were treated with DMEM containing 1% horse serum. For Giemsa staining, cells were washed with PBS, fixed with ice-cold methanol for 15 min and stained with 10% Giemsa for 1hr. Photomicrographs are representative of a minimum of 3 independent experiments.

Isolation and differentiation of skeletal muscle precursor cells. Skeletal muscle precursor cells were isolated essentially as described (Megeney et al. 1996). Lower hind limb muscles from C57BL/6 female mice aged 6-8 weeks (Charles River laboratories, Saint-Constant, Canada) were dissected and digested with dispase and collagenase (Roche). Isolated cells were plated on Matrigel-coated dishes and allowed to grow in DMEM containing 20% FBS, 10% HS (Invitrogen), with penicillin and streptomycin (Wisent) in the presence of 10ng/ml bFGF and 2ng/ml HGF (Peprotech) as indicated in figure legends. Differentiation was achieved by changing the medium of 70% confluent myoblasts cultures to DMEM containing 2% FBS and 10% HS. To observe the natural

changes in C/EBPβ during the spontaneous differentiation of isolated SCs, cultures were maintained in DMEM containing 10% FBS, and differentiated in DMEM containing 2% horse serum.

Western Analysis. To assess expression of myoblast and myotube markers, the following antibodies were used: anti-MyoD (M-318 and 5.8a), anti-Myf-5 (C-20), anti-myogenin (M-225), anti-myosin heavy chain (H-300), anti-C/EBPβ (C-19), and anti-tubulin (B-7) (all Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclophilin B (Abcam ab16045), anti-Pax7, anti-myogenin and anti-β-tubulin (DSHB). Chemiluminescence images were captured using the Luminescent Image Analyzer LAS-4000 (Fujifilm Life Science).

RT-qPCR. For RT-qPCR, RNA was extracted using the RNeasy kit (Qiagen), remaining DNA was digested with DNase (Ambion), and RNA was reverse transcribed using iScript kit (BioRad) according to manufacturer's instructions. Real-time PCR reactions were performed with Quantitect SYBR green (Qiagen) on a Mx3005p thermocycler (Stratagene). Primers were designed to span an intron when possible and sequences are available upon request. Relative fold induction was determined using the ΔΔCt method (Livak and Schmittgen, 2001) following normalization with 18S rRNA.

Indirect Immunofluorescence staining. Indirect immunofluorescence was performed on paraffin embedded and frozen sections of muscle, fixed in 4% paraformaldehyde. Cell cultures were fixed with ice-cold methanol. Detection was performed according to standard procedures using the following antibodies: anti-C/EBPβ (C-19), (Santa Cruz Biotechnology), anti-Pax7 and anti-MF20 (DSHB), anti-mouse-Cy5 (Invitrogen) and

DyLight™ 549 (Piercenet), anti-rabbit DyLight™ 488 (Piercenet) Conjugates (Donkey Anti-Mouse or anti-rabbit IgG (H+L)) (Thermo Scientific).

Analysis of reporter gene expression. For Pax7 reporter assays, C2C12 cells were transfected with 3.8kb Pax7 promoter-luciferase reporter construct (Lang et al. 2009) and a constitutively active RSV-β-galactosidase reporter in the presence or absence of mammalian expression plasmids for C/EPBβ using FuGene HD (Promega). Cells were grown under growth conditions for 24 hours, then collected for luciferase assays or switched to differentiation media for another 24 hours. Luciferase assays were performed according to standard protocol, and corrected for transfection efficiency with β-gal enzyme activity. Error bars represent the standard error of the mean of a minimum of 3 experiments.

Myogenin promoter activity was measured by transfecting C2C12 cells with a -2.0kb Myogenin-luciferase reporter construct (Liu et al. 2010) and a constitutively active RSV-β-galactosidase reporter in the presence or absence of mammalian expression plasmids for C/EPBβ and/or MyoD using FuGene HD (Promega). Cells were growth in growth conditions for 24 hours then switched to differentiation media for another 24 hours (DM). Samples were collected and luciferase assays were performed according to standard protocol, and corrected for transfection efficiency with β-gal enzyme activity. Error bars represent the standard error of the mean of a minimum of 3 experiments.

Chromatin Immunoprecipitation (ChIP) Assay. Freshly isolated SCs were purified by selective plating and were grown in growth media containing 10% FBS for four days and ChIP was performed as described (Wiper-Bergeron et al. 2003) using C/EBPβ (C-19) (Santa Cruz Biotechnology) for precipitation or a type matched non-specific antibody at

4°C overnight. DNA fragments were purified using the Qiaquick PCR purification kit™ (Qiagen) and amplified by PCR using primers to amplify -695 to -465 of the murine Pax7 promoter using qPCR. The primer sequences used were: forward 5'-CCCGAACTGGCCCCCTTTCC-3' and reverse 5'-TCCCCCGGAGGACTGGAACG-3'. Error bars represent the standard error of the mean of 3 experiments.

Myogenic Conversion Assay. C2C12 myoblasts retrovirally transduced with empty vector (pLXSN) or C/EBPβ were transiently transfected with a mammalian expression plasmid for MyoD and pEGFPplasmid using FuGene HD (Promega), as indicated in the figure legend. Cells were then induced to differentiate in 2% horse serum for 5 days. Cells were then fixed and stained for myosin heavy chain (MF20; DSHB) or harvested for protein. Error bars represent the standard error of the mean of 3 experiments.

C57BL/6 and C/EBPβ conditional knockout mice. C57BL/6 female mice aged 6-8 weeks were obtained through Charles River laboratories. A mouse bearing C/EBPβ-floxed allele was created previously (Sterneck et al. 2006) and homozygous progeny (C/EBPβ$^{fl/fl}$) were obtained by breeding heterozygous progenitors. C/EBPβ$^{fl/fl}$ mice were crossed with mice bearing the Pax7-CreER^{tm} allele (Nishijo et al. 2009). All animals were maintained in a controlled facility at 22ºC with 30% relative humidity on a 12-hours light/dark cycle and provided food and water *ad libitum*. *In vivo* activation of CreER^{tm} was achieved by 5 daily intraperitoneal injections of 1.5mg of tamoxifen (Sigma) dissolved in corn oil. Mice were aged 2-3 months and no weight differences were noted between same-sex littermates. In utero activation of CreER^{tm} was achieved by a single gavage of 2.5mg of tamoxifen of pregnant dams when pregnancy was at E15.5. In culture

activation of CreER^{tm} was achieved by a 72hrs treatment with 2µM of 4-OH tamoxifen (Sigma).

For $BaCl_2$ injury, mice were anesthetized with isofluorane before the procedure. Legs were shaved and washed with an antiseptic solution, after which 50µl of 1.2% $BaCl_2$ in PBS or PBS alone was injected intra-muscularly into the TA. For all animal work, following sacrifice, hindlimb muscles from homozygous null animals and wild type littermates aged 5 weeks were flash frozen in isopentane and sectioned for indirect immunofluorescence or processed for isolation of primary myoblasts. For histological analysis, a minimum of 400 fibers from the TA muscle were measured on a minimum of 2 cross sections separated by at least 50µm. All animal handling procedures conformed to the guidelines established by the University of Ottawa Animal Care Service and the Canadian Council on Animal Care.

Results

C/EBPβ is expressed in muscle SCs in vivo.

We first sought to quantify C/EBPβ expression in muscle extracts. As differential initiation of translation of the *Cebpb* mRNA results in 3 proteins with identical carboxy termini and variable amino termini, we investigated the expression of the two activating isoforms of C/EBPβ, known as LAP* (Liver Activating Protein, full-length isoform), and LAP, which lacks the first 21 amino acids but contains all of the activation domains, as well as that of the dominant negative isoform Liver Inhibitory Protein (LIP) in whole muscle extracts. Western analysis of C/EBPβ expression in C57BL/6 mouse extensor digitorum longus (EDL) and soleus (Sol.) muscles revealed C/EBPβ expression in both muscles, with the LAP isoform most predominant in both muscles (Figure 5A).

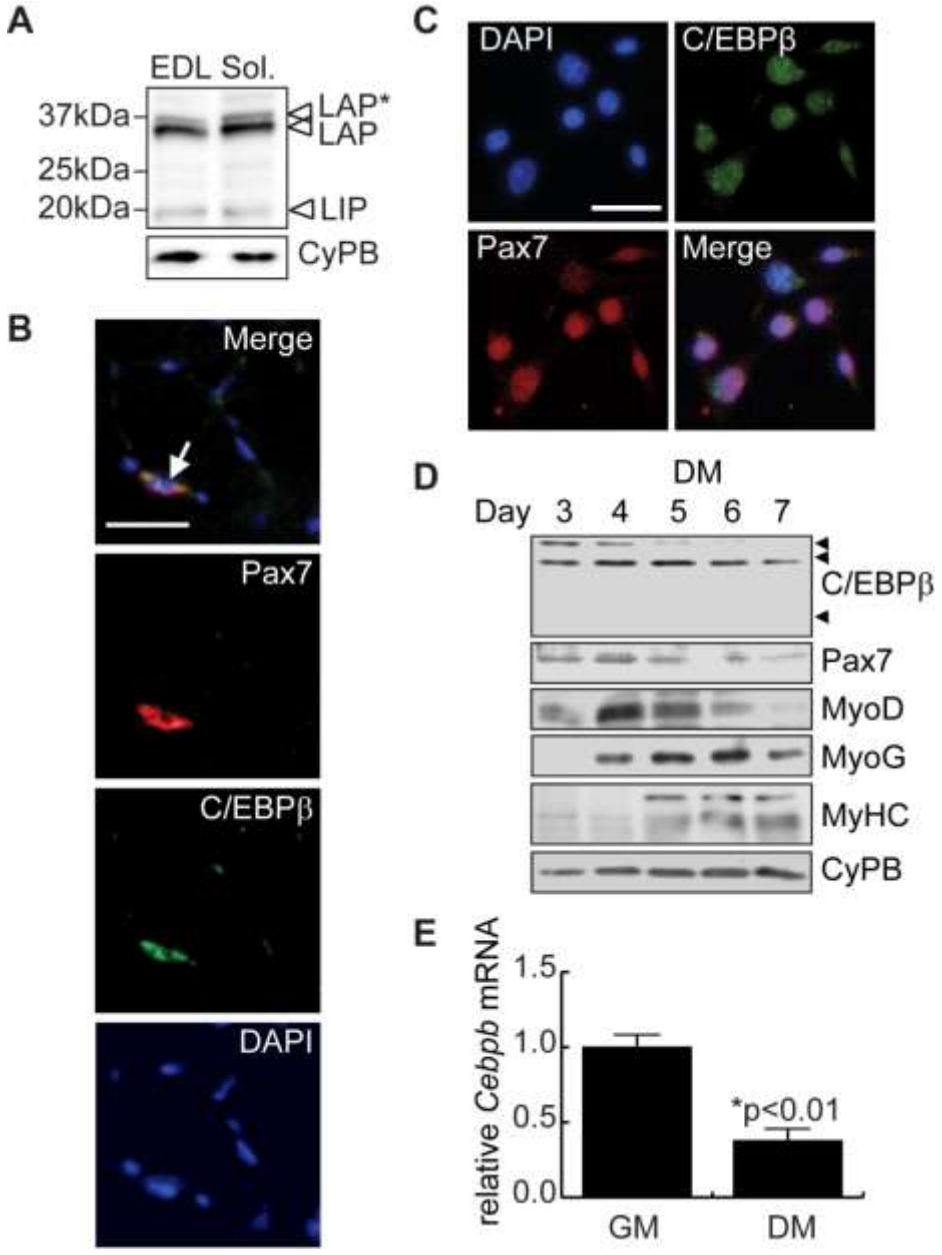

Figure 5. C/EBPβ is expressed in muscle SCs. (A) Western analysis of C/EBPβ expression in C57BL/6 mouse extensor digitorum longus (EDL) and soleus (Sol.) muscle. Positions of the LAP*, LAP and LIP isoforms of C/EBPβ are indicated. Cyclophilin B (CyPB) expression is used as a loading control. **(B)** Indirect immunofluorescence of Pax7 and C/EBPβ expression in C57BL/6 mouse tibialis anterior

muscle. DAPI staining reveals nuclei, Satellite cell, as determined by Pax7 staining is indicated with a white arrow. Data is representative of 3 independent experiments performed on a minimum of 3 male C57BL/6 mice aged 4-6 weeks. Scale bar = 50 μm. **(C)** Expression of Pax7 and C/EBPβ in primary myoblasts cultured in growth medium as detected by indirect immunofluorescence. Nuclei are visualized with DAPI stain. Scale bar = 50 μm. Failure to add primary antibodies to the samples did not result in any fluorescent signal. **(D)** Western analysis of C/EBPβ, Pax7, and myogenic marker expression in skeletal muscle SCs three days post-isolation cultured in growth medium lacking FGF/HGF and during differentiation following transfer to low serum conditions on day 4. The expected migration of the three isoforms of C/EBPβ (LAP*, LAP, and LIP) are indicated with arrowheads. MyoG, myogenin; MyHC, myosin heavy chain. Cyclophilin B (CyPB) is a loading control. **(E)** Relative *Cebpb* mRNA expression in SCs isolated from female C57BL/6 mouse hindlimb (age 6-8 weeks) cultured in growth medium (GM) in the absence of growth factors (day 3 post-isolation) or after incubation in differentiation medium (DM) for 3 days. Error bars are the standard error of the mean for three independent experiments. P-value was calculated using a Student's t-test assuming equal variance.

Further analysis by indirect immunofluorescence indicated that C/EBPβ expression was not equally distributed throughout the muscle fiber, but rather was localized to $Pax7^+$ cells found at the perimeter of muscle fibers, corresponding to muscle SCs (SCs) (Figure 5B). When primary myoblasts were harvested and cultured in growth medium in the presence of both fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) to prevent differentiation, we noted that all of the $Pax7^+$ cells were also positive for C/EBPβ expression by indirect immunofluorescence, suggesting that C/EBPβ expression was present in myogenic precursor cells (Figure 5C).

To explore the expression of C/EBPβ during activation and differentiation of SCs, SCs were harvested from hindlimb muscle by enzymatic digestion and selective plating, and cultured in growth medium without addition of growth factors for several days, allowing cells to adhere and proliferate. This method was chosen to allow us to observe changes in C/EBPβ expression in the pre-myoblastic cell, the myoblast and the myocyte as the cells spontaneously progressed through these stages. Distinct waves of myoblast and myocyte marker expression were observed using this method. Four days after isolation (day 0 = day of harvest), cultures were switched to differentiation medium and allowed to differentiate for 3 days. Western analysis revealed that SC cultures initially expressed the LAP* and LAP isoforms of C/EBPβ which decreased as cells progressed through differentiation (Figure 5D). This pattern of downregulation mirrored the loss of Pax7 expression as the cells progressed through differentiation (Figure 5D). Indeed, the decline in C/EBPβ and Pax7 expression coincided with an increase in both MyoD and myogenin expression upon switching to differentiation medium on day 4 (Figure 5D). Upregulation of these myogenic factors resulted in the robust induction of myosin heavy

chain expression on day 5 (Figure 5D). As differentiation progressed, MyoD levels were observed to decrease and to return to baseline (Figure 5D).

In accordance with these results, RT-qPCR analysis demonstrated that *Cebpb* mRNA expression in cells cultured in differentiation medium (low serum) for 3 days (7 days post-isolation) decreased by approximately 60% as compared to isolated SCs cultured in growth medium on day 3 post-isolation (Figure 5E). Consistent with our results, microarray analyses comparing the gene expression profiles of quiescent SCs to activated differentiating cells revealed that *Cebpb* expression was reduced by 92% in activated cells (Fukada et al. 2007).

C/EBPβ inhibits the differentiation of myoblasts.

To evaluate the roles of C/EBPβ expression in SCs during differentiation, we retrovirally transduced freshly isolated SCs from the hindlimb of C57BL/6 mice aged 6-8 weeks (n=3) to express C/EBPβ or with empty virus (pLXSN) and allowed these cells to differentiate in low serum conditions for 3 days. Ectopic expression of C/EBPβ was confirmed by western blotting, and it was noted that the LAP isoform was the most prominent one expressed (Figure 6A). We then analyzed myogenic gene expression in these cultures following differentiation. Ectopic C/EBPβ potently inhibited the differentiation of these cells as evidenced by a decrease in MyoD, myogenin and myosin heavy chain (MyHC) expression, without affecting Myf5 levels (Figure 6B), suggesting that the cells remained committed to the myogenic lineage but failed to differentiate efficiently. The undifferentiated phenotype observed in C/EBPβ-overexpressing cells was further supported by the increased expression of the SC and myoblast marker Pax7

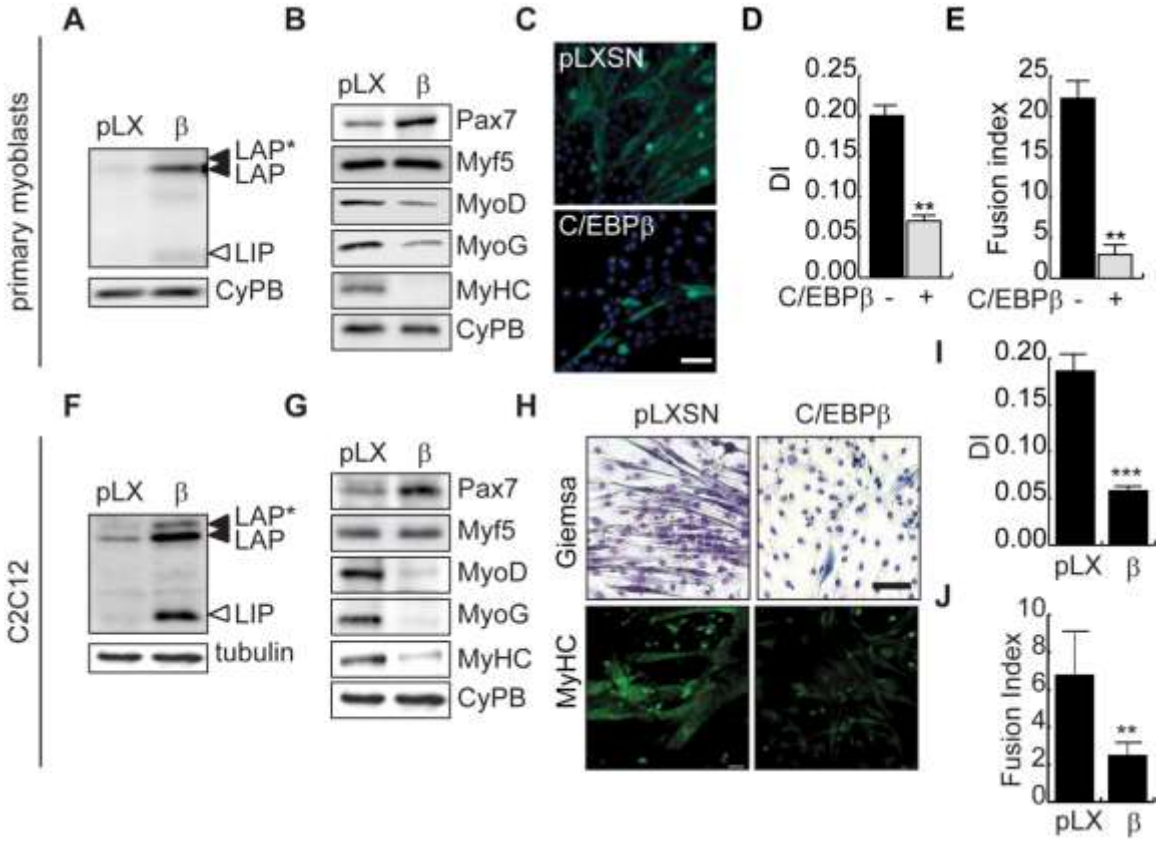

Figure 6. C/EBPβ inhibits the differentiation of myoblasts. (A) Western analysis of C/EBPβ isoform expression in SCs isolated from 3 male C57BL/6 mice in 3 independent trials and retrovirally transduced to express full-length C/EBPβ or with empty vector (pLX). Cells were induced to differentiate in low serum for 3 days before protein analysis. Cyclophilin B (CyPB) is shown as a loading control. **(B)** Western analysis of myogenic protein expression in primary cells harvested, transduced and differentiated as in (A). MyoG, myogenin; MyHC, myosin heavy chain. Cyclophilin B (CyPB) is shown as a loading control. **(C)** Indirect immunofluorescence of MyHC expression (as detected using MF-20 antibody) in primary myoblasts cultures transduced and differentiated as in (A). Scale bar = 100 μm. **(D)** Differentiation index (DI, #myonuclei/# total nuclei) from five random fields of cells differentiated and stained as in (C) **p<0.01, n=3. **(E)** Fusion

index (FI, #myonuclei/#myotubes) from experiment as in (C). **(F)** Western analysis of C/EBPβ isoform expression in C2C12 cells retrovirally transduced to express C/EBPβ or with empty virus (pLX) and induced to differentiate in low serum conditions for 5 days. B-tubulin expression is used as a loading control. **(G)** Western analysis of myogenic protein expression in C2C12 cultures transduced and induced to differentiate as in (F). MyoG, myogenin; MyHC, myosin heavy chain. Cyclophilin B (CyPB) is shown as a loading control. **(H)** Bright field images of Giemsa-stained (top) and MyHC immunostained C2C12 cells transduced and differentiated as in (F). Scale bar = 100 μm**.** **(I)** Differentiation index of cells in (H) and calculated as in (D), ***$p<0.001$, n=3. **(J)** Fusion index from cells differentiated and stained as in (H), **$p<0.01$, n=3.

(Figure 6B). Given that C/EBPβ has been implicated in the regulation of cell proliferation in other systems, and that reduced cell proliferation could impact the efficiency of differentiation and fusion, we measured cell number in proliferating cultures using crystal violet (Figure S1A) (Johnson 2005). Equal cell numbers were plated for both control and test lines and cell density was evaluated 48 and 72 hours after plating. We did not measure any differences in cell number.

Evaluation of myosin heavy chain expression by immunofluorescence revealed that there were fewer MyHC positive cells in C/EBPβ-overexpressing cultures as compared to empty virus controls with a 4-fold reduction in the differentiation index (#myonuclei/total nuclei) 3 days after induction to differentiate (Figure 6C,D). Of the MyHC-positive cells present in the C/EBPβ-overexpressing cultures, the myotubes were smaller such that the fusion index was reduced by approximately 10-fold in C/EBPβ-overexpressing cells as compared to empty virus controls (Figure 6C,D). Taken together, these results support the notion that ectopic C/EBPβ induces a blockade of differentiation.

The effect of overexpressing C/EBPβ in C2C12 cells was similar to that of primary myoblasts. Retroviral transduction to express ectopic C/EBPβ in C2C12 myoblasts resulted in a robust increase in both LAP* and LAP isoforms of C/EBPβ protein levels 5 days after the induction of differentiation (Figure 6F). The inhibitory LIP isoform was not detected in control cultures, though it was detected in C/EBPβ-overexpressing cultures, in contrast to our results in SCs (Figure 6A,F). In this model, following 5 days in differentiation medium, we noted an increase in Pax7 expression and a concomitant decrease in MyoD, myogenin and MyHC expression (Figure 6G). Ectopic expression of C/EBPβ also inhibited myoblast fusion as evidenced by Giemsa staining (Figure 6H).

While empty vector controls displayed numerous purple-stained myotubes, C/EBPβ-overexpressing cells had only a few small myocyte-like cells (Figure 6H). Immunostaining for myosin heavy chain revealed that while some MyHC positive cells existed in C/EBPβ-overexpressing cells, their fusion was limited (Figure 6H). While approximately 20% of the empty virus control culture cells underwent differentiation to become $MyHC^{+}$, only 5% of C/EBPβ overexpressing cells did (Figure 6I). Furthermore, the fusion index of differentiated cells was also reduced by C/EBPβ overexpression, consistent with our results in SCs (Figure 6J). As in SC cultures, cell growth was not perturbed by C/EBPβ expression in C2C12 myoblasts (Figure S1B).

Given that the phenotype produced in C/EBPβ-overexpressing myoblasts closely resembled that of the *Myod1* null (Sabourin et al. 1999), we sought to determine if the blockade in differentiation could be rescued by ectopic MyoD expression. C2C12 myoblasts retrovirally transduced with empty vector or to express C/EBPβ were transiently transfected following selection to express MyoD along with GFP and then induced to differentiate for 4 days in low serum conditions (Figure S2A). Under these conditions, overexpression of C/EBPβ resulted in a 50% reduction in the differentiation index with ectopic expression of MyoD restoring the differentiation index to the level of controls (Figure S2B). Furthermore, transient overexpression of MyoD resulted in a rescue of myogenin expression (Figure S2C), suggesting that C/EBPβ acts to inhibit differentiation at the level of MyoD expression and/or activity. Of note, transfection efficiencies were extremely low, resulting in less than 20% of the cells being transfected (Figure S2D,E). However, despite inefficient transfection of MyoD into C/EBPβ-

overexpressing cultures, MyoD was able to rescue the differentiation blockade imposed by C/EBPβ, suggesting that ectopic C/EBPβ inhibits myogenesis at the level of MyoD.

Pax7 is a target of C/EBPβ and represses myogenic gene expression.

Since C/EBPβ inhibits MyoD protein expression and myogenesis in both primary myoblasts and C2C12 cells, we next analyzed myogenic transcript levels by RT-qPCR in C2C12 cells retrovirally transduced to express C/EBPβ and differentiated for 5 days in low serum conditions. *Cebpb* transcript levels increased 20-fold in these cells as compared to empty vector controls (Figure 7A). Despite an important reduction of MyoD protein expression in C/EBPβ overexpressing cultures, *Myod1* mRNA levels were unaffected, as was *Myf5* expression. However, significant decreases in *Myog* (myogenin), and both the embryonic (*Myh3*) and perinatal/adult (*Myh*) myosin heavy chain isoforms 1, 2, 8, and 13 were measured in C/EBPβ overexpressing cells (Figure 7B). We also observed a significant increase in *Fbxo32*/atrogin-1 expression in C/EBPβ-overexpressing cells, an E3 ubiquitin ligase known to be regulated by C/EBPβ in myofibers and to promote the degradation of MyoD protein by the 26S proteasome (Tintignac et al. 2005; Zhang et al. 2011) (Figure 7B). Thus, we hypothesized that the increase in atrogin-1 expression contributed to the loss of MyoD protein seen in differentiating myoblast cultures overexpressing C/EBPβ. To test this, we treated C/EBPβ-overexpressing C2C12 cells and empty virus controls with the proteasome inhibitor MG132 for 2 hours prior to harvest and evaluated MyoD levels by western blotting (Figure S3A). Despite the increase in *Fbxo32* expression, blocking proteasome activity with MG132 resulted in only modest and variable increases in MyoD expression

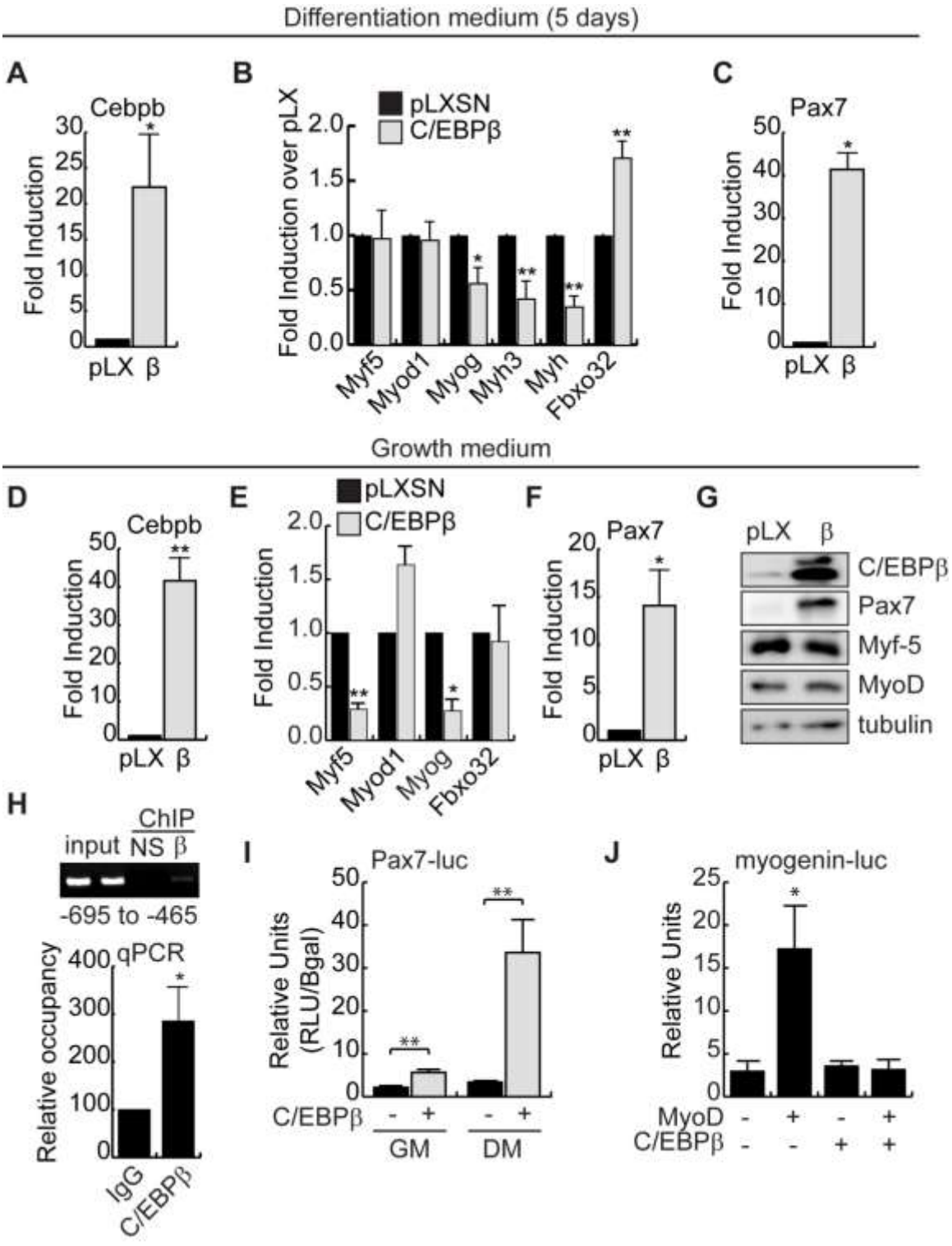

Figure 7. C/EBPβ regulates myogenic gene expression. (A) RT-qPCR analysis of *Cebpb* expression in C2C12 cells retrovirally transduced to express C/EBPβ or with

empty virus and differentiated in low serum conditions for 5 days. *p<0.01 as determined using a paired t-test, error bars are the standard error of the mean. **(B)** RT-qPCR analysis of myogenic gene expression in C2C12 transduced and differentiated as in (A). Error bars are the standard error of the mean. *p<0.05, **p<0.01 as determined using a paired t-test. *Myh* represents an amplicon common to Myh1, 2, 8, and 13. **(C)** RT-qPCR analysis of *Pax7* expression in C2C12 transduced and differentiated as in (A), *p<0.05 as determined by a paired t-test. **(D)** RT-qPCR analysis of *Cebpb* expression in C2C12 retrovirally transduced to express C/EBPβ or with empty virus (pLX) and cultured in growth conditions. **(E)** RT-qPCR analysis of myogenic gene expression in C2C12 cells transduced as in (D), *p<0.05, **p<0.01. **(F)** RT-qPCR analysis of *Pax7* expression in C2C12 transduced as in (D). **(G)** Western analysis of myogenic marker expression in C2C12 cells transduced as in (D) cultured in growth conditions. Only the LAP* and LAP isoforms for C/EBPβ are shown (see Fig. 2F). **(H)** ChIP analysis of C/EBPβ occupancy on the mouse *Pax7* promoter region -695/-465 in freshly isolated SCs as determined by gel analysis (top) and by qPCR (bottom). qPCR data is shown as enrichment over pulldown with a type-matched non-specific antibody (IgG). *p<0.05 as determined using a paired t-test. **(I)** *Pax7* promoter activity in a transient transcription assay where the mouse –3800/+21 *Pax7* promoter drives the expression of luciferase. The reporter construct and a mammalian expression vector for C/EBPβ were transiently transfected into C2C12 cells and luciferase activity was measured in growth medium (GM) and in differentiation medium (DM). **p<0.01, n=3. **(J)** Myogenin promoter activity as measured in (I) under DM conditions using a -2kb myogenin-luc reporter construct. *p<0.05, n=3.

under differentiation conditions, suggesting that while atrogin-1 may contribute to reducing MyoD expression in C/EBPβ-overexpressing cells, it is unlikely to be the principal mechanism (Figure S3).

In addition to induction of *Fbxo32* expression, *Pax7* expression was also increased 40-fold in C/EBPβ stable cell lines (Figure 7C), consistent with our western analysis (Figure 6G). High levels of Pax7 expression have been correlated with the self-renewal of SCs and the inhibition of differentiation and thus could explain the phenotype evoked by forced C/EBPβ expression in myoblasts (Olguin & Olwin 2004). However, given that Pax7 levels drop as differentiation progresses, the high Pax7 levels could also represent a culture that fails to differentiate as in the case of C/EBPβ-overexpressing cells. We thus assessed myogenic marker expression in C/EBPβ-overexpressing cultures under growth conditions, where differentiation is inhibited (Figure 7D-G). In growth medium, RT-qPCR analysis revealed that *Cebpb* was expressed 40-fold over empty vector controls (Figure 7D), and both *Myf5* and *Myog* expression was significantly decreased (Figure 7E). Despite a modest increase in *Myod1* expression, this effect was not statistically significant (Figure 7E). *Fbxo32* expression was also unaffected by C/EBPβ overexpression under growth conditions (Figure 7E), which corresponded with no change in MyoD protein expression (Figure 7G). Thus, while overexpression of C/EBPβ can promote loss of MyoD protein under differentiation conditions, it did not in growth medium. These results suggest that C/EBPβ activity could be sensitive to serum levels resulting in differential regulation of protein expression in growth and differentiation media.

Despite some differences in gene expression in growth and differentiation media, the expression of *Pax7* was still significantly increased 12-fold in C2C12 cells stably expressing C/EBPβ (Figure 7F). Western analysis of myogenic protein markers echoed the RT-qPCR results, with increased Pax7 expression, and unchanged MyoD expression in C/EBPβ overexpressing cells (Figure 7G). Interestingly, Myf5 levels were only moderately decreased by C/EBPβ overexpression (Figure 7G).

To determine if the regulation of Pax7 expression by C/EBPβ was direct, we first performed *in silico* analysis of the Pax7 proximal promoter to identify potential C/EBP response elements, revealing one putative element (TTGCACA) at position -590/-583. Less stringent algorithms also predicted elements in the following regions: -3006/-2808, -2646/-1955 and -1845/-933. Chromatin immunoprecipitation analysis performed in freshly isolated SCs in growth conditions (where endogenous C/EBPβ levels are high) revealed that C/EBPβ did occupy the Pax7 promoter at position -695/-465 (containing the putative element at -590/-583) but none of the other predicted sites (Figure 7H). Further, transient reporter assays performed in C2C12 myoblasts in both growth and differentiation conditions revealed that C/EBPβ can activate the Pax7 promoter, most robustly in low serum conditions (Figure 7I). Thus, the activation of Pax7 expression by C/EBPβ likely contributes, at least in part, to the C/EBPβ-dependent inhibition of myogenesis.

In addition to compromising myogenesis through the activation of both atrogin-1 and Pax7 expression in myoblasts, C/EBPβ also interferes with MyoD transcriptional activity. Reporter assays using the -2kb myogenin promoter revealed that co-expression of C/EBPβ with MyoD blocked the activation of the myogenin promoter by MyoD (Figure

7J). It remains unclear if this inhibition is mediated by a direct effect of C/EBPβ on MyoD or rather via the previously described inhibition of MyoD activity by high levels of Pax7 (Olguin et al. 2007). Taken together, these results suggest that C/EBPβ expression can abrogate myogenesis through at least two converging mechanisms at the level of MyoD expression and function.

Loss of Cebpb expression results in precocious differentiation under high serum conditions.

To evaluate the importance of changes in *Cebpb* expression during the differentiation of primary myoblasts, we generated a conditional knock-out mouse model by breeding a floxed mouse ($Cebpb^{fl/fl}$) with a $Pax7^{CreERtm}$ mouse (Nishijo et al. 2009) (Figure 8A). The floxed *Cebpb* mouse was bred to heterozygosity for the $Pax7^{CreERtm}$ allele, and stud males were crossed with $Cebpb^{fl/fl}$ females (Figure 8B). The $Pax7^{CreERtm}$ allele expresses, by virtue of a bicistronic expression cassette, a tamoxifen-inducible Cre under the control of the *Pax7* promoter, thereby not affecting *Pax7* expression (Nishijo et al. 2009; Sterneck et al. 2006). Both experimental ($Cebpb^{-/-}Pax7^{CreER/+}$) and control mice ($Cebpb^{fl/fl}Pax7^{+/+}$) were generated at Mendelian ratios. *Cebpb* excision was accomplished in CreER-expressing floxed animals by daily i.p. injections of tamoxifen for 5 days. This treatment reduced the percentage of $Pax7^{+}$/C/EBPβ^{+} double positive cells by approximately 74% one week after the end of treatment (Figure 8C). The percentage of $Pax7^{+}$ nuclei in muscle sections was unaffected by *Cebpb* excision at the time of harvest (fl/fl: 1.83±0.52%; -/-: 1.81±0.99%). This excision rate is consistent with other reports using this strain of Cre mouse (Nishijo et al. 2009). Following isolation of SCs, floxed and null

cultures were maintained in growth medium including FGF/HGF and under these conditions only minimal differentiation was observed (Figure 8D, top). However, *Cebpb*$^{-/-}$ cultures underwent more differentiation than floxed controls, though still only restricted to a small fraction of cells. Withdrawal from growth factors in high serum conditions resulted in the stimulation of differentiation of the *Cebpb*$^{fl/fl}$ control cultures as measured by immunostaining for MyHC, while the *Cebpb*$^{-/-}$ cultures differentiated robustly producing large multinucleated fibers (Figure 8D, bottom). Furthermore, both the differentiation and fusion indices were significantly increased in null cells as compared to floxed controls upon withdrawal of growth factors (Figure 8E,F). In accordance with these observations, western analysis of myogenic markers revealed that withdrawal from the growth factors decreased C/EBPβ expression in floxed controls (Figure 8G). Furthermore, reduced C/EBPβ expression correlated with a decrease in Pax7 expression and a concomitant increase in myogenin expression (Figure 8G). Interestingly, loss of C/EBPβ expression in complete growth medium resulted in an increase in MyoD protein expression, supporting our hypothesis that C/EBPβ is a regulator of MyoD expression (Figure 8G). MyoD levels were lowest in the most differentiated of cultures, *Cebpb*$^{-/-}$ cells withdrawn from growth factors (Figure 8G).

Among the growth factors included in growth media to prevent early myogenesis, HGF has been shown to regulate C/EBPβ expression (Shen et al. 1997). Withdrawal of HGF from the growth medium for 48 hrs decreased *Cebpb* expression by 50% (Figure S4A). This decrease in *Cebpb* expression correlated with a modest yet significant decrease in *Pax7* mRNA expression and an increase in *Myog* expression, suggesting enhanced differentiation in the withdrawn cultures consistent with our results in the

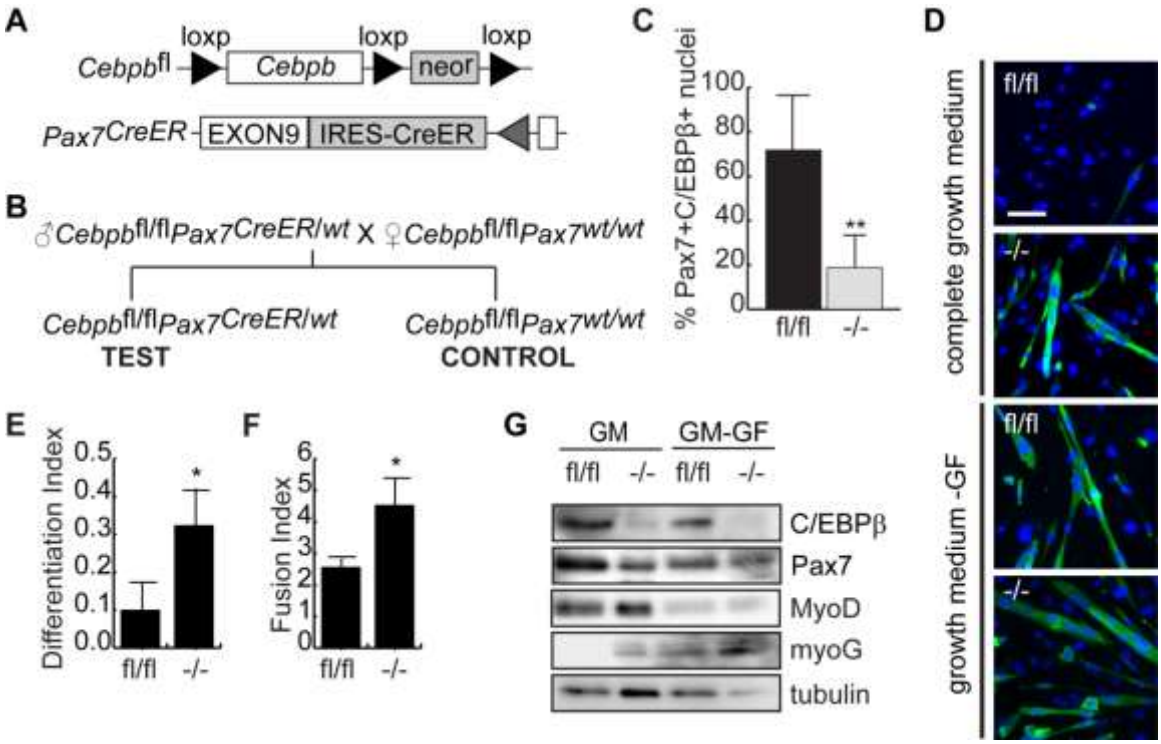

Figure 8. Loss of *Cebpb* expression in Pax7$^+$ cells results in precocious differentiation under high serum conditions. (A) Schematic representation of the transgenic animals used to generate a tamoxifen-inducible excision of *Cebpb* in Pax7$^+$ cells. **(B)** Breeding strategy used to generate *Cebpb* conditional knockout progeny for experimentation. **(C)** Excision efficiency in Cre$^+$ SCs isolated from 11.5 week old mice following 5 i.p. injections of tamoxifen one week prior to sacrifice, calculated as the percentage of Pax7+ cells isolated by enzymatic digestion from skeletal muscle that are also C/EBPβ$^+$ by immunocytochemistry in floxed controls (fl/fl) and Cre$^+$ (-/-) cells. Error bars are the standard deviations. **p<0.01 **(D)** Immunostaining of myosin heavy chain expression in primary myoblasts cells isolated as in (C) and cultured in complete growth medium or the absence of growth factors (-GF). Images are representative of 3 independent trials from two pairs of mice. Scale bar = 100 μm. **(E)** Differentiation index calculated from 3 random images per trial and 3 trials of the images in (D). *p<0.05, n=3.

Error bars are the standard deviation. **(F)** Fusion index from experiment as in (D). *$p<0.05$, n=3. **(G)** Western analysis of C/EBPβ and myogenic marker expression in primary myoblasts derived from conditional nulls (-/-) and from floxed littermate controls (fl/fl) grown in growth medium in the presence (GM) or absence (GM-GF) of FGF/HGF.

Cebpb conditional null cultures (Figure S4A). At the protein level, C/EBPβ and Pax7 protein levels were decreased following withdrawal from HGF (Figure S4B). Taken together, these data suggest that HGF may act to restrain differentiation of primary myoblast cultures in part by maintaining the expression of C/EBPβ.

Loss of Cebpb expression in Pax7^{+} cells results in increased cell fusion under differentiation conditions.

SCs were isolated from *Cebpb*$^{fl/fl}$*Pax7*$^{CreER+/-}$ and *Cebpb*$^{fl/fl}$*Pax7*$^{+/+}$ muscle and were maintained in culture in growth medium containing 20% FBS and FGF/HGF to inhibit differentiation. To induce excision of *Cebpb*, cells were treated with 4-hydroxy-tamoxifen for 3 days to activate the Cre recombinase. 4-OH-tamoxifen treatment resulted in a 45% excision of *Cebpb* in isolated genomic DNA and in a robust decrease in C/EBPβ LAP protein and mRNA expression (Figure 9A,D,E). To induce differentiation, cultures were switched to low serum conditions for two days, after which immunostaining for MyHC revealed enhanced myotube size in *Cebpb*$^{-/-}$ cells as compared to the floxed controls with a 50% increase in fusion index (Figure 9C, top). Loss of *Cebpb* expression also resulted in only a mild (17%), though not significant increase in differentiated index (Figure 9C, bottom). Indeed, the levels of differentiation achieved in these experiments surpassed 85%, making the determination of enhanced differentiation difficult. Nonetheless, in accordance with these observations, western analysis of myogenic marker expression 2 days following the induction to differentiate indicated that null cells expressed comparable levels of myogenic proteins (MyoD, myogenin, MyHC) as controls (Figure 9D). Analysis of relative mRNA expression by

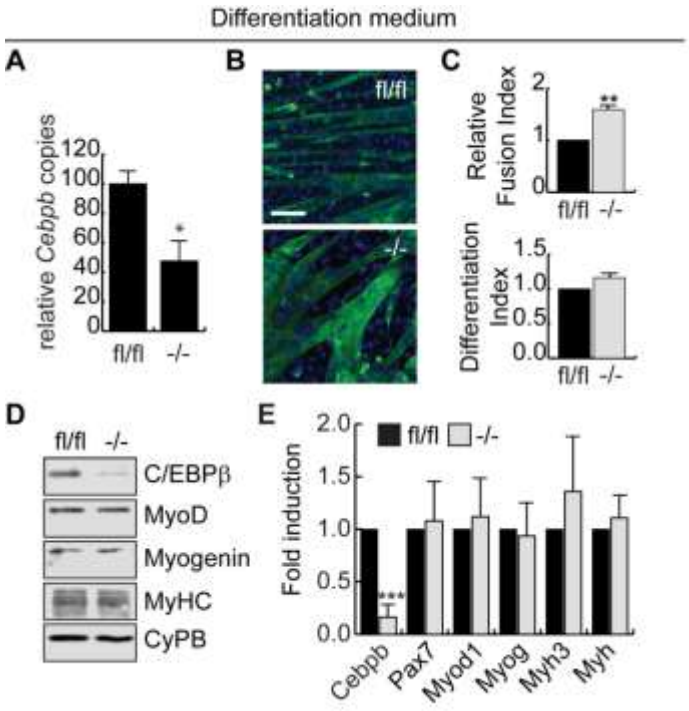

Figure 9. Loss of *Cebpb* expression in Pax7+ cells results in increased cell fusion under differentiation conditions. Primary myoblasts from female *Cebpb*$^{fl/fl}$*CreER-/-* and *Cebpb*$^{fl/flCreER+/-}$ hindlimb muscle (aged 8-12 weeks) treated in culture with 2μM 4-hydroxy-tamoxifen for 3 days following isolation to excise *Cebpb*. Only the Cre-expressing cells excised *Cebpb* (-/-) with an efficiency of approximately 55%. **(A)** Confirmation of efficient excision of *Cebpb* as measured by gel densitometry with amplification of *Cebpb* from genomic DNA. **(B)** Immunocytochemical analysis of MyHC expression in myotubes generated from primary myoblasts derived from control (fl/fl) and excised (-/-) littermates following differentiation in low serum conditions for 2 days. Scale bar = 100 μm. **(C)** Fusion and differentiation indices (shown relative to floxed controls) calculated from cells differentiated as in (D). **p<0.01, n=4 as determined using a paired t-test. **(D)** Western analysis of C/EBPβ and myogenic marker expression in primary myoblasts differentiated as in (B). Cyclophilin B (CyPB) is used as

a loading control. **(E)** RT-qPCR analysis of myogenic marker expression in control (fl/fl) and excised (-/-) SC cultures differentiated as in (B). *Myh* represents an amplicon common to Myh1, 2, 8,and 13. ***$p<0.001$, n=4.

RT-qPCR revealed that while *Cebpb* levels were dramatically reduced in null cells, all other genes tested in *Cebpb*$^{-/-}$ cells were comparable to floxed controls.

Satellite cell-specific loss of Cebpb expression results in muscle fiber hypertrophy.

Given the robust precocious differentiation of *Cebpb*$^{-/-}$ SCs in culture and the enhanced fusion observed, we investigated the histology of *Cebpb*$^{-/-}$ muscle following excision of *Cebpb in utero* induced by a single tamoxifen gavage of pregnant dams at E15.5. Mice were sacrificed at postnatal day 21 and the muscle phenotype was characterized. Excision, as measured by dual immunofluorescence staining of TA muscle sections for Pax7 and C/EBPβ indicated a significant 50% decrease in double positive cells (Figure 10A). The total number of Pax7^{+} cells in the muscle was unaffected in null mice at this time point (Figure 10B). Histologically, no apparent differences were observed in female *Cebpb*$^{-/-}$ tibialis anterior muscle as compared to sex-matched littermates, while fiber size was perceptibly larger in the male nulls (Figure 10C,D). Consistent with these observations, the average fiber cross-sectional area of female mice muscle fibers was not different from littermate controls, whereas the conditional knockout males had cross-sectional areas one third larger than controls (Figure 10D). The increase in cross-sectional area was not accompanied by changes in overall fiber number (Figure 10E). When fiber cross-sectional areas were plotted as a distribution, it was noted that for both female and male mice, there was a shift of the distribution towards larger fiber sizes in null animals as compared to littermate controls (Figure 10F,G). Indeed, despite no difference in average cross-sectional area in female mice, there was both an increase in larger fibers and a significant decrease in smaller fibers, suggesting that there

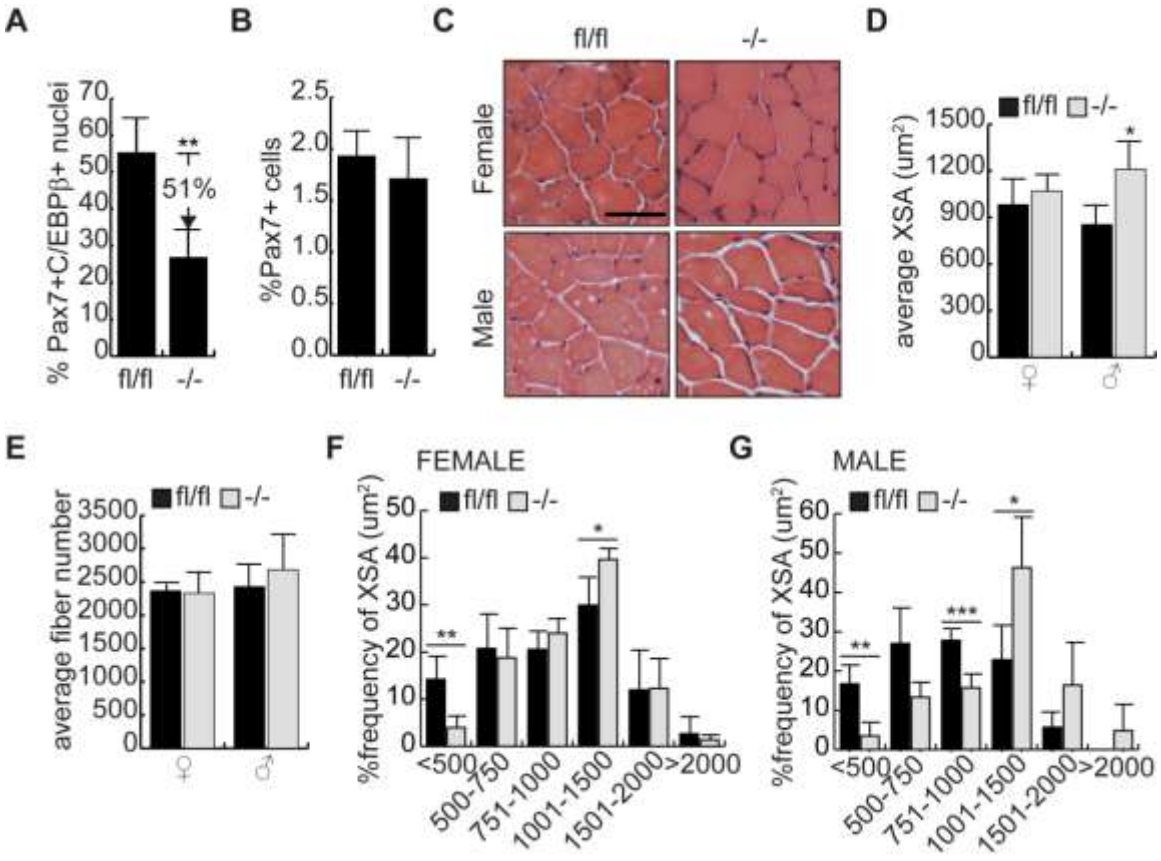

Figure 10. *Cebpb* conditional knockouts display fiber hypertrophy. (A) Percent of Pax7^{+} cells also positive for C/EBPβ in *Cebpb*$^{fl/fl}$ and *Cebpb*$^{-/-}$ in 21 day old mice following a single 2.5mg tamoxifen gavage of pregnant dams at E15.5 as determined by fluorescent IHC on cross sections of the TA muscle. n≥3 animals of each genotype. **(B)** Percentage of Pax7+ nuclei relative to total nuclei in sections from TA muscle derived from 21 day old *Cebpb*$^{fl/fl}$ and *Cebpb*$^{-/-}$ mice following maternal gavage at E15.5. Error bars are the standard deviations. **(C)** Representative bright field images of tibialis anterior cross sections from control female and male *Cebpb*$^{fl/fl}$ (fl/fl) and conditional null *Cebpb*$^{-/-}$ *Pax7*$^{Cre/+}$ (-/-) animals at postnatal day 21 stained with hematoxylin and eosin. Scale bar = 100 μm. **(D)** Average cross-sectional areas of muscle fibers from female (♀) and male (♂) control (fl/fl) and conditional null (-/-) animals. For each group n≥3. Error bars are

the standard deviation. *p<0.02. **(E)** Average fiber number for the tibialis anterior muscles of control and conditional null mice (n≥3 for each group). **(F)** Frequency distribution of fiber size in female control and conditional null mice. *p<0.05, **p<0.02. Error bars are the standard deviation. **(G)** Frequency distribution of fiber size in male control and conditional null mice. *p<0.05, **p<0.02, ***p<0.01. Error bars are the standard deviation.

is a modest fiber hypertrophy in the null female animals (Figure 10F). The shift in fiber size distribution was even more pronounced in male mice (Figure 10G). As the mice aged to postnatal day 56, the trend towards larger fiber sizes in null mice became more marked, though the sample sizes (2 animals per genotype) was small (Figure S5). These results indicate that loss of *Cebpb* expression in SCs promotes fiber hypertrophy, which may result from enhanced differentiation and fusion.

Loss of Cebpb expression in SCs promotes healing after muscle injury.

To evaluate the regenerative capacity of conditional null muscle, we induced the excision of *Cebpb* in floxed animals by daily i.p. injections of tamoxifen for 5 days. Excision was confirmed in 74% of $Pax7^{+}$ cells (Figure 8C). One week following completion of treatment, male mice were subjected to a single $BaCl_2$ injection to the left TA muscle and allowed to recover for one week. After sacrifice, muscle sections were analyzed for the extent of repair. This short time frame did not permit the development of fiber hypertrophy in uninjured Cre-expressing muscle (Figure 11A). Examination under light microscopy revealed that the healing process, as determined by the presence of centrally located nuclei, was more advanced in null animals as compared to littermate controls not expressing the Cre (Figure 11A). Indeed, the average cross sectional area of regenerating fibers (as defined by the presence of a centrally located nuclei) was increased by 30% in conditional null animals as compared to floxed controls (Figure 11B). Frequency distribution of regenerating fiber cross-sectional areas revealed that there was a pronounced shift in fiber size towards larger fibers in the conditional nulls as compared to littermate controls (Figure 11C), suggesting that repair was more advanced

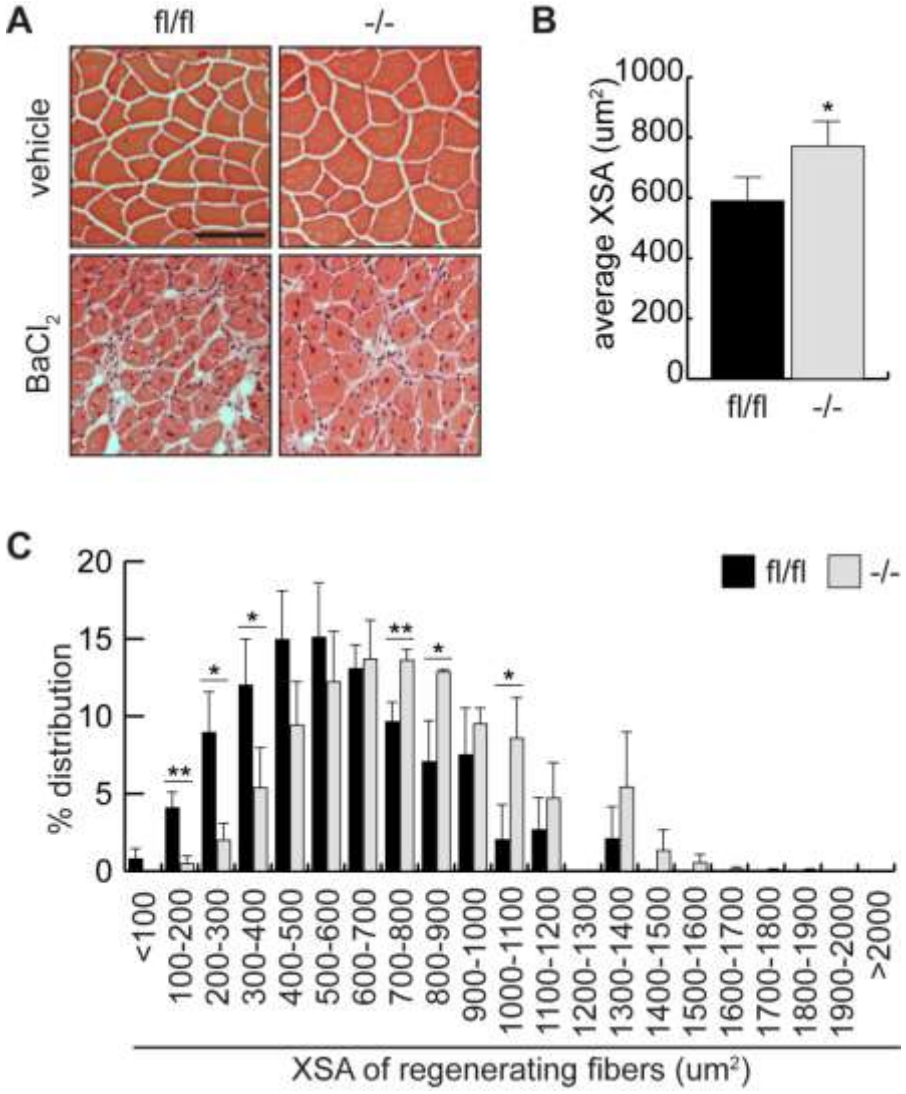

Figure 11. Muscle repair following $BaCl_2$ injury is enhanced in *Cebpb* conditional knockout animals. Four male *Cebpb*$^{fl/fl}$*Pax7*$^{+/+}$ and *Cebpb*$^{-/-}$*Pax7*$^{Cre/+}$ mice were induced to excise *Cebpb* by 5 daily i.p. injections of tamoxifen (see Fig4C). Mice were injured with $BaCl_2$ one week after excision. **(A)** Representative brightfield images of injured ($BaCl_2$) and uninjured (vehicle) TA muscle from control (fl/fl) and conditional null (-/-) mice 7 days post-injury. Data is representative of four experimental pairs aged 2-3 months old. Scale bar = 100 μm. **(B)** Average cross sectional area of regenerating fibers

from injured TA muscle in control (fl/fl) and conditional null (-/-) male mice. Error bars are the standard error of the mean, n= 4 animals per group, $p<0.05$. **(C)** Distribution of cross-section areas of regenerating fibers (defined as those with centrally located nuclei) from injured TA of mice treated as in (A). *$p<0.05$, **$p<0.01$, n=4 per group. Error bars are the standard error of the mean.

in the null animals. These observations are consistent with our culture work, and taken together, support the notion that the loss of *Cebpb* in the muscle SCs promotes a more robust differentiation program.

Discussion

Ectopic expression of C/EBPβ produces a differentiation defect that partially recapitulates the phenotype of *Myod1*$^{-/-}$ skeletal muscle precursor cells by impinging on MyoD expression and activity through at least two converging mechanisms: the stimulation of proteasomal degradation of MyoD, the upregulation of Pax7 expression and the interference with MyoD transcriptional activity (Megeney et al. 1996; Sabourin et al. 1999). In both *Myod1*$^{-/-}$ myoblasts and myoblasts over-expressing C/EBPβ, myogenic marker expression is limited and fusion inhibited. In culture, loss of C/EBPβ expression results in precocious differentiation in high serum conditions which normally restrains differentiation. *In vivo*, we observe larger caliber fibers in the conditional null mice as compared to their littermate controls and more efficient repair. These observations define a novel role for C/EBPβ as a transcription factor important not only for the commitment of stem cells to adipose or osteoblast lineages, but also in myogenesis as a tissue satellite cell marker, a regulator of MyoD expression and an inhibitor of differentiation (Cao et al. 1991; Smink et al. 2009).

Based on our results, we propose that C/EBPβ expression in SCs acts to maintain the undifferentiated state. Indeed, many similarities exist between the myogenic precursor marker Pax7 and C/EBPβ expression and function. Both factors are expressed in SCs and their expression is downregulated as cells progress towards the differentiated state.

Expression of either factor correlates with the maintenance of the undifferentiated state, and our results indicate that the ectopic expression of C/EBPβ in myoblasts results in increased Pax7 expression in both growth and differentiation conditions. Thus, the parallel course of C/EBPβ and Pax7 expression may be due to direct regulation of Pax7 expression by C/EBPβ. Given that Pax7 is only required until juvenile age for proper functioning of SCs, it is interesting to speculate that C/EBPβ may play a role in the maintenance of SCs in the adult, a hypothesis with many important implications for the development of treatments for muscular atrophies (Lepper et al. 2009; Shea et al. 2010). Treatments that would prevent the loss of C/EBPβ in primary myoblasts would be predicted to maintain the undifferentiated state of these cells, and could conceivably have tremendous therapeutic potential by improving myoblast engraftment and satellite cell niche repopulation.

It is interesting to note that C/EBPβ has a different effect on myogenic regulatory factor expression, particularly MyoD, under high serum (GM) versus low serum (DM) conditions. It has previously been shown that HGF induces *Cebpb* expression and C/EBPβ DNA binding activity in hepatocytes and that this induction is enhanced by serum (Cho & Kim 2003; Shen et al. 1997). In addition to being regulated by HGF, C/EBPβ has also been shown to be rapidly and transiently phosphorylated by growth hormone (GH). GH-induced phosphorylation of C/EBPβ was required for its transcriptional activity in fibroblasts cells (Piwien-Pilipuk et al. 2002). It would be interesting to investigate serum- and growth factor-dependent regulation of C/EBPβ expression and post-translationnal modifications during the process of myogenesis. This

could lead to a better understanding of the mechanisms by which C/EBPβ regulates satellite cell function.

Misexpression of C/EBPβ in SCs in pathological conditions would be predicted to impair normal repair mechanisms. C/EBPβ expression can be induced by lipopolysaccharides, IL-6 and IL-1 and its nuclear localization is promoted by TNFα (Akira et al. 1990; Poli et al. 1990; Yin et al. 1996). Thus, systemic inflammation could result in increases in C/EBPβ expression in muscle SCs. Given that the activation of MyoD expression in SCs is required for their differentiation and regeneration, our data suggests that C/EBPβ levels must first be downregulated for differentiation to occur and thus signals that prevent this downregulation event could trigger a failure of muscle regeneration. Indeed, both aging (sarcopenia) and sepsis can trigger increases in muscle C/EBPβ expression and muscle wasting (Giresi et al. 2005; Penner et al. 2011; Yang et al. 2005). Assessment of engraftment efficiency, self-renewal and differentiation of muscle stem cells expressing ectopic C/EBPβ into skeletal muscle would address many of these questions.

Loss of C/EBPβ expression in $Pax7^+$ SCs not only permitted the differentiation of myoblasts in high serum conditions in the absence of growth factors, but also enhanced their fusion in culture and resulted in muscle fiber hypertrophy *in vivo*, suggesting that the expression of C/EBPβ may, in addition to controlling the efficiency of differentiation, contribute to the inhibition of fusion. Kruppel-like factors 2 and 4 have been shown to have critical functions for fusion during myogenesis, as knockdown of both these factors in primary myoblasts abrogated the fusion of myocytes (Sunadome et al. 2011). Interestingly, C/EBPβ is a known repressor of Klf4 expression in pre-adipocytes, thereby

linking the loss of *Cebpb* expression to the control of myocyte fusion that should be further explored (Birsoy et al. 2008).

With this new role as an inhibitor of myogenesis, C/EBPβ emerges as a central regulator of mesenchymal differentiation in the post-natal organism, acting to promote the formation of fat mass at the expense of lean mass (Rosen et al. 2000; Wiper-Bergeron, Salem, et al. 2007; Wiper-Bergeron, St-Louis, et al. 2007). Thus signaling pathways that impinge on C/EBPβ activity could force lineage decisions in multipotent stem cells in this manner. Forced expression of C/EBPβ in muscle SCs and C2C12 myoblasts did not promote adipoconversion in our experiments, but rather restrained myogenesis. This may be due to the unaffected Myf5 expression observed, which would act to maintain commitment to the myogenic lineage. However, persistent expression of C/EBPβ in SCs coupled with a permissive environment such as signaling that promotes C/EBPβ transcriptional activity could conceivably force the differentiation of SCs into adipocytes (Asakura et al. 2001; Shefer et al. 2004). Indeed, many examples of muscle wasting including sarcopenia and Duchenne's muscular dystrophy are characterized by an abnormal accumulation of fat tissue within the muscle (Argilés et al. n.d.; Chamberlain et al. 2007).

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Disclosure of potential conflict of interest

Dr. Charles Keller has received honoraria from Novartis within the last 12 months. Dr. Charles Keller holds intellectual property rights and ownership interests in Numira Biosciences. Dr. Charles Keller has received research funding from Eli Lilly,Johnson & Johnson, and Blueprint within the last 12 months.

CHAPTER THREE: CCAAT/ENHANCER BINDING PROTEIN β IS REQUIRED FOR SATELLITE CELL SELF-RENEWAL

CCAAT/Enhancer Binding Protein β is required for satellite cell self-renewal

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Abstract

Background: Post-natal growth and repair of skeletal muscle relies upon a population of quiescent muscle precursor cells, called satellite cells. These cells can be activated to proliferate and differentiate into new myofibers, as well as self-renew to replenish the satellite cell population. The balance between differentiation and self-renewal is critical to maintain muscle tissue homeostasis and alterations in this equilibrium can lead to chronic muscle degeneration. The transcription factor CCAAT/Enhancer Binding Protein beta (C/EBPβ) is expressed in $Pax7^+$ satellite cells of healthy muscle and is downregulated during myoblast differentiation. Persistent expression of C/EBPβ upregulates Pax7, inhibits MyoD and blocks myogenic differentiation.

Methods: Using genetic tools to conditionally abrogate C/EBPβ expression in $Pax7^+$ cells, we examined the role of C/EBPβ in self-renewal of satellite cells during muscle regeneration.

Result: We found that loss of C/EBPβ leads to precocious differentiation at the expense of self-renewal in primary myoblast and myofiber cultures. After a single muscle injury, C/EBPβ-deficient satellite cells failed to self-renew resulting in a reduction of satellite cells available for future rounds of regeneration. After a second round of injury, muscle regeneration was impaired in C/EBPβ conditional knockout mice compared to wild-type control mice.

Conclusions: These findings demonstrate that C/EBPβ is a novel regulator of satellite cell self-renewal during muscle regeneration.

Keywords: C/EBPβ, satellite cell, self-renewal, Pax7, MyoD

Background

Skeletal muscle has a remarkable capacity to regenerate after injury, which depends on the presence of muscle progenitor cells, called satellite cells (SC) (Collins et al. 2005; Sambasivan et al. 2011). In adult muscle, satellite cells make up 2-7% of all myonuclei and express the paired-box transcription factor Pax7 (Seale et al. 2000; Fukada et al. 2007; Kuang & Rudnicki 2008; Gnocchi et al. 2009). After myofiber damage, SCs quickly upregulate the myogenic regulatory factor MyoD and re-enter the cell cycle to give rise to a population of transient-amplifying cells (Yablonka-Reuveni & Rivera 1994; Chen & Goldhamer 1999; Zammit et al. 2006). Subsequently, they downregulate Pax7, irreversibly withdraw from the cell cycle, upregulate myogenin expression, commit to terminal differentiation and fuse to repair damaged fibers or to create new ones (Hasty et al. 1993; Zammit et al. 2004; Tedesco et al. 2010; Chen & Goldhamer 1999). While a large majority of myoblasts will differentiate into myocytes, a small percentage of myoblasts escape differentiation by maintaining Pax7 expression, downregulating MyoD expression, and returning to mitotic quiescence (Motohashi & Asakura 2014; Collins et al. 2005). As such, the expression of Pax7 and MyoD allows for classification of SCs as self-renewing ($Pax7^{+}/MyoD^{-}$), proliferating ($Pax7^{+}/MyoD^{+}$) or differentiating ($Pax7^{-}/MyoD^{+}$) (Halevy et al. 2004; Olguin & Olwin 2004; Zammit et al. 2004; Motohashi & Asakura 2014). Self-renewal is important for maintaining the precursor pool. Transplantation of SCs or single muscle fibers can contribute to the repair of hundreds of new muscle fibers (Sacco et al. 2008; Collins et al. 2005) and SCs can replenish the stem cell niche (Montarras, Morgan, Collins, Frédéric Relaix, et al. 2005).

CCAAT/Enhancer Binding Proteins (C/EBPs) are a family of transcription factors that regulate cellular growth and differentiation, metabolism, and inflammation (Ramji & Foka 2002). In skeletal muscle, C/EBPβ expression is localized to $Pax7^{+}$ SCs of healthy muscle and is rapidly downregulated upon induction to differentiate (Marchildon et al. 2012; D. Fu et al. 2015). *In vivo*, loss of C/EBPβ expression in SCs resulted in larger muscle fiber cross-sectional area, fewer fibers and improved repair after a single acute muscle injury (Marchildon et al. 2012; D. Fu et al. 2015). When overexpressed in the murine myoblast cell line C2C12 or in primary myoblasts, C/EBPβ reduced MyoD and other myogenic protein levels during differentiation, in addition to reducing myoblast fusion. C/EBPβ also increased Pax7 protein expression suggesting that C/EBPβ acts to maintain myogenic progenitors in an undifferentiated state (Lamarche et al. 2015; Marchildon et al. 2012).

In this study, we examined the role of C/EBPβ in SC self-renewal using conditional knockout mice ($Cebpb^{fl/fl}Pax7^{CreER/+}$) in which C/EBPβ is knocked down in $Pax7^{+}$ cells following activation of the CreER recombinase with tamoxifen (Sterneck et al. 2006; Nishijo et al. 2009; Marchildon et al. 2012; D. Fu et al. 2015; Lamarche et al. 2015). We show that loss of C/EBPβ in SCs results in precocious differentiation at the expense of self-renewal demonstrated in primary myoblasts and myofiber cultures. Finally, C/EBPβ-deficient SCs are unable to self-renew after muscle injury leading to a reduction in the SC pool. When subjected to a second round of injury, muscle regeneration is impaired in C/EBPβ conditional knockout mice due to the reduced number of SCs after the first round of injury. Taken together, these results establish C/EBPβ as a novel regulator of SC homeostasis.

Methods

Mice and Animal Care. All animal work was performed in accordance with the guideline set out by the Canadian Council on Animal Care and was approved by the University of Ottawa Animal Care Committee. A mouse bearing a C/EBPβ-floxed allele (C/EBPβ$^{fl/fl}$) (Sterneck et al. 2006) was crossed with mice bearing the Pax7-CreER^{tm} allele (Nishijo et al. 2009) to generate C/EBPβ$^{fl/fl}$ (wild-type, WT) and conditional null C/EBPβ$^{-/-}$Pax7$^{CreER-/+}$ (C/EBPβ$^{-/-}$) animals as previously described (Marchildon et al. 2012). All animals were housed in a controlled facility (22°C with 30% relative humidity on a 12 hours light/dark cycle) and provided with food and water ad libitum. *In vivo* induction of CreER^{tm} activity, for myofiber and muscle injury experiments, was accomplished by performing daily intraperitoneal injections of 3mg/40g of tamoxifen (dissolved in corn oil; Sigma-Aldrich) for five days.

For single $BaCl_2$ injury, mice were anesthetized with isofluorane and hind limbs were shaved before the procedure. C/EBPβ$^{fl/fl}$ and C/EBPβ$^{-/-}$Pax7$^{CreER-/+}$ mice aged 8-10 weeks were injected with 50ul of 1.2% $BaCl_2$ in PBS into the left tibialis anterior (TA) muscle. Mice were sacrificed 4, 7 and 42 days post injury (dpi) and the TA was collected, embedded in Tissue-Tek OCT compound, flash frozen in isopentane cooled by liquid nitrogen and sectioned (8um thick) for immunofluorescence. For double $BaCl_2$ injury, mice were allowed to recover from a $BaCl_2$ injury for 21 days before a second injury to the same TA. Mice were sacrificed 7 and 21 days after the second injury.

Preparation and Differentiation of Primary Myoblasts. Primary myoblasts were isolated as described previously (Marchildon et al. 2012). Briefly, hind limb muscles of

adult (6 to 8 weeks of age) mice were dissected and digested with collagenase/dispase (Roche). After digestion, the muscle slurry was filtered through a 70uM cell strainer to remove undigested muscle. Cells were washed with serum-free media and then enriched for myoblasts by selective plating. Primary myoblasts were grown on matrigel-coated plates in growth media (DMEM (Wisent) containing 20% FBS, 10% HS (Invitrogen) with penicillin and streptomycin (Wisent)) supplemented with 10ng/ml basic fibroblast growth factor (bFGF) and 2ng/ml human growth factor (HGF) (Peprotech). Differentiation was induced by changing the media of confluent cultures to differentiation media (DMEM containing 2% FBS and 10% HS) for 48 hours. To induce $CreER^{tm}$ activity in culture, primary myoblasts from wild-type and conditional knockout animals were treated with 4-OH tamoxifen (2uM dissolved in 100% ethanol; Sigma-Aldrich) for 48 hours.

Limited Trypsinization. Myotubes were separated from reserve cells in differentiated WT and $C/EBP\beta^{-/-}$ myoblast cultures as previously described (Kitzmann et al. 1998). Briefly, myoblasts were plated on 10cm culture plates, cultured in GM until confluent and then induced to differentiate in DM for 48 hours. Following differentiation, myotubes were removed from reserve cells by limited trypsinization (0.15% trypsin for 5 minutes). Reserve cells, which remained attached to the culture plate, were removed with 0.25% trypsin. Reserve cells were counted and then re-plated to be induced for differentiation.

Isolation and Culture of Single EDL Myofibers. Myofibers were isolated from extensor digitorum longus (EDL) muscle as described previously (Pasut et al. 2013). Briefly, EDLs were removed from adult (6-8 weeks of age) mice and digested with collagenase type I (2mg/mL in DMEM; Sigma-Aldrich). Muscles were transferred to horse serum-coated plates and myofibers were separated by trituration using heat-polished glass Pasteur pipettes. Fibers were incubated for 72 hours in DMEM supplemented with 15% FBS and 2% chick embryo extract at 37°C, 5% CO_2.

Immunofluorescence. Myofibers were fixed in 4% paraformaldehyde (PFA) in PBS, 1% glycine and blocked in PBS containing 0.2% Triton X-100 (BioShop), 2% BSA, 5% goat serum (Cedarlane), 1% azide. Myoblasts were fixed in 2% PFA in PBS and blocked in PBS containing 0.3% Triton X-100 and 10% goat serum. Cryosections were thawed at room temperature, fixed in 4% PFA and processed for antigen retrieval in citrate buffer at 95°C for 20 minutes. Sections were permeabilized with PBS containing 0.5% Triton X-100 and blocked in PBS containing 0.1% Triton X-100, 5% donkey serum (Cederlane) prior to incubation with primary antibody overnight at 4°C. Cells were washed with PBS and incubated in biotin anti-mouse (when indicated) or secondary antibodies conjugated to a fluorescent dye (Cy3, Alexa 488 or Alexa Flour 647; all from Jackson ImmunoResearch). Nuclei were counterstained with DAPI (0.5ug/ml). Primary antibodies used were: Pax7-c (DSHB), MYH (H-300; Santa Cruz), MyoD (C-20; Santa Cruz), myogenin (M-225; Santa Cruz), and Laminin (AL-4; Millipore).

Image Acquisition. Digital images of stained myoblasts, myofibers and muscle sections were acquired at room temperature using a microscope (Leica DM 3000B) Infinity-3 camera (Lumenera) and Infinity Capture imaging software (Lumenera). Images were composed and edited in paint.net.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). A student's t-test was used in all experiments comparing wild-type and C/EBPβ$^{-/-}$ conditions. The cutoff for significance was $p<0.05$. All experiments are representative of a minimum of three biological replicated and data is presented as a mean ± standard error mean (SEM).

Results

C/EBPβ-deficient satellite cells display increased differentiation

To investigate the functional consequences of disrupting C/EBPβ expression on SC function *in vitro,* we isolated SCs from conditional knockout mice (*Cebpb*$^{fl/fl}$*Pax7*$^{CreER/+}$) in which C/EBPβ expression is abrogated in Pax7^{+} cells following activation of the CreER recombinase with tamoxifen. SC-derived myoblasts (*Cebpb*$^{fl/fl}$*Pax7*$^{+/+}$ (wild-type; WT) and C/EBPβ$^{-/-}$) were cultured in high serum (GM) for 24 hours and excision was confirmed by RT-qPCR, with *Cebpb* expression reduced to ~25% of controls (Figure 12A). Isolated myoblasts were stained for Pax7 and MyoD (Figure 12B). As C/EBPβ is a known regulator of Pax7 and MyoD protein expression, the proportion of self-renewing (Pax7^{+}/MyoD^{-}), proliferating (Pax7^{+}/MyoD^{+}) and differentiating (Pax7^{-}/MyoD^{+}) cells was assessed in WT and C/EBPβ$^{-/-}$ myoblasts in

growth medium. The percentage of $Pax7^{+}/MyoD^{-}$ (self-renewing) cells was decreased in $C/EBP\beta^{-/-}$ myoblasts compared to WT myoblasts (2.9% vs. 5.2%) (Figure 12C). There was no significant change in the population of $Pax7^{+}/MyoD^{+}$ proliferating cells between $C/EBP\beta^{-/-}$ myoblasts and WT myoblasts in GM (Figure 12D); however, there were significantly more $Pax7^{-}/MyoD^{+}$ differentiating cells in C/EBPβ-deficient myoblasts as compared to controls (16.2% vs. 8.8%) (Figure 12E).

We next examined if C/EBPβ-deficient myoblasts differentiated further down the myogenic pathway to express myogenin. There was a significant ~2-fold increase in the percentage of myogenin-positive cells in $C/EBP\beta^{-/-}$ myoblasts as compared to WT myoblasts (Figure 12F,G) suggesting that C/EBPβ-deficient cells are more apt to precociously differentiate under growth conditions, consistent with our previous findings (Marchildon et al. 2012).

Myoblasts (WT and $C/EBP\beta^{-/-}$) grown in GM for 24 hours were also harvested for mRNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR) of Notch receptor and target gene expression. The Notch pathway is an established regulator of SC self-renewal and homeostasis (Fukada et al. 2011; Mourikis et al. 2012; Bjornson et al. 2012; Wen et al. 2012), thus we examined whether the expression of Notch receptors (*Notch1, Notch2, Notch3*) and Notch target genes (*Hey1, Heyl*) were disrupted in $C/EBP\beta^{-/-}$ myoblasts. The expression of Notch2 and Notch3 as well as both target genes were significantly downregulated in myoblasts deficient in C/EBPβ compared to wild-type myoblasts in GM (Figure 12H), suggesting that the self-renewal pathway is perturbed in the absence of C/EBPβ.

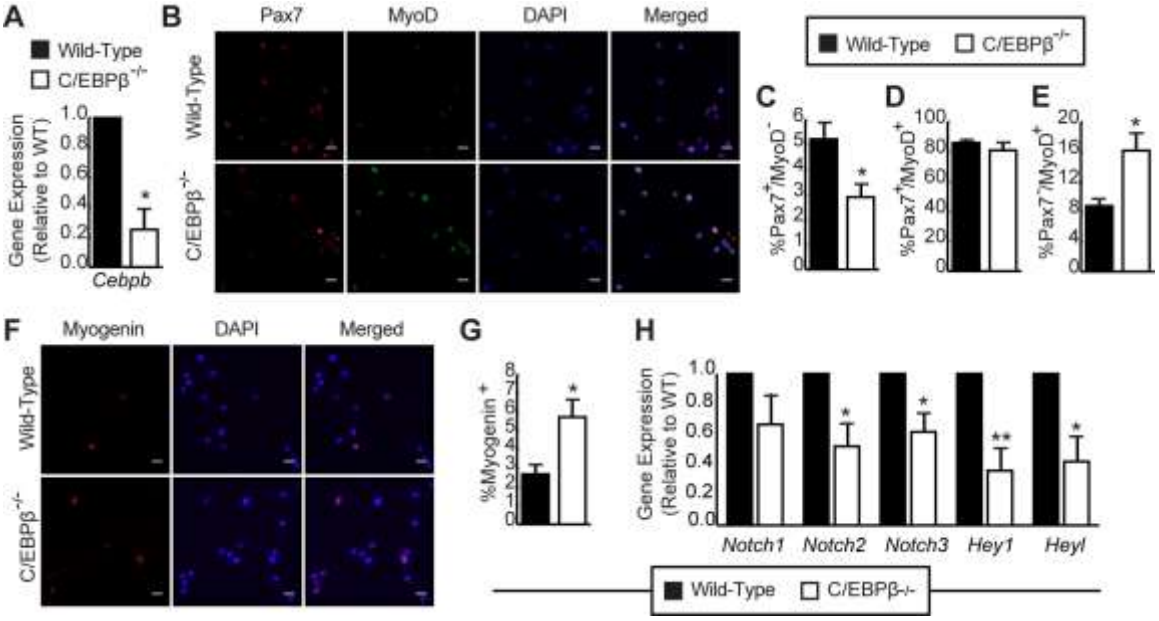

Figure 12. Loss of C/EBPβ reduces the proportion of Pax7$^+$ cells in myogenic cultures. (A) RT-qPCR analysis of *Cebpb* expression in primary myoblasts isolated from C/EBPβ conditional knockout mice (C/EBPβ$^{-/-}$) or wild-type non-Cre expressing littermates (WT) subjected to *in vitro* 4'OH-TAM treatment and cultured in growth medium (GM) (n=3). **(B)** Representative pictures of myoblasts cultured under growth conditions for 24 hours stained for Pax7 (red) and MyoD (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20um. Percentage of **(C)** self-renewing (Pax7$^+$/MyoD$^-$), **(D)** proliferating (Pax7$^+$/MyoD$^+$) and **(E)** differentiating (Pax7$^-$/MyoD$^+$) WT (black bars) and C/EBPβ$^{-/-}$ (white bars) myoblasts cultured as in (A) as determined by immunocytochemistry (n=4). **(F)** Representative pictures of cells cultured as in (A) and immunostained for myogenin (red). Nuclei were counterstained with DAPI (blue). Scale bar: 20um. **(G)** Percentage of myogenin$^+$ cells relative to total nuclei (n=4). (H) RT-qPCR analysis of Notch receptor expression (*Notch1, Notch2, Notch3*) and Notch

target gene expression (*Hey1*, *Heyl*) in myoblasts cultured for 24h in GM (n=3). For all panels, data is the mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

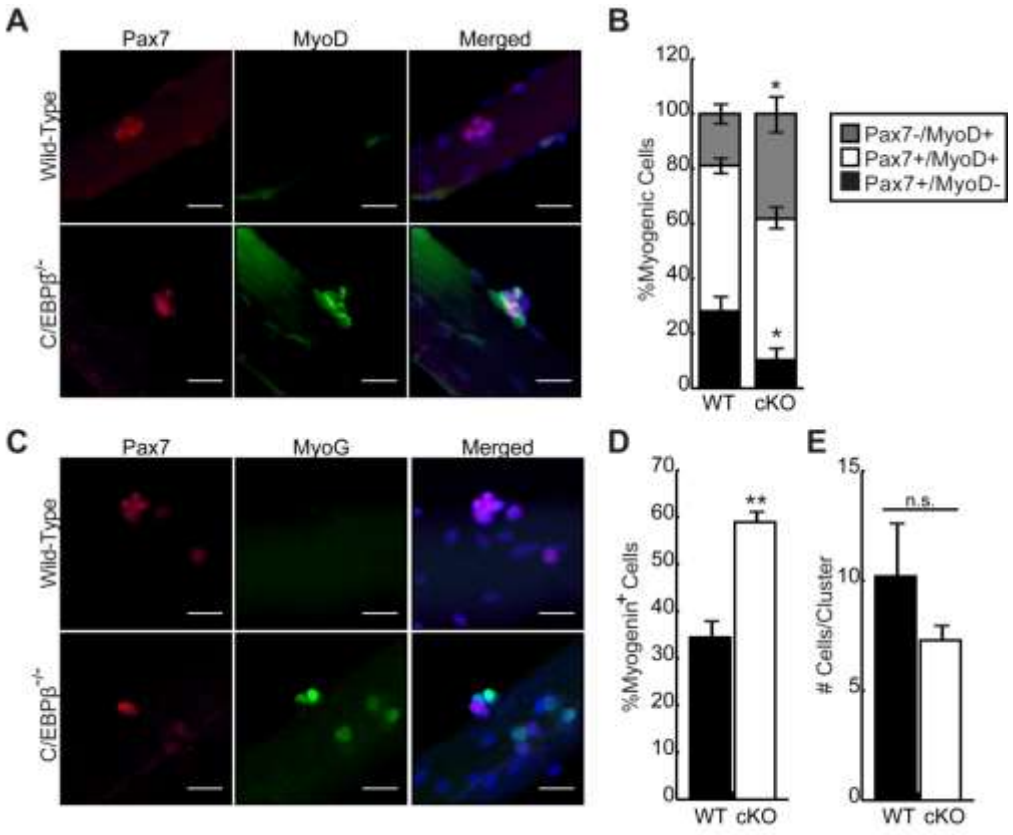

Figure 13. Loss of C/EBPβ inhibits self-renewal of satellite cells and promotes differentiation. C/EBPβ$^{fl/fl}$Pax7$^{+/+}$ (wild-type) and conditional null C/EBPβ$^{-/-}$Pax7$^{CreER-/+}$ (C/EBPβ$^{-/-}$) mice were injected daily with tamoxifen for 5 days. One week after the last injection, myofibers were isolated from the EDL of each mouse and cultured for three days in suspension before immunostaining. **(A)** Representative pictures of myofibers stained for Pax7 (red) and MyoD (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20um. **(B)** Quantification of self-renewing (Pax7^{+}/MyoD^{-}), proliferating (Pax7^{+}/MyoD^{+}) and differentiating (Pax7^{-}/MyoD^{+}) myoblasts on single myofibers (n=4 mouse pairs). **(C)** Representative pictures of myofibers stained for Pax7 (red) and myogenin (green). Nuclei were counterstained with DAPI (blue). Scale bar: 20um. **(D)** Quantification of myogenin^{+} cells as a percentage of total nuclei (n=3 mouse pairs). **(E)**

Total number of cells per cluster (n=3 mouse pairs). For all panels, data is mean ± SEM, *p<0.05, **p<0.01, n.s. not significant.

To further investigate C/EBPβ-dependent alteration in cell fate choices, we used single myofiber preparations isolated from the extensor digitorum longus (EDL) muscles of wild type (WT) and conditional null (C/EBPβ$^{-/-}$) animals one week after daily tamoxifen (3mg/40g for 5 days) injections to induce excision. Myofibers from WT and C/EBPβ$^{-/-}$ mice were stained for Pax7 and MyoD and counterstained with DAPI to determine the number of self-renewing (Pax7^{+}/MyoD^{-}), activated (Pax7^{+}/MyoD^{+}) and differentiated (Pax7^{-}/MyoD^{+}) SCs. Myofibers from C/EBPβ$^{-/-}$ animals had significantly fewer Pax7^{+}/MyoD^{-} SCs (10.4%) and more Pax7^{-}/MyoD^{+} SCs (38.3%) compared to WT EDL myofibers (28.1% and 18.9%, respectively) (Figure 13A,B). Furthermore, when immunolabelled for Pax7 and myogenin, myofibers from C/EBPβ$^{-/-}$ animals had significantly more myogenin^{+} cells (59%) compared to control EDL myofibers (34%) (Figure 13C,D). There were no changes in the number of satellite cells per cluster on WT and C/EBPβ$^{-/-}$ myofibers, confirming that loss of C/EBPβ does not inhibit SC activation (Figure 13E). These results further demonstrate that C/EBPβ promotes SC self-renewal and that loss of C/EBPβ in SCs leads to increased differentiation.

C/EBPβ is required for the generation of reserve cells *in vitro*

To examine the role of C/EBPβ in SC self-renewal during myogenic differentiation, SC-derived myoblasts (wild-type and C/EBPβ$^{-/-}$) were cultured in high serum (GM) for 24 hours and then switched to low serum (differentiation media; DM) for 48 hours to induce differentiation. Terminally differentiated myoblasts were fixed and stained for myosin heavy chain (MyHC) and Pax7 expression to quantify myoblast fusion and reserve cell formation, respectively (Figure 14A) (Yoshida et al. 1998; Kitzmann et

al. 1998). Differentiated C/EBPβ-null cultures had fewer $Pax7^{+}$ reserve cells (2.8%) compared to control cultures (8.2%) (Figure 14A,B) consistent with the reduction of $Pax7^{+}$/$MyoD^{-}$ cells observed in GM (Figure 12B). $Pax7^{+}$ reserve cells can be separated from myotubes by limited trypsinization and differentiated again to study self-renewal dynamics in a cell population (Kitzmann et al. 1998; Yoshida et al. 1998). To determine whether C/EBP$\beta^{-/-}$ reserve cells function normally, we collected reserve cells by limited trypsinization, and after expansion and equal plating, induced them to differentiate. After each round of differentiation, we counted the total number of reserve cells. After each passage, despite equal plating for each round of differentiation, there were fewer C/EBP$\beta^{-/-}$ reserve cells compared to wild-type cells (Figure 14C). Further, after each round, the number of reserve cells trended towards a decrease in the C/EBP$\beta^{-/-}$ cultures, but did not achieve statistical significance (Figure 14C). These data suggests that C/EBPβ is required for maintenance of reserve cells *in vitro*.

Differentiated myoblast (WT and C/EBP$\beta^{-/-}$) cultures were also harvested for gene expression analysis. RT-qPCR confirmed excision of *Cebpb* in myoblasts isolated from C/EBP$\beta^{-/-}$ animals (Figure 14D). As in GM cultures, the expression of Notch receptors (*Notch1, Notch2, Notch3*) and Notch target genes (*Hey1, Heyl*) was disrupted in C/EBP$\beta^{-/-}$ myoblast (Figure 14E).

C/EBPβ is required to restore the muscle satellite cell pool during regeneration.

To confirm the reduced self-renewal potential of C/EBP$\beta^{-/-}$ satellite cells *in vivo*, an acute injury was induced by $BaCl_2$ injection into the TA muscle of C/EBP$\beta^{fl/fl}$$Pax7^{+/+}$ (wild-type) and conditional null C/EBP$\beta^{-/-}$$Pax7^{CreER-/+}$ (C/EBP$\beta^{-/-}$) animals one week after

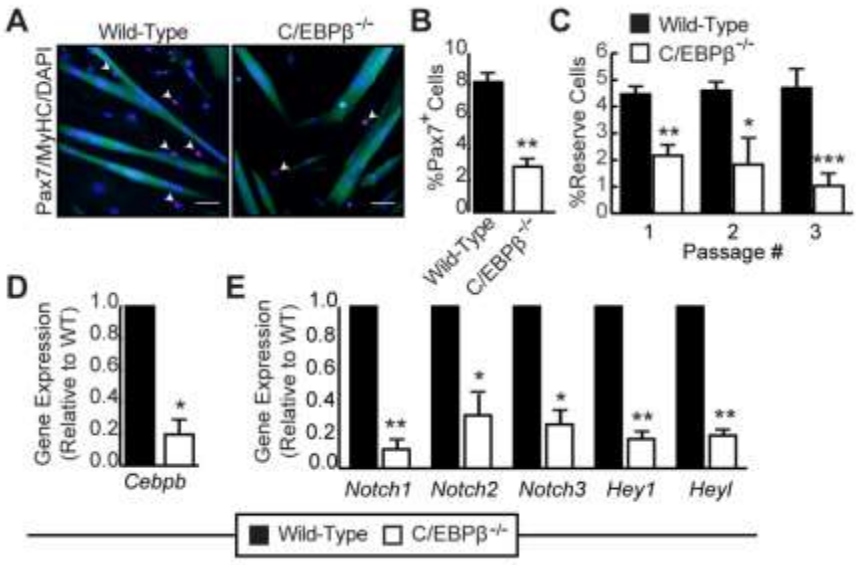

Figure 14. Loss of C/EBPβ leads to a reduction in reserve cells *in vitro*. Primary myoblasts isolated from C/EBPβ conditional knockout mice (C/EBPβ$^{-/-}$) or wild-type non-Cre expressing littermates were subjected to *in vitro* 4'OH-TAM treatment, cultured in GM, plated and transferred to differentiation media for 48h. **(A)** Representative pictures of cells stained for myosin heavy chain (MyHC; green) and Pax7 (red). Nuclei were counterstained with DAPI (blue). Scale bar: 50um. **(B)** Pax7^{+} cells were counted and represented as a percentage of total nuclei (n=3). **(C)** Reserve cells were collected and counted following limited trypsinization after 48h in differentiation media. After counting, reserve cells were expanded, re-plated at equal densities and induced to differentiate in low serum for another 48h. The number of reserve cells was counted after each round of differentiation and represented as a percentage of total nuclei (n=3). **(D)** RT-qPCR analysis of *Cebpb* expression in myoblasts cultured for 48h in DM (n=3). **(E)** RT-qPCR analysis of Notch receptor expression (*Notch1, Notch2, Notch3*) and Notch target gene expression (*Hey1, Heyl*) (n=3). Data is represented as a mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

daily tamoxifen (3mg/40g for 5 days) administration. TA muscles were collected 0, 4, 7 and 42 days post injury (dpi) (Figure 15A). Immunostaining of injured TA muscle at 0, 4 and 7 dpi for Pax7 revealed that there were no changes in the number of $Pax7^+$ cells in C/EBPβ-deficient muscle compared to wild-type muscle (Figure 15B,C), suggesting that C/EBPβ-deficient myoblasts expand efficiently after muscle injury.

During postnatal regeneration, the size of the $Pax7^+$ satellite cell pool reaches homeostatic levels observed in uninjured muscle 40 days after injury (Shea et al. 2010). In wild-type muscle, $Pax7^+$ cell numbers returned to non-injured levels, however, there were significantly fewer $Pax7^+$ cells in C/EBPβ-null muscle (Figure 15B,D). These findings suggest that C/EBPβ is essential for directing cells towards self-renewal after injury.

Loss of C/EBPβ expression cripples the long-term regenerative response.

As SC self-renewal is essential for efficient repair of tissue after injury, we examined muscle regeneration in wild-type and conditional null ($C/EBP\beta^{-/-}$) animals after two serial $BaCl_2$ injuries separated by 21 days. TA muscles were collected 7 and 21 days after the second injury for analysis (Figure 16A). One week following double injury (7 dpi), TA muscle sections were stained for embryonic myosin heavy chain (eMyHC) to measure the size of newly regenerated fibers. Contrary to what was observed after a single injury (Marchildon et al. 2012), the average cross-sectional area of $eMyHC^+$ fibers was significantly smaller in $C/EBP\beta^{-/-}$ muscle (348 um) compared to wild-type muscle (661 um) (Figure 16B,C). There were also fewer $Pax7^+$ cells in C/EBPβ-deficient muscle compared to wild-type muscle (Figure 16D). To confirm that the smaller fiber size in

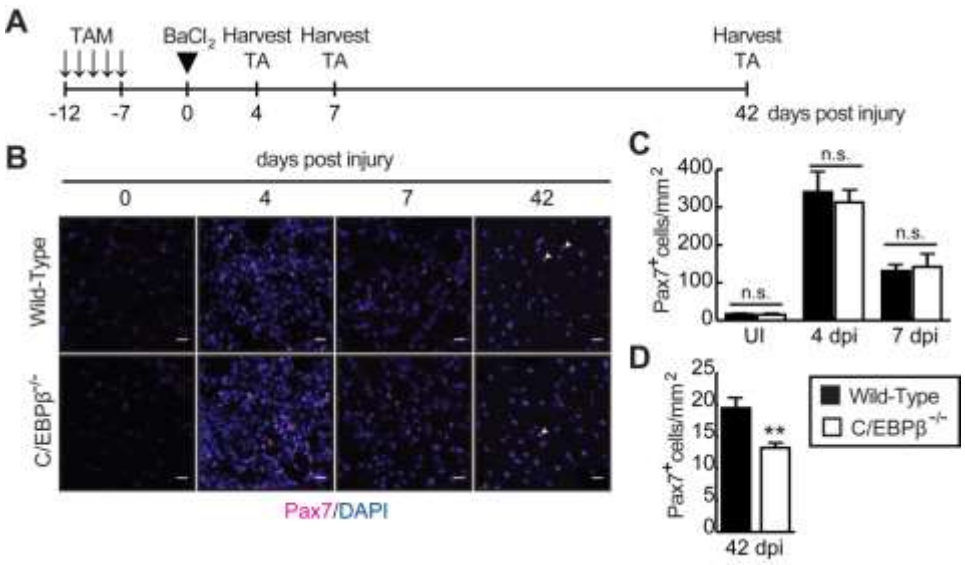

Figure 15. C/EBPβ is required for restoring the muscle stem cell pool after acute injury. (A) Experimental design. C/EBPβ$^{fl/fl}$ Pax7$^{+/+}$ (wild-type) and conditional null C/EBPβ$^{-/-}$Pax7$^{CreER-/+}$ (C/EBP β$^{-/-}$) mice were injected daily with tamoxifen for 5 days. One week after the last injection, the left tibialis anterior (TA) muscle was injured by intramuscular injection of 50ul of 1.2% $BaCl_2$. Muscle was analysed at 0, 4, 7 and 42 days post injury (dpi). **(B)** Cross-sections of injured TA 0, 4, 7 and 42 dpi were immunostained for Pax7 (red) and counterstained with DAPI (blue). Representative pictures are shown. Scale bar: 20um. **(C)** Pax7$^+$ nuclei were counted at 0, 4 and 7 dpi and represented as total Pax7$^+$ cells per area (mm^2) (n≥3 mouse pairs). **(D)** Pax7$^+$ nuclei were counted in TA sections 42 dpi and represented as total Pax7$^+$ cells per area (mm^2) (n=4 mouse pairs). For all panels, data is represented as mean ± SEM, **p<0.01, n.s. not significant.

C/EBPβ-null animals was not due to a delay in regeneration, we assessed the average cross-sectional area of fibers 21 days after secondary injury (21 dpi). Consistent with our 7 dpi findings, muscle fibers from C/EBPβ$^{-/-}$ animals were smaller (1353 um) compared to wild-type fibers (1703 um) (Figure 16E,F) and the percentage of Pax7 SCs remained lower in C/EBPβ-deficient muscle (Figure 16G). Taken together, these results strongly suggest that C/EBPβ is essential for SC self-renewal that is necessary for continual muscle regeneration.

Discussion

While most activated satellite cells will progress through myogenic differentiation, a small fraction escape, return to quiescence and self-renew to maintain the muscle stem cell population, marked by loss of MyoD expression. In addition to downregulation of MyoD expression, it is known that the SC population itself is heterogeneous and can undergo both asymmetrical and symmetrical divisions in the context of the niche, with asymmetric distribution of cellular proteins and chromatin templates which regulate the balance between SC maintenance and regenerative potential. Analysis of myogenic regulatory factor (Myf5 and MyoD) expression in proliferating SCs revealed asymmetric expression of these factors in newly divided daughter cells (Kuang et al. 2007; Troy et al. 2012; Liu et al. 2012). These multiple mechanisms implicated in the maintenance of the skeletal muscle stem cell pool underlie the importance of these cells for muscle homeostasis. Too many cells destined towards repair results in rapid depletion of the SC compartment as observed with loss of C/EBPβ, while enhanced self-renewal restrains regeneration, with both disequilibria leading to

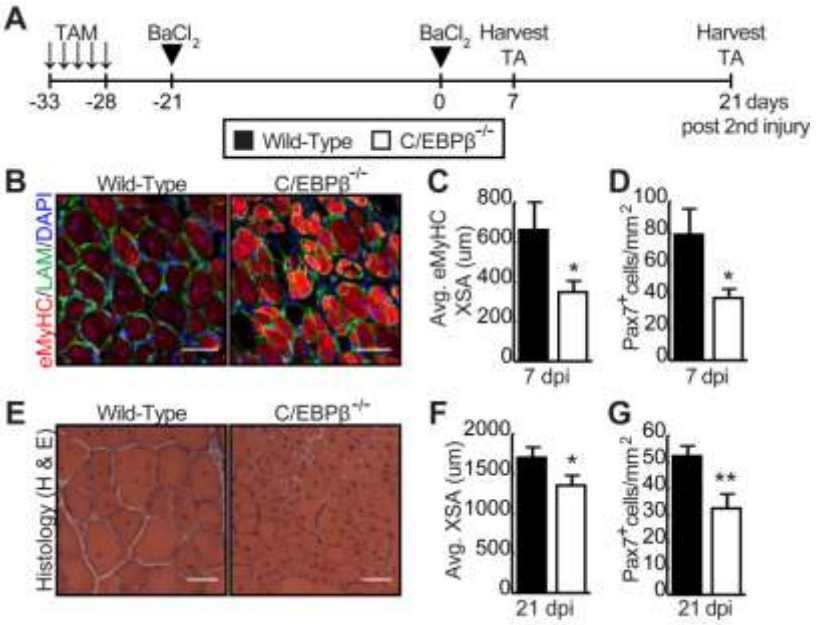

Figure 16. Loss of C/EBPβ expression in satellite cells decreases regenerative capacity following serial injury. (A) Experimental design. C/EBPβ$^{fl/fl}$ Pax7$^{+/+}$ (wild-type) and conditional null C/EBPβ$^{-/-}$Pax7$^{CreER-/+}$ (C/EBP β$^{-/-}$) mice were injected daily with tamoxifen for 5 days. One week after the last injection, the left tibialis anterior (TA) muscle was injured using 50ul of 1.2% $BaCl_2$ (1st injury). 21 days after the initial injury, the same TA muscle was injured again using 50ul of 1.2% $BaCl_2$ (2nd injury). Muscle was analysed at 7 and 21 days after the 2nd injury (dpi). **(B)** Cross-sections of injured TA 7 dpi were stained with embryonic myosin heavy chain (eMyHC; red) and laminin (LAM; green). Nuclei were counterstained with DAPI (blue). Representative pictures are shown. Scale bar: 50um. **(C)** Average cross-sectional area of eMyHC^{+} fibers from TA sections stained as in (B) (n=5 mouse pairs). **(D)** Pax7^{+} nuclei were counted in 7 dpi muscle and represented as total Pax7^{+} cells per area (mm^2) (n=4 mouse pairs). **(E)** Cross-sections of injured TA 21 dpi were stained for hematoxylin (nuclei; purple) and eosin (pink) (H & E). Representative pictures are shown. Scale bar: 50um. **(F)** Average cross-sectional area

of myofibers from TA sections stained as in (E) (n=6 mouse pairs). **(G)** $Pax7^{+}$ nuclei were counted in 21 dpi muscle and represented as total $Pax7^{+}$ cells per area (mm^2) (n=3 mouse pairs). Data is represented as mean ± SEM, *$p<0.05$, **$p<0.01$.

muscle atrophy. Recently, loss of dystrophin expression in SCs in Duchenne muscular dystrophy was shown to reduce the available pool of SCs for repair, contributing to the pathogenesis of this disease (Dumont et al. 2015).

While self-renewal is important for maintaining the precursor pool, the mechanisms by which some SCs evade differentiation remain poorly understood. In this study we identify C/EBPβ as a novel regulator of SC self-renewal, demonstrating that in its absence, $Pax7^{+}/MyoD^{-}$ cells are generated in lesser number, resulting in a reduction of the satellite cell pool. While muscle in the conditional nulls repairs with greater efficiency after a single injury due to an increased propensity to differentiate (Marchildon et al. 2012), this repair results in a significant decrease in the SC population that cannot support repair after a second injury. However, using our conditional model, we observed a reduction in the satellite cell pool, rather than depletion, likely because of incomplete excision of *Cebpb* and therefore the contribution of recombination escapers.

The mechanisms by which C/EBPβ expression promotes self-renewal and inhibits myogenesis remain unknown. While C/EBPβ is normally downregulated in early differentiation, a step that is required for full expression of MyoD and differentiation to occur, C/EBPβ can also regulate Pax7 expression (Marchildon et al. 2012). While loss of C/EBPβ expression does not affect the progression towards the $Pax7^{+}/MyoD^{+}$ state, it remains unclear whether C/EBPβ is asymmetrically distributed in proliferating myoblasts or whether its expression is re-initialized in a small population of Pax7+ cells destined to self-renew. Indeed, while C/EBPβ expression does not appear to be required for Pax7 expression (Marchildon et al. 2012), it can force Pax7 to be expressed under differentiation conditions, and this mechanism could prevent myoblasts from completing

myogenic differentiation. Many studies have demonstrated that adult skeletal muscles lacking Pax7-expressing satellite cells cannot regenerate (Lepper et al. 2011; Murphy et al. 2011; Sambasivan et al. 2011).

A number of signaling pathways have been implicated in SC self-renewal. Recent literature suggests that Notch signaling is crucial for SCs to self-renew and return to quiescence (Mourikis et al. 2012; Bjornson et al. 2012; Wen et al. 2012; Fukada et al. 2011). In adult muscle, the absence of Notch signaling induced spontaneous activation and differentiation of SCs which lead to a reduction in self-renewal and consequently impaired muscle regeneration (Mourikis et al. 2012; Bjornson et al. 2012). Furthermore, overexpression of Notch Intracellular Domain ($NICD^{OE}$) upregulates Pax7, downregulates MyoD and inhibits S-phase entry of primary myoblasts (Wen et al. 2012). SC specific $NICD^{OE}$ also impaired regeneration of skeletal muscle (Wen et al. 2012) and loss of downstream Notch target genes Hesr1 (Hey1) and Hesr3 (Heyl) led to precocious differentiation, reduced SC self-renewal and a reduced SC pool (Fukada et al. 2011). Interestingly, we show that loss of C/EBPβ in SC-derived myoblasts results in a significant downregulation in genes involved in the Notch signaling pathway (Figure 1 and 3) suggesting one possible mechanism by which C/EBPβ regulates SC self-renewal is through regulation of Notch. Indeed, reduced expression of Notch signaling genes and poor repopulation of mammary stem cells has been observed in mammary glands lacking C/EBPβ (LaMarca et al. 2010).

Conclusions

C/EBPβ-deficient satellite cells are unable to efficiently self-renew after muscle injury, leading to a reduction in the SC pool, and impaired regenerative capacity after serial injury. Taken together, our results establish C/EBPβ as a novel regulator of SC homeostasis that promotes differentiation at the expense of self-renewal. For example, while increased expression of C/EBPβ in cachectic muscle inhibits repair of wasting fibers, C/EBPβ protects the stem cells from apoptosis. The characterization of transcription factors controlling SC homeostasis provides important insights into the molecular mechanisms regulating skeletal muscle regeneration and further insight into the regulation of C/EBPβ function and its biological role in the context of healthy and diseased muscle can fuel the development of novel therapeutic approaches for the treatment of muscle atrophies.

Abbreviations: SC, satellite cell, C/EBPβ, CCAAT/Enhancer Binding Protein beta; Pax7, paired box protein 7; MyoD, myogenic differentiation factor 1; GM, growth media; WT, wild-type; PBS, phosphate-buffered saline; PFA, paraformaldehyde; MyoG, myogenin; EDL, extensor digitorum longus; IHC, immunohistochemistry; TA, tibialis anterior; DM, differentiation media; MyHC, myosin heavy chain; mRNA, messenger ribonucleic acid; RT-qPCR, real time quantitative polymerase chain reaction; dpi, days post injury; eMyHC, embryonic myosin heavy chain; IBMX, 3-isobuty-1-methylxanthine

Declarations

Ethics Approval. The animal experiments were approved by the University of Ottawa's Animal Care Committee (protocols CMM-2165-R1- A1, CMM-2166-R1) respecting the standards for ethical use of animals stipulated by the Canadian Council on Animal Care.

Competing Interests. The authors declare that they have no competing interests.

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Author Contributions. NLT: conception and design, collection, assembly, analysis and interpretation of data, manuscript writing, and final approval of manuscript; FM: conception and design, collection and assembly of data, data analysis and interpretation, and final approval of manuscript; DF: collection and assembly of data, data analysis and interpretation, and final approval of manuscript; NWB: conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript.

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CHAPTER FOUR: CCAAT/ENHANCER BINDING PROTEIN B IS REQUIRED TO MAINTAIN MUSCLE SATELLITE CELL QUIESCENCE

CCAAT/Enhancer Binding Protein β is required to maintain muscle satellite cell quiescence

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Author Contributions.

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Hamood AlSuidais: Collection and assembly of data, data analysis and interpretation and final approval of manuscript

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Nadine Wiper-Bergeron: conception and design, financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript

Abstract

Regeneration of skeletal muscle depends on resident muscle stem cells called satellite cells which, in healthy muscle remain quiescent (non-cycling). Loss of satellite cell quiescence can result in spontaneous activation and differentiation leading to depletion of the stem cell pool. Here, we show that the transcription factor CCAAT/Enhancer Binding Protein beta (C/EBPβ) is required to maintain quiescence in satellite cells. We show that C/EBPβ is upregulated in non-cycling myoblasts. Forced expression of C/EBPβ in myoblasts decreased the number cycling myoblasts, whereas loss of C/EBPβ resulted in an increase in cycling cells. C/EBPβ-mediated induction of quiescence was dependant on caveolin-1, and we demonstrate that caveolin-1 is a direct transcriptional target of C/EBPβ. Finally, loss of C/EBPβ in the satellite cells of resting muscle resulted in their premature activation and differentiation leading to stem cell pool depletion. Our study provides new insight into the mechanisms regulating satellite cell quiescence and homeostasis.

Introduction

Most adult mammalian tissues contain resident stem cells. These cells are an important reserve for tissue regeneration providing differentiated cells while able to repopulate the stem cell pool (self-renewal). In tissues with low turnover such as skeletal muscle, adult stem cells remain quiescent (in G0 or G1 state of cell cycle) for extended periods of time (Li & Clevers 2010; Cotsarelis et al. 1990; Arai et al. 2004; Schultz et al. 1978). Loss or dysregulation of quiescence in stem cells can result in an imbalance characterized by an increase in differentiated progenitor cells and reduced self-renewal leading to depletion of the stem cell pool (Orford & Scadden 2008; Cheung et al. 2012). For example, loss of caveolin-1, a major structural component of caveolae, in hematopoietic stem cells disrupted quiescence and inhibited their ability to differentiation into mature blood cells (Bai et al. 2014). Thus, identification of the molecular regulators of stem cell quiescence can contribute to our understanding of tissue homeostasis.

The remarkable capacity of skeletal muscle to regenerate after injury depends on muscle stem cells, called satellite cells (SC) (Collins et al. 2005; Sambasivan et al. 2011). These cells make up 2-7% of all myonuclei and are maintained in a quiescent state in healthy (resting) muscle (White et al. 2010; Schultz et al. 1978). Upon muscle injury SCs activate, quickly re-entering the cell-cycle to proliferate and give rise to committed fusion-competent myoblasts (Chen & Goldhamer 1999; Yablonka-Reuveni & Rivera 1994; Zammit et al. 2006). Activated satellite cells that do not differentiate undergo self-renewal and return to mitotic quiescence (Motohashi & Asakura 2014; Almada & Wagers 2016; Collins et al. 2005). Despite the importance of muscle stem cell quiescence in

muscle homeostasis, the mechanisms by which satellite cells maintain quiescence remains poorly understood.

CCAAT/Enhancer Binding Protein β (C/EBPβ) is a member of the C/EBP family of bzip transcription factors that are involved in a number of cellular processes such as growth and differentiation (Ramji & Foka 2002). C/EBPβ's function in cell growth is very complex and highly context specific, having both inhibitory and stimulatory effects on proliferation. For example, C/EBPβ-null mice have enhanced hematopoiesis and mild epidermal hyperplasia, suggesting that C/EBPβ inhibits expansion of lymphoid cells and keratinocytes respectively (Screpanti et al. 1995; Zhu et al. 1999). By contrast, C/EBPβ-null mammary epithelial cells have impaired proliferation and disrupted ductal morphogenesis (Robinson et al. 1998) and C/EBPβ has been shown to be essential for mitotic clonal expansion during adipogenesis (Tang et al. 2003; J.-W. Zhang et al. 2004), suggesting that the regulation of proliferation by C/EBPβ is highly cell type specific .

In skeletal muscle, C/EBPβ expression is localized to satellite cells of resting muscle and *Cebpb* mRNA is highly expressed in quiescent SCs (Marchildon et al. 2012; Fukada et al. 2007). When satellite cell-derived myoblasts are isolated and induced to differentiate, C/EBPβ expression is rapidly downregulated (D. Fu et al. 2015; Marchildon et al. 2012). Persistent expression of C/EBPβ in myoblasts blocks differentiation locking cells in a Pax7 high, MyoD low, undifferentiated state (Lamarche et al. 2015; Marchildon et al. 2012).

In this study, we examined the role of C/EBPβ in the establishment of satellite cell quiescence. We demonstrate that C/EBPβ is upregulated in non-cycling myoblasts and when overexpressed, C/EBPβ can promote cell cycle arrest. Furthermore, C/EBPβ-

mediated cell cycle arrest requires upregulation of caveolin-1, which we identify as a direct transcriptional target of C/EBPβ. Loss of C/EBPβ in satellite cells *in vivo* results in precocious activation and differentiation in uninjured muscle, suggesting that C/EBPβ is required to maintain SC quiescence.

Results and discussion

C/EBPβ regulates cell cycle progression in myoblasts

Overexpression of C/EBPβ has been shown to increase Pax7 expression while decreasing MyoD protein expression (Marchildon et al. 2012) and this profile coupled with low Ki67 expression is characteristic of cells that self-renew under differentiation conditions to return to quiescence (Motohashi & Asakura 2014). To determine if C/EBPβ is regulated during the transition from activation to quiescence, we induced quiescence in C2C12 myoblasts using methionine-depleted media, which halts the cell cycle and prevents myogenic differentiation (Kitzmann et al. 1998). G0/G1 arrest was confirmed by cell cycle analysis, with approximately 93% of cells in G0/G1 versus approximately 63% in growth medium (GM) (Figure 17A). C2C12 myoblasts cultured in methionine-depleted cells had increased p27 and p21 expression as compared to proliferating cells in growth medium, as well as lower levels of Cyclin D1, consistent with growth arrest (Figure 17B). Further, C/EBPβ expression was higher in methionine-depleted cells when compared to proliferating C2C12s. Culture in low serum conditions to induce differentiation resulted in stimulation of p27 and p21 expression and a drop in Cyclin D1 levels as well as loss of C/EBPβ expression, as expected. Thus, C/EBPβ is

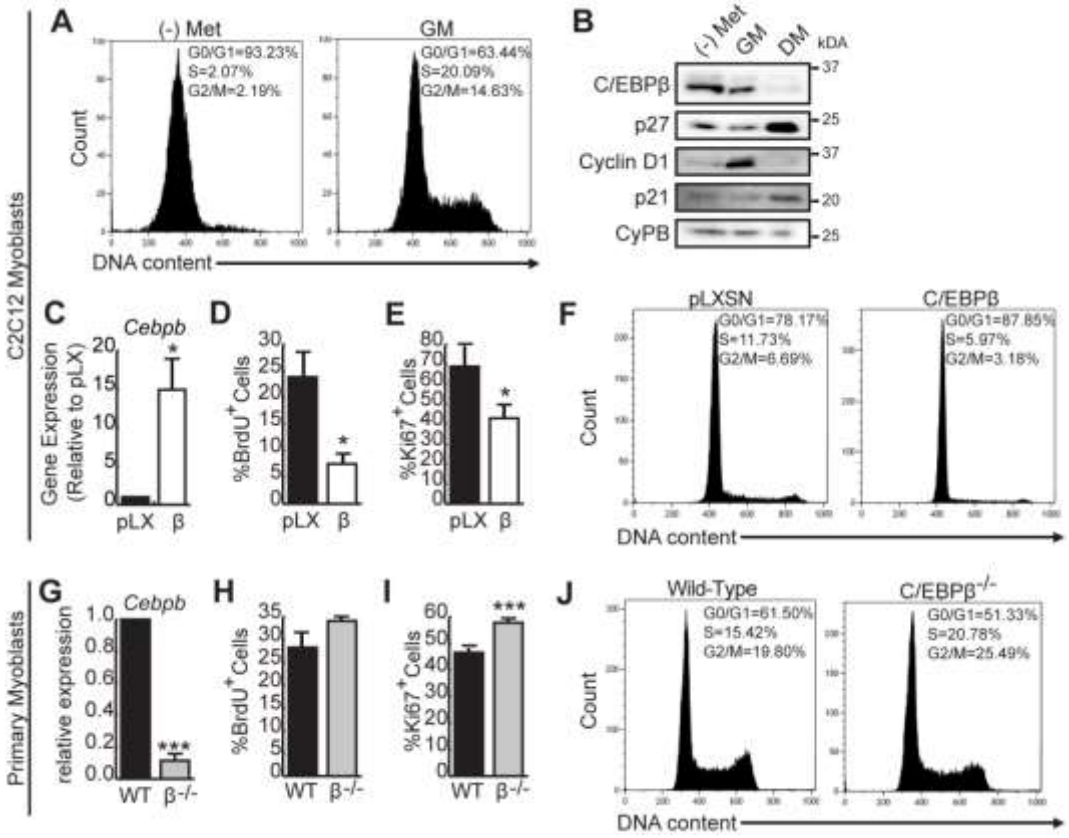

Figure 17. C/EBPβ regulates cell cycle progression in myoblasts. (A) Flow cytometric analysis of DNA content in C2C12 myoblasts cultured in methionine-depleted media ((-) Met) or growth media (GM). **(B)** Representative western blots of C/EBPβ and cell cycle marker expression from cells cultured as in (A) or in differentiation medium for 24 hours (DM). Cyclophilin B (CyPB) is a loading control. **(C)** *Cebpb* expression in C2C12s retrovirally transduced to express C/EBPβ (β) or with empty virus (pLX) cultured in growth medium. **(D)** $BrdU^+$ cells as the percentage of total nuclei in C2C12 cells transduced to express C/EBPβ (β) or with empty virus (pLX) and pulsed with 10uM BrdU for 24h in growth medium. **(E)** Percentage of $Ki67^+$ cells cultured as in (D), relative to total nuclei. **(F)** Representative histograms of propidium iodide fluorescence for cell cycle analysis for cells transduced as in (C). **(G)** *Cebpb* expression in primary myoblasts isolated from $Cebpb^{fl/fl}Pax7^{+/+}$ (wild-type, WT) and conditional null $Cebpb^{-/-}$

Pax7$^{CreER/+}$ ($\beta^{-/-}$) mice. **(H)** Percent $BrdU^{+}$ primary myoblasts relative to total nuclei. Myoblasts were pulsed for 6 hours with 10uM BrdU in GM. **(I)** Percent $Ki67^{+}$ primary myoblasts relative to total nuclei. **(J)** Representative histograms of propidium iodide fluorescence for cell cycle analysis for wild-type (left) and C/EBP$\beta^{-/-}$ (right) primary myoblasts. For panels C-E and G-I, data is the mean ± s.e.m, n=3, *$p<0.05$, ***$p<0.001$.

stimulated following induction of mitotic quiescence in myoblasts but not during cell cycle exit due to differentiation (Figure 17B).

To determine if C/EBPβ expression modifies myoblast cell cycle kinetics to promote quiescence, C2C12s retrovirally transduced with empty vector (pLXSN) or to express C/EBPβ were grown under high serum conditions (growth media) and pulsed with BrdU for 24 hours. In cells overexpressing C/EBPβ (Figure 17C), the number of $BrdU^{+}$ cells was significantly reduced by ~70% as compared to control myoblasts suggesting that ectopic C/EBPβ expression can reduce cell proliferation (Figure 17D). Further, only 43% of cells were cycling ($Ki67^{+}$) in C/EBPβ-overexpressing cultures whereas, 69% of cells were cycling in pLXSN controls (Figure 17E), suggesting that C/EBPβ promotes cell cycle exit. Flow cytometric cell cycle analysis revealed that C/EBPβ-overexpressing cells had a greater percentage of cells in the G0/G1 phase and fewer cells in the S and G2/M phases compared to control myoblasts (Fiure 17F). Taken together, these results indicate that C/EBPβ can induce cell cycle exit in C2C12 myoblasts under growth conditions.

To examine the effect of C/EBPβ-deficiency on myoblast proliferation, we isolated SCs from conditional knockout mice ($Cebpb^{fl/fl}Pax7^{CreER/+}$) in which C/EBPβ expression is abrogated in $Pax7^{+}$ cells following activation of the CreER recombinase with tamoxifen (Figure 17G). SC-derived myoblasts from $C/EBP\beta^{-/-}$ and wild-type control ($Cebpb^{fl/fl}Pax7^{+/+}$, WT) were cultured in high serum (GM) for 24 hours and cell proliferation was assessed by BrdU incorporation and Ki67 immunostaining. While BrdU incorporation was unchanged with loss of C/EBPβ in primary myoblasts, Ki67 staining revealed a larger fraction of cycling cells in $C/EBP\beta^{-/-}$ cultures (58%) as compared to control cultures (46%) (Figure 17H,I). Flow cytometric cell cycle analysis revealed that

C/EBPβ-deficient cells had fewer cells in the G0/G1 phase and more cells in the S and G2/M phase compared to control myoblasts (Figure 17J), consistent with a role for C/EBPβ in the establishment of quiescence.

The upregulation of Pax7 can inhibit BrdU incorporation in myoblasts and Pax7 expression is regulated by C/EBPβ (Olguin & Olwin 2004; Marchildon et al. 2012), suggesting that C/EBPβ may regulate the cell cycle at least in part through regulation of Pax7 levels. C/EBPβ can induce cell cycle arrest in other cell types such as keratinocytes, where overexpression of C/EBPβ inhibited the number of $BrdU^+$ cells and C/EBPβ-deficient keratinocytes were resistant to calcium-induced growth arrest (Zhu et al. 1999) We observed the same decrease in cells that could incorporate BrdU when full length C/EBPβ overexpressed in C2C12 myoblasts.

C/EBPβ regulates caveolin-1 expression.

To identify mediators of C/EBPβ-induced cell cycle arrest, we performed RT-qPCR and western blot analysis on extracts from C2C12s overexpressing C/EBPβ grown under high serum conditions for 24 hours. We analyzed expression of cell cycle regulators that have previously been implicated in satellite cell quiescence and activation. C/EBPβ-overexpression significantly inhibited *Ccnd1* gene expression and stimulated *Cav1* gene expression, without affecting *Cdkn1a* or *Cdkn1b* levels (Figure 18A). Consistent with changes in gene expression, Cyclin D1 protein was downregulated in cells overexpressing C/EBPβ while caveolin-1 and p27 expression was upregulated

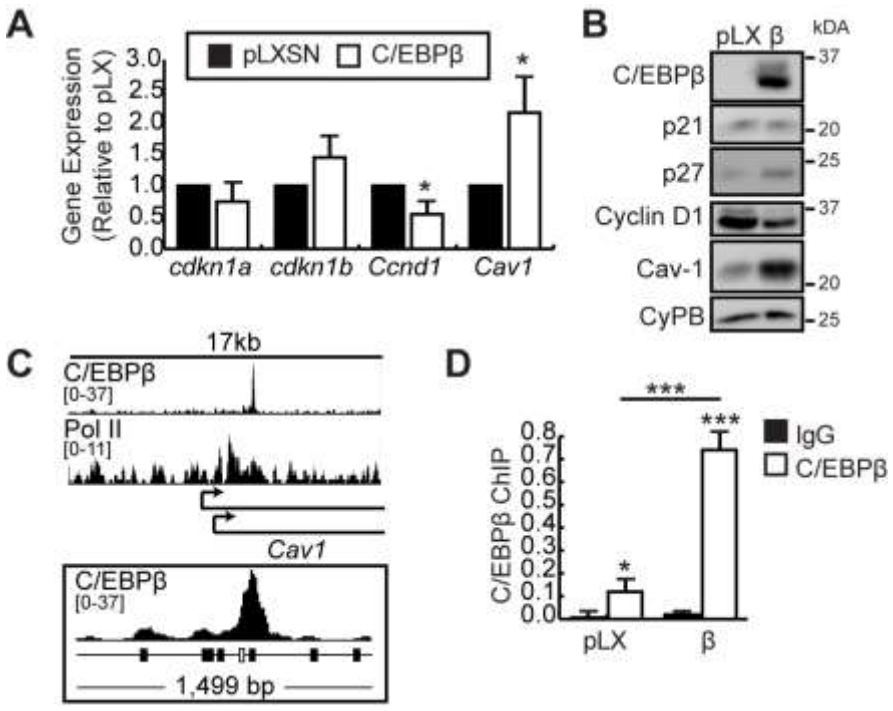

Figure 18. C/EBPβ regulates Caveolin-1 expression. (A) C2C12s retrovirally transduced to express C/EBPβ (β) or with empty virus (pLX) were cultured in growth medium for 24 hours and analyzed by RT-qPCR for *Cdkn1a, Cdkn1b, Ccnd1* and *Cav1* expression. **(B)** Representative western blot of C/EBPβ, p21, p27, Cyclin D1, and Caveolin-1 (Cav-1) expression in cells cultured as in (A). Cyclophilin B (CyPB) is a loading control. **(C)** *Cav1* regulatory region (Chr6:17,250,000-17,226,000) with C/EBPβ and RNA Pol II binding peaks indicated. Region Chr6:17,258,600-17,260,000 is expanded in the inset to show putative C/EBP binding motifs (black boxes) and the TBP motif (white box). **(D)** ChIP analysis of C/EBPβ occupancy on the *Cav1* promoter in C2C12s retrovirally transduced to express empty vector (pLX) or C/EBPβ (β) in GM. RT-qPCR data is shown as the percentage of enrichment in relation to the 10% input of each condition (n=4). For panels A and D, data is the mean ± s.e.m, n≥3, *p<0.05.

(Figure 18B). p27 is considered a marker of G_0 and is upregulated in muscle precursor cells that exit the cell cycle for self-renewal (Fortier et al. 2013). Further, the low levels of cyclin D1 expression are consistent with arrest in G0. Expression of p21, which normally occurs with cell cycle exit for differentiation, was not affected by C/EBPβ overexpression (Figure 18B).

Caveolin-1 is a structural protein component of caveolae. Expressed in quiescent satellite cells, caveolin-1 levels decrease upon muscle injury and differentiation (Volonte et al. 2005; Gnocchi et al. 2009). Caveolin-1 overexpression inhibits myogenesis *in vitro* and muscle regeneration *in vivo* (Volonte et al. 2005). The caveolin-1 null mouse has expansion of several stem cell populations including mammary stem cells, neural stem cells and intestinal crypt stem cells (Jasmin et al. 2009; Sotgia et al. 2005; Li et al. 2005) and overexpression of caveolin-1 in myogenic progenitor cells inhibits BrdU incorporation (Volonte et al. 2005). Caveolin-1 is, therefore, an inhibitor of stem cell proliferation *in vivo* and an interesting candidate for the induction of quiescence by C/EBPβ in myoblasts. We examined whether the regulation of Caveolin-1 expression by C/EBPβ was direct. *In silico* analysis of the *Cav1* regulatory region identified 12 C/EBPβ conserved binding sites. Using publicly available C/EBPβ ChIP-seq reads from C2C12 myoblasts (GSE36024), we identified a C/EBPβ binding peak 25bp upstream of a TATA-binding protein (TBP) motif, in close proximity to 6 of the predicted C/EBPβ binding sites (Figure 18C). Chromatin immunoprecipitation (ChIP) analysis performed in C/EBPβ-overexpressing C2C12s revealed that C/EBPβ can occupy the *Cav1* regulatory region (Figure 18D). C/EBPβ and caveolin-1 share a similar expression pattern in satellite cells, both inhibit myogenesis and promote myoblast migration (Marchildon et

al. 2015; Lala-Tabbert et al. 2016; Volonte et al. 2005). In addition to common roles in myogenesis, genetic ablation of both C/EBPβ and caveolin-1 results in lipodystrophy (Wang et al. 2000; Liu et al. 1999; Schrauwen et al. 2015), suggesting that regulation of caveolin-1 expression by C/EBPβ may extend beyond skeletal muscle. Thus, the activation of caveolin-1 expression by C/EBPβ likely contributes to the C/EBPβ-mediated cell cycle arrest.

C/EBPβ-mediated quiescence is dependent on Caveolin-1 expression.

Since the phenotype of caveolin-1-overexpressing myoblasts resembles that of C/EBPβ-overexpressing myoblasts, we considered the possibility that C/EBPβ inhibits proliferation of myoblasts through stimulation of caveolin-1 expression. Control (pLX) and C/EBPβ-overexpressing C2C12 cells were lentivirally transduced to express a scrambled shRNA construct (shCntrl) or a shRNA construct targeting caveolin-1 (shCav1). RT-qPCR and western blot analysis confirmed C/EBPβ overexpression and knockdown of caveolin-1 (Figure 19A-C). At the protein level we did not see significant knockdown of caveolin-1 in pLX/shCav1 cells, most likely due to the already low levels of caveolin-1 observed in these cells (Figure 19C). However, C/EBPβ was unable to stimulate caveolin-1 expression in C/EBPβ/shCav1 cells indicating that the shRNA construct is efficient in targeting caveolin-1 (Figure 19C). C/EBPβ-overexpressing cells have lower levels of Cyclin D1 regardless of caveolin-1 protein levels (Figure 19C). To determine if C/EBPβ inhibits myoblast proliferation through caveolin-1, C2C12s were cultured in GM for 24 hours and pulsed with BrdU. After a 24 hour BrdU pulse, cells were stained and $BrdU^+$ cells were counted. While overexpression of C/EBPβ resulted in a decrease in the percentage of $BrdU^+$ cells in C/EBPβ/shCntrl cells as compared to

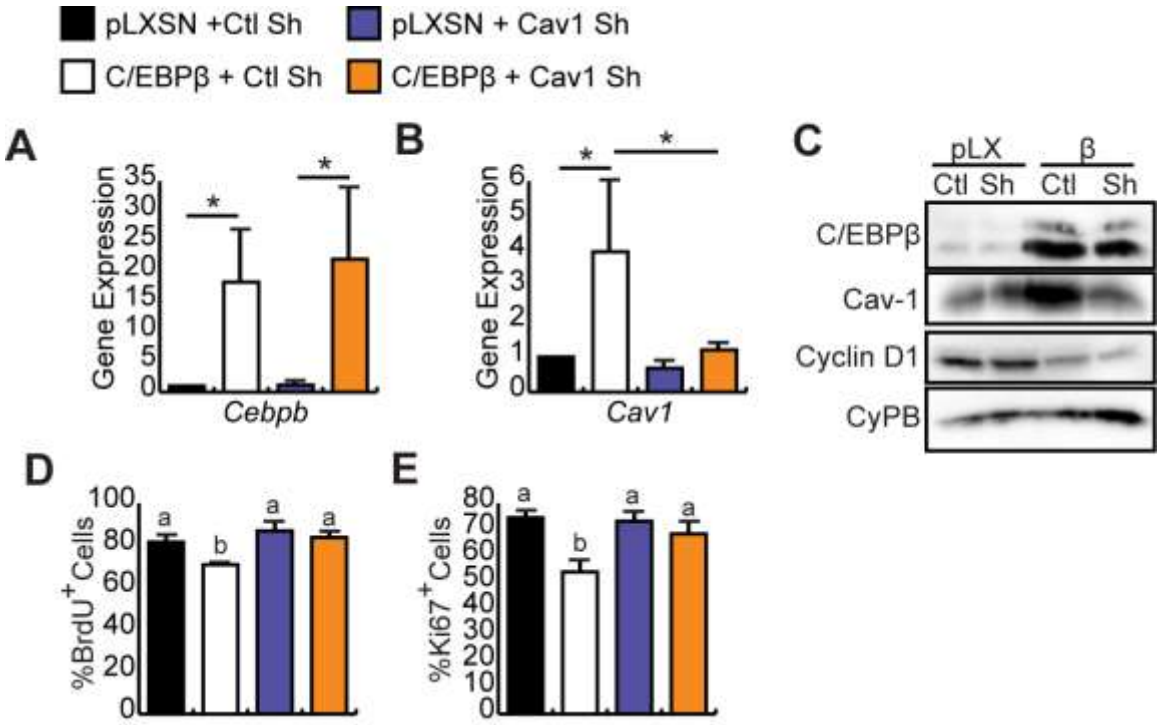

Figure 19. C/EBPβ-mediated quiescence is dependent on Caveolin-1 expression. C2C12 myoblasts expressing C/EBPβ (β) and controls (pLX) were transduced with lentiviral particles to express a control shRNA or a shRNA directed against caveolin-1 to create pooled stable cell lines. **(A)** RT-qPCR analysis of *Cebpb* and **(B)** *Cav1* expression in cells cultured in GM. **(C)** Representative western blot of C/EBPβ, Caveolin-1 (Cav-1) and Cyclin D1 expression in cells cultured as in (A). Cyclophilin B (CyPB) is a loading control. **(D)** Cells were cultured as in (A) and pulsed with BrdU for 24 hours. $BrdU^+$ cells were counted and represented as the percentage of total nuclei. **(E)** Cells cultured as in (A) were immunostained for Ki67 and shown as the percent $Ki67^+$ cells relative to total nuclei. For panels D-E, data is the mean ± s.e.m, n=3, *$p<0.05$, means with different letters are significantly different.

pLX/shCntrl cells, this was restored when caveolin-1 was lost in C/EBPβ/shCav1 cells (Figure 19D). Further, the number of proliferating ($Ki67^{+}$) cells was increased in C/EBPβ/shCav1 cells (68%) compared to C/EBPβ/shCntrl cells (Figure 19E). These results suggest that C/EBPβ induces quiescence in myoblasts through upregulation of caveolin-1.

The mechanism by which caveolin-1 induces quiescence in myoblasts is unknown. Since cyclin D1 expression was not rescued with loss of caveolin-1 in C/EBPβ-overexpressing cells, caveolin-1 likely induces quiescence through a different pathway. Caveolins can regulate cell signalling by cell surface receptor endocytosis, receptor sequestration in calveolae and interaction with downstream signalling molecules (Baker & Tuan 2013). Caveolin-1 can inhibit extracellular signal-regulated kinase (ERK) activation by HGF (Volonte et al. 2005), and inhibition of ERK1/2 activity is known to inhibit myoblast proliferation (Jones et al. 2001). Interestingly, ERK1/2 activity can also result in C/EBPβ protein phosphorylation, which stimulates its transcriptional activity (Nakajima et al. 1993; Hu et al. 2001), resulting in a presumptive increase in caveolin-1 expression which would negatively feedback on this signalling loop. As such, caveolin-1 could induce quiescence in C/EBPβ-overexpressing cells by inhibiting ERK activation.

C/EBPβ is required to maintain quiescence in vivo

To confirm the regulation of quiescence *in vivo*, the SC population was assessed in $C/EBP\beta^{fl/fl}Pax7^{+/+}$ (wild-type) and conditional null $C/EBP\beta^{-/-}Pax7^{CreER-/+}$ ($C/EBP\beta^{-/-}$) muscle following excision of C/EBPβ *in utero* by a single tamoxifen gavage of pregnant dams at E15.5. Mice were sacrificed at postnatal day 21 (p21) or 56 (p56) and the number of $Pax7^{+}$ cells was scored. At p21, the number of $Pax7^{+}$ cells in $C/EBP\beta^{-/-}$ muscle and

control muscle were comparable, suggesting that C/EBPβ is not required for postnatal growth (Figure 20A,B). However at p56, the number of $Pax7^{+}$ cells was significantly reduced in C/EBP$\beta^{-/-}$ muscle as compared to WT littermates (Figure 20A,B). To examine the cell cycle state of these $Pax7^{+}$ cells, we performed dual immunohistochemistry (IHC) of WT and C/EBP$\beta^{-/-}$ tibialis anterior (TA) muscle sections for Pax7 and Ki67. We expected the majority of $Pax7^{+}$ satellite cells to be quiescent ($Ki67^{-}$). Indeed, in WT and C/EBP$\beta^{-/-}$ muscle at p21, the majority of Pax7+ cells were quiescent ($Ki67^{-}$) and there were no differences between WT and C/EBP$\beta^{-/-}$ muscle (Fig. 20C). However at p56, approximately 26% of the $Pax7^{+}$ cells were also $Ki67^{+}$ in WT muscle while in C/EBP$\beta^{-/-}$ TA muscle sections, there were significantly more cycling ($Ki67^{+}$) $Pax7^{+}$ satellite cells, increasing this population to approximately 53% (Fig. 20C). Since no significant changes were noted with loss of C/EBPβ at postnatal day 21, remaining analysis was performed at p56.

Muscle sections at p56 were co-immunostained for Pax7 and MyoD, and while only 17% of $Pax7^{+}$ cells were also $MyoD^{+}$ in WT muscle, 43% of $Pax7^{+}$ cells also expressed MyoD in C/EBP$\beta^{-/-}$ muscle, suggesting that the SCs that are present in C/EBP$\beta^{-/-}$ muscle are more activated than in controls (Figure 20D). Resting muscle sections were also stained for myogenin (MyoG) to determine their progression towards terminal differentiation. There was a significant increase in $MyoG^{+}$ cells in C/EBP$\beta^{-/-}$ muscle (1.7%) compared to WT muscle (0.8%) in resting muscle (Figure 20E). Taken together these results suggest that C/EBPβ is required to maintain the quiescence of SCs in the niche and that loss of C/EBPβ results in spontaneous activation and precocious differentiation.

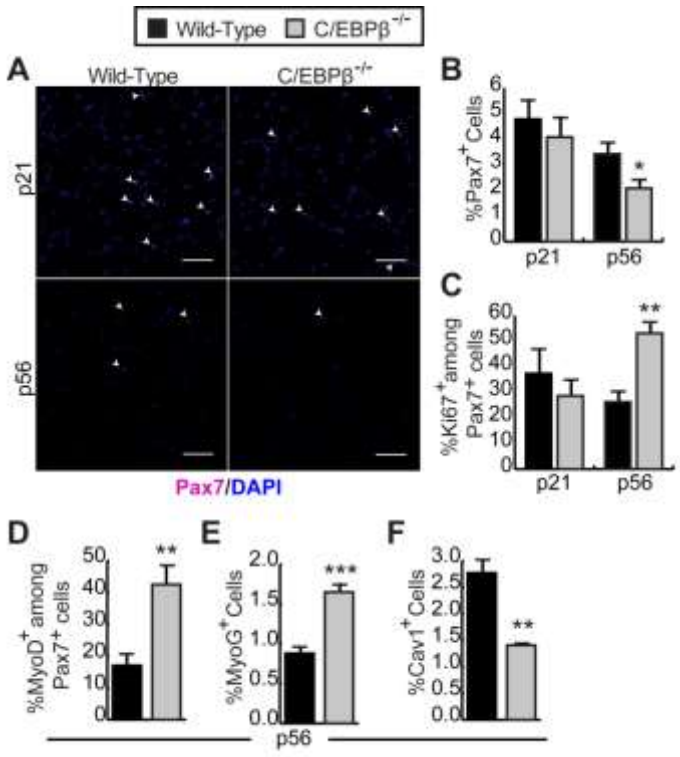

Figure 20. C/EBPβ is required to maintain satellite cell quiescence in adult muscle. TA muscles from 21- (p21) and 56-day-old mice (p56) were collected following a single tamoxifen gavage of pregnant dams at E15.5. **(A)** Representative pictures of $Pax7^+$ cells in TA muscle sections using DAPI as a nuclear counterstain. Scale bar = 50 μm. **(B)** Percentage of $Pax7^+$ cells relative to total nuclei at p21 and p56. **(C)** Percentage of $Ki67^+$/Pax7+ cells relative to total $Pax7^+$ population at p21 and p56. **(D)** Percentage of $MyoD^+$/$Pax7^+$ cells relative to total $Pax7^+$ cells at p56. **(E)** Percentage of myogenin$^+$ ($MyoG^+$) cells relative to total nuclei in TA cross-sections at p56. **(F)** Percentage of caveolin-1$^+$ ($Cav1^+$) cells relative to total nuclei in TA cross-sections at p56. For panels A-E, data is the mean ± s.e.m, n≥3 mouse pairs, *p<0.05, **p<0.01, ***p<0.001.

Given that caveolin-1 is a target of C/EBPβ (Figure 20E) and that caveolin-1 is required for C/EBPβ-induced quiescence, TA muscle sections were immunostained for caveolin-1 (Cav1) in WT and C/EBPβ$^{-/-}$ muscle. Consistent with our *in vitro* data, a significant reduction in the number of Cav1^{+} cells in C/EBPβ$^{-/-}$ muscle was observed as compared to wild-type (Figure 20F), confirming the regulation of caveolin-1 expression by C/EBPβ *in vivo*.

Caveolin-1 expression has been implicated in the maintenance of quiescence in hematopoietic stem cells and in its absence, expansion of the stem cell pool occurs (Bai et al. 2014). Herein, we describe a similar function for C/EBPβ in muscle satellite cells and place C/EBPβ as a transcriptional regulator of *Cav1* expression. Given the importance of caveolin-1 in the regulation of proliferation in a number of adult stem cell populations (Razani et al. 2001; Head et al. 2010; Volonte et al. 2005; Bai et al. 2014) and the near ubiquitous expression of C/EBPβ, the mechanism described herein may be more generally conserved.

Quiescence is considered an efficient approach to long term maintenance of adult stem cell populations, and the molecular mechanisms governing the induction of quiescence likely also regulate the activation of stem cell populations and their self-renewal. In skeletal muscle, aging triggers satellite cell dysfunction characterized by a switch from mitotic quiescence to a pre-senescent state, resulting in blunted regenerative responses and the development of sarcopenia (Sousa-Victor et al. 2014). Interestingly, while C/EBPβ expression is required for the maintenance of the quiescent state, increased *Cebpb* expression is observed in sarcopenia (Giresi et al. 2005). Given that C/EBPβ is required for the development of oncogene-induced senescence of H-ras^{V12} transformed

fibroblasts (Salotti et al. 2015), misexpression of C/EBPβ with aging may contribute to the transition to senescence in sarcopenia.

Material and methods

Mice and Animal Care. All animal work was performed in accordance with the guidelines set out by the Canadian Council on Animal Care and was approved by the University of Ottawa Animal Care Committee. C/EBPβ$^{fl/fl}$ and conditional null C/EBPβ$^{-/-}$ Pax7$^{CreER-/+}$ (cKO) mice were previously described (Marchildon et al. 2012). Activation of CreER^{tm} *in utero* was achieved by a single gavage of 2.5 mg of tamoxifen (dissolved in corn oil) of pregnant dams when pregnancy was at E15.5. All animals were housed in a controlled facility (22°C with 30% relative humidity on a 12 hours light/dark cycle) and provided with food and water ad libitum.

Primary and C2C12 Myoblast Culture. Primary myoblasts were isolated as described previously (Marchildon et al. 2012). Briefly, hind limb muscles of adult (6 to 8 weeks of age) mice were dissected and digested with collagenase/dispase (Roche), and enriched by selective plating. Primary myoblasts were cultured in growth media (Dulbecco's Modified Eagle Medium containing 20% fetal bovine serum (FBS), 10% horse serum (HS)) supplemented with 10ng/ml basic fibroblast growth factor (bFGF) and 2ng/ml human growth factor (HGF) (Peprotech). To induce CreER^{tm} activity in culture, primary myoblasts were treated with 4-OH tamoxifen (2uM dissolved in 100% ethanol; Sigma-Aldrich) for 48 hours. When required, 10uM BrdU (Sigma-Aldrich) was added 6 hours before fixation.

C2C12 murine myoblasts were retrovirally transduced with empty vector (pLXSN) or to express C/EBPβ (pLXSN-C/EBPβ) and selected based on G418 resistance and maintained in growth medium (DMEM with 10% FBS). Finally, pLXSN and pLXSN-C/EBPβ expressing C2C12s were transduced with lentiviral particles targeting caveolin-1 (shCav1) or non-targeting control (shCntrl) (Santa Cruz Biotechnology) to create pLXSN/shCntrl, pLXSN/shCav1, pLXSN-C/EBPβ/shCntrl and pLXSN-C/EBPβ/shCav1 pooled stable lines.

Induction of mitotic quiescence was achieved by a 48 hour culture in methionine-depleted DMEM supplemented with 1% FBS for 48 hours as previously described (Zhang et al. 2010; Kitzmann et al. 1998). Differentiation was induced by culturing confluent cells in DMEM supplemented with 2% HS for 24 hours. When required, 10uM BrdU (Sigma-Aldrich) was added 24 hours before fixation.

Flow Cytometry. Primary myoblast and C2C12 myoblast cultures were fixed with cold 70% ethanol overnight. Cells were then washed and stained with propidium iodide (50ug/ml; Molecular Probes) and analyzed in a CyAN^{TM} ADP Analyzer with data analysis using Kaluza® Flow Analysis Software (Beckman Coulter).

Western Analysis. Whole cell extracts from C2C12s and primary myoblasts were resolved on a 12% SDS-PAGE gel, transferred to a PVDF membrane and probed with specific antibodies: C/EBPβ (E299; Abcam), Caveolin-1 (N-20; Santa Cruz), p21 (C-19; Santa Cruz), p27 (Y236; Abcam) , Cyclin D1 (DCS-6; Santa Cruz) and Cyclophilin B

(Abcam). Chemiluminescence was detected with the ChemiDoc™ MP System (Bio-Rad Laboratories).

Real-time quantitative PCR. Total RNA was isolated from C2C12s and primary myoblasts using the RNeasy kit (QIAGEN), treated with DNase (Ambion) and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories) following manufacturer's protocols. Real-time quantitative PCR reactions were performed using iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories) on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories). The ΔΔCt method was used to determine relative fold induction after normalization with 18S rRNA.

Chromatin Immunoprecipitation. C2C12 myoblasts were crosslinked for 30 min at room temperature with 1% formaldehyde and sonicated with a Diagenode bioruptor®. Chromatin immunoprecipitation (ChIP) analysis was performed as described (Wiper-Bergeron et al. 2003) using antibodies against C/EBPβ (C-19; Santa Cruz) or rabbit IgG as negative control. Protein G conjugated Dynabeads (Invitrogen) were used to pull down immunoconjugates and DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen). A standard curve was generated using input DNA for each immunoprecipitate and quantification was determined as the abundance of target DNA to the input DNA and presented as fold difference to negative control (IgG). Primer sequences for qPCR amplification were: Forward: ATGGAGCCTCCCTGTTGTTG and Reverse: ATGTGTGGGGACCCAAATCC.

Immunofluorescence. Myoblasts were fixed in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and blocked with 0.3% Triton X-100 and 10% goat serum. Cryosections were fixed in 4% PFA and processed for antigen retrieval in citrate buffer at 95°C for 20 minutes. Sections were permeabilized with 0.5% Triton X-100 PBS and blocked in 0.1% Triton X-100, 5% donkey serum (Cederlane) prior to incubation with primary antibody overnight at 4°C. Cells and sections were then washed with PBS and incubated with biotin anti-mouse (when indicated) or secondary antibodies conjugated to a fluorescent dye (Cy3, Alexa 488 or Alexa Flour 647; all from Jackson ImmunoResearch). Nuclei were counterstained with DAPI (0.5ug/ml). Primary antibodies used were: Pax7-c (DSHB), MyoD (C-20; Santa Cruz), myogenin (M-225; Santa Cruz), BrdU (Biotin; Abcam), Ki67 (Abcam), myogenin (F5d, DSHB), caveolin-1 (N-20; Santa Cruz) and Laminin (AL-4; Millipore).

Microscope Image Acquisition. Digital images were taken at room temperature with a Leica DM 3000B microscope and Infinity-3 camera (Lumenera). The Infinity Capture imaging software (Lumenera) was used to take images which were later composed and edited in paint.net.

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Children's Cancer Therapy Development Institute (Beaverton, OR) and the Cebpb$^{fl/fl}$ mouse was a kind gift from Dr. Esta Sterneck at the Center for Cancer Research at the National Institutes of Health.

Abbreviations: SC, satellite cell, C/EBPβ, CCAAT/Enhancer Binding Protein beta; Pax7, paired box protein 7; MyoD, myogenic differentiation factor 1; MyoG, myogenin; GM, growth media; WT, wild-type; BrdU, bromodeoxyuridine; Cav1, caveolin-1; IHC, immunohistochemistry; TA, tibialis anterior; mRNA, messenger ribonucleic acid; RT-qPCR, real time quantitative polymerase chain reaction; PI, propidium iodine; FBS, fetal bovine serum; HS, horse serum; bFGF, basic fibroblast growth factor; HGF, human growth factor; SEM, standard error of the mean; ChIP, chromatin immunoprecipitation

CHAPTER FIVE: INDUCTION OF C/EBPβ EXPRESSION WITH THE PHOSPHODIESTERASE INHIBITOR ISOBUTYLMETHYLXANTHINE IMPROVES MYOBLAST ENGRAFTMENT INTO DYSTROPHIC MUSCLE

Induction of C/EBPβ Expression with the Phosphodiesterase Inhibitor Isobutylmethylxanthine Improves Myoblast Engraftment into Dystrophic Muscle

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Keywords: CCAAT-Enhancer-Binding Protein-beta, phosphodiesterase inhibitors, stem cell transplantation, muscular dystrophies, Pax7 transcription factor, skeletal muscle satellite cells

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Running Head: C/EBPβ improves myoblast transplantation outcomes.

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Author contributions:

Neena Lala-Tabbert: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript

Dechen Fu: Collection and assembly of data, final approval of manuscript

Nadine Wiper-Bergeron: Conception and design, financial support, manuscript writing, final approval of manuscript

Abstract:

Duchenne muscular dystrophy (DMD), caused by mutations in the dystrophin gene, is the most common muscular dystrophy. Characterized by rounds of muscle degeneration and regeneration, DMD features progressive muscle wasting and is fatal. One approach for treatment is transplantation of muscle progenitor cells to repair and restore dystrophin expression to damaged muscle. However, the success of this approach has been limited by difficulties in isolating large numbers of myogenic progenitors with strong regenerative potential, poor engraftment, poor survival of donor cells and limited migration in the diseased muscle. We demonstrate that induction of the transcription factor CCAAT/Enhancer Binding Protein beta (C/EBPβ) using the cAMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) results in enhanced myoblast expansion in culture and increased satellite cell marker expression. When equal numbers of IBMX-treated cells were transplanted into dystrophic muscle, they contributed to muscle repair more efficiently than vehicle-treated cells and engrafted into the satellite cell niche in higher numbers, demonstrating improved cell migration from the site of injury and enhanced survival after transplantation. Thus, pharmacological stimulation of C/EBPβ expression reprograms myoblasts to a more stem cell-like state, promotes expansion in culture and improves engraftment such that better transplantation outcomes are achieved.

Introduction

Post-natal growth and repair of skeletal muscle depends on muscle precursor cells, termed satellite cells (Mauro 1961; McCullagh & Perlingeiro 2014). These normally quiescent cells activate upon injury, re-enter the cell cycle, upregulate the myogenic factor MyoD, differentiate and fuse to give rise to new myofibers or to repair damaged ones (Yin et al. 2013). Satellite cells also have the ability to self-renew to re-populate the satellite cell niche (Motohashi & Asakura 2014). Pax7 is the canonical marker of satellite cells; however, satellite cells appear to exist as a heterogeneous population expressing different cell surface markers including the cell adhesion protein M-cadherin, the chemokine receptor CXCR4, and the heparan sulfate proteoglycans, syndecan-3 and -4 (Seale et al. 2004; Wernig et al. 2004; Cornelison et al. 2001; Ratajczak et al. 2003; Motohashi & Asakura 2014). A small subpopulation of satellite cells, the satellite side population, are characterized by the expression of ABCG2 and Sca-1 and their ability to exclude Hoechst dye 33342 (Tanaka et al. 2009).

Duchenne muscular dystrophy (DMD) is the most common muscular dystrophy affecting 1 in 3,500 male births and is caused by spontaneous or inherited mutations in the dystrophin gene (Ray PN, Belfall B, Duff C, Logan C, Vanora K, Thompson MW, Sylvester JL, Gorski JL 1985; Rahimov & Kunkel 2013). The absence of dystrophin leads to membrane fragility and degeneration of the muscle fiber (Allikian & McNally 2007). To date, no cure for DMD exists, underscoring the need to identify the mechanisms controlling myogenic potential, migration in the host musculature, and repopulation of the satellite cell niche. One promising approach for treatment of DMD is

transplantation of satellite cells or muscle progenitor cells expressing dystrophin into damaged muscle.

The use of satellite cell-derived myoblasts as a therapeutic approach for DMD has been limited by failure of the transplanted cells to migrate from the site of injection, low donor cell survival rates, insufficient cell fusion and poor repopulation of the stem cell niche, necessary for sustained repair and long-term cure (Skuk et al. 2004). Adding to the challenge of cell-based therapies, large numbers of satellite cells are difficult to isolate from muscle biopsies, necessitating their expansion in culture which in turn reduces their regenerative potential (Montarras, Morgan, Collins, Frédéric Relaix, et al. 2005). Thus, to be a viable treatment for DMD, a suitable progenitor cell must be isolated and expanded without loss of stem cell properties, permitting efficient repair, homing to the satellite cell niche and self-renewal for sustained therapeutic benefit.

The transcription factor, CCAAT/Enhancer Binding Protein beta (C/EBPβ), is an important regulator of mesenchymal stem cell fate and has been shown to be upregulated in muscle wasting such as sarcopenia (Giresi et al. 2005). Our work has shown that in healthy muscle, C/EBPβ expression is localized to $Pax7^{+}$ satellite cells and its expression is downregulated during myogenesis in parallel with Pax7 (D. Fu et al. 2015; Marchildon et al. 2012). Forced expression of C/EBPβ in myoblasts stimulates Pax7 expression, reduces MyoD protein expression, and inhibits myogenesis. As such, through stimulation of Pax7 and inhibition of MyoD, C/EBPβ acts to maintain satellite cells in an undifferentiated state (Marchildon et al. 2012).

Phosphodiesterases are a class of enzymes that are responsible for controlling the cellular concentration of cyclic adenosine monophosphate (cAMP) and cyclic guanosine

monophosphate (cGMP) by cleaving their phosphodiester bond to yield 5'-cyclic nucleotides (5' AMP or 5'-GMP, respectively) (Azevedo et al. 2014; Maurice et al. 2014). Inhibitors of phosphodiesterases (PDEi) are a class of drugs that are widely used for various pharmacological properties including anti-inflammatory, vasodilator and antithrombotic functions (Rahimi et al. 2010; Nieder et al. 2005; Rendell & Bamisedun 1992). Pre-clinical studies have demonstrated that PDE5A inhibitors (sildenafil and tadalafil), which prevent the breakdown of cGMP, protect skeletal muscle of mdx mice from contraction-induced injury (Asai et al. 2007) and restore blood supply to their muscles after exercise (Kobayashi et al. 2008). Currently, PDE5A inhibitors are in clinical trials.

IBMX (isobutylmethylxanthine) is a methyl-xanthine non-selective PDEi that increases cellular cAMP and cGMP levels. Increased intracellular cAMP levels, leads to activation of protein kinase A (PKA) and the phosphorylation of cAMP response element-binding protein (CREB) (Goethe et al. 2007; M Niehof et al. 1997; J. W. Zhang et al. 2004; Lechner et al. 2013). Phosphorylated CREB stimulates C/EBPβ expression by binding to the cAMP response elements (CRE) in the *Cebpb* promoter (M Niehof et al. 1997). IBMX has been extensively used to transiently stimulate C/EBPβ expression in preadipocytes in culture to induce adipogenesis (Cao et al. 1991; M Niehof et al. 1997; J. W. Zhang et al. 2004; Lechner et al. 2013). In this study, we cultured satellite cell-derived myoblasts in IBMX with the goal of transiently stimulating C/EBPβ expression to stimulate Pax7 expression, reduce MyoD expression and improve the suitability of these cells prior to transplantation into dystrophic muscle. We demonstrate that treatment of cultured myoblasts with IBMX for 5 days increases their proliferation in culture and

the expression of satellite cell markers associated with efficient engraftment, improves cell survival upon transplantation and enhances migration of myoblasts in a C/EBPβ-dependent manner. Treatment of myoblasts with low doses of IBMX was sufficient to significantly improve their ability to repair dystrophic muscle and to engraft into the satellite cell niche. IBMX treatment should be considered as a simple yet effective method to reprogram myoblasts to a more stem cell-like state that correlates with better transplantation outcomes for muscle degenerative disorders.

Materials and Methods

Mice and Animal Care. C57BL/6-Tg(UBC-GFP)30Scha/J mice (GFP mice) aged 6 weeks, C57BL/6J aged 6 weeks and C57BL/10ScSn-mdx/J mice (mdx mice) aged 4 weeks were obtained from The Jackson Laboratory. A mouse bearing a C/EBPβ-floxed allele was created previously (Sterneck et al. 2006) and homozygous progeny (C/EBPβ$^{fl/fl}$) were crossed with mice bearing the Pax7-CreER^{tm} allele (Nishijo et al. 2009) to generate wild-type (WT; C/EBPβ$^{fl/fl}$Pax7$^{CreER-/-}$) and conditional knockout (cKO; C/EBPβ$^{-/-}$Pax7$^{CreER-/+}$) animals as previously described (Marchildon et al. 2012). To induce CreER^{tm} activity, WT and cKO animals were subjected to daily intraperitoneal injections of tamoxifen (3mg/40g; Sigma) for 5 days. All animals were housed in a controlled facility (22°C with 30% relative humidity on a 12 hours light/dark cycle) and provided with food and water ad libitum. Animals were bred and handled as recommended by the guidelines established by the University of Ottawa Animal Care Service and the Canadian Council on Animal Care.

Preparation and Culture of Primary Myoblasts and C2C12 Myoblasts. Satellite cell-derived myoblasts were isolated as described (Megeney et al. 1996; Marchildon et al. 2012). Briefly, hind limb muscles of adult (aged 6-8 weeks) C57BL/6, GFP, WT or cKO mice were dissected and digested with collagenase/dispase (Roche Diagnostics) and the muscle slurry was filtered through a 70um cell strainer to remove undigested muscle. Cells were washed with serum-free media (SFM) and enriched for myoblasts by selective plating. Primary myoblasts were grown on matrigel-coated plates in growth media (GM; DMEM containing 20% FBS, 10% HS) supplemented with basic fibroblast growth factor (bFGF; 10ng/ml) and hepatocyte growth factor (HGF; 2ng/ml) (Peprotech). For in vitro and transplantation studies, 3-isobutyl-1-methylxanthine (IBMX; Sigma) was used at a concentration of 30uM or 300uM and potassium hydroxide (0.35N) was used as the vehicle control. When required, 10uM BrdU (Sigma) was added 6 hours before fixation. For differentiation, primary myoblasts were induced to differentiate (DM; DMEM containing 2% FBS, 10% HS) in the presence or absence of IBMX for 2 days.

Cell Growth Curve. Myoblasts cultured under growth conditions for up to 5 days in the absence or presence of IBMX were subjected to crystal violet staining (0.03%) at days 0, 1, 3, and 5. The following day, the dye was eluted with 1% SDS and read in a 96-well plate by a SpectraMax® Plus 384 microplate reader at 570nm. Cell number was expressed as the optical density at 570nm.

Scratch Wound Assay. In vitro migration assays were performed by disruption of confluent myoblast monolayers as previously described (Goetsch & Niesler 2011).

IBMX-treated myoblasts were trypsinized and re-plated at equal densities. The next day, confluent myoblasts were treated with mitomycin C (Sigma; 50ug/ml) for 2 hours prior to wounding the monolayer with a sterile pipette tip. Cells were washed twice with PBS and switched to DM in the absence or presence of AMD3100 (Sigma; 10uM). Pictures were taken at 0h and 6h. The percentage of wound closure was calculated as follows: (width of 0h wound gap – width of 6h wound gap)/width of 0h wound gap x 100%.

Western Analysis. Whole cell extracts were resolved on a 12% SDS-PAGE gel and after transfer to PVDF membrane, probed with specific antibodies: C/EBPβ (C-19; Santa Cruz), C/EBPβ (1H7; Thermo Scientific), C/EBPβ (E299; Abcam), Pax7-c (DSHB), MyoD (5.8A; Santa Cruz), MHC (MF-20; DSHB) and Cyclophilin B (Abcam). Chemiluminescence was detected with the Luminescent Image Analyzer LAS-4000 (Fujifilm Life Science).

Real-time quantitative PCR. Total RNA was isolated from cultured myoblast using the RNeasy kit (QIAGEN), treated with DNase (Ambion) and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad Laboratories) following manufacturer's protocols. Real-time quantitative PCR reactions were performed using iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories) on an Mx3005p thermocycler (Stratagene). Primer sequences can be found in supplemental Table S1.

Myoblast Transplantation. Muscle regeneration in mdx mice aged 5 weeks was induced by injecting 30ul of 10uM cardiotoxin (CTX; Sigma) into the mid-belly of both tibialis

anterior (TA) muscles. The next day, 10^5 vehicle- or IBMX-treated GFP myoblasts were injected into the right and left TA of the CTX-injured TA muscle, respectively. Mice were sacrificed 2 months after transplantation and the TAs were collected, embedded in OCT compound, flash frozen in isopentane cooled by liquid nitrogen and sectioned (8um thick) for GFP fluorescence and immunohistochemistry. To assess survival of transplanted cells, IBMX-treated WT and cKO myoblasts were stained with CFSE (Sigma) and injected into the TA of CTX-injured mdx mice.

Immunostaining and Antibodies. PFA-fixed myoblasts were permeabilized in PBS containing 0.3% Triton X-100 and 10% goat serum (Cedarlane). Cryosections were thawed at room temperature, fixed in 4% PFA, and permeabilized with PBS containing 0.5% Triton X-100 and blocked in PBS containing 0.1% Triton X-100, 5% donkey serum (Cedarlane). For Pax7 staining, sections were processed for antigen retrieval at 95°C for 20 min in citrate buffer prior to permeabilization. Blocking was followed by incubation in primary antibody solution overnight at 4°C. Primary antibodies used in this study were as follows: MF-20 (DSHB), Pax7-c (DSHB), MyoD (C-20; Santa Cruz), GFP (Abcam), BrdU (Biotin; Abcam), Ki67 (Abcam), and Dystrophin (Abcam). Detection was accomplished with secondary antibodies conjugated to a fluorescent dye (Cy3, Alexa 488 or Alexa Flour 647; all from Jackson ImmunoResearch). In vivo apoptosis was measured using the In Situ Cell Death Detection Kit, TMR red (Roche) as per manufacturer's protocol. The staining was completed by counterstaining the nuclei with DAPI (0.5ug/ml).

Flow Cytometry. Satellite-cell derived myoblasts pre-treated with vehicle or 30uM IBMX were detached with Cell Dissociation Buffer (Invitrogen). Cells were stained with human M-Cadherin (R&D) on ice for 30 minutes, followed by incubation for 30 minutes with Sheep IgG APC-conjugated secondary antibody, FITC rat anti-mouse CD184 (CXCR4; BD) and PE mouse anti-human CD338 (ABCG2; BD). Cells were analyzed in a CyAN^{TM} ADP Analyzer and data was analyzed using Kaluza® Flow Analysis Software (Beckman Coulter).

Statistical Analysis. Statistical analysis was performed using the GraphPad Prism 5.0 Software. A two-tailed or one-tailed student's t-test was performed when comparing a single experimental condition to the control condition. One-way ANOVA followed by Tukey's post-hoc test was used when comparing 3 or more experimental variables. All experiments are representative of a minimum of three biological replicates and are presented as the mean ± standard error mean (SEM). Significance is indicated as n.s., not significant, *$p<0.05$, **$p<0.01$, or ***$p<0.001$.

Results

IBMX upregulates C/EBPβ expression and reversibly inhibits myogenesis. C/EBPβ maintains myoblasts in an undifferentiated state, and thus genetic overexpression of this factor is of no use for cell therapy purposes (Marchildon et al. 2012). Given that C/EBPβ expression can be induced by increased cAMP signaling via CREB (M Niehof et al. 1997), the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) was used to induce C/EBPβ expression in primary mouse myoblasts. Primary myoblasts isolated from C57BL/6 mouse hindlimb were treated with increasing doses of IBMX (from 30 to 300

uM) for 24 hours in growth medium. As seen in preadipocyte cultures, treatment of myoblasts with as low as 30 uM IBMX induced *Cebpb* mRNA expression (Figure 21A). Low doses of IBMX (30-300 uM) did not affect cell numbers after a 2 day treatment, suggesting that these doses were not toxic to the myoblasts (Figure 21B). However, 1000 uM IBMX significantly reduced cell numbers, suggesting toxicity at this dose. Consistent with previous reports, higher *Cebpb* expression correlated with a decrease in the efficiency of differentiation that was dose dependent, with significant reductions in the differentiation index observed for all doses tested (Figure 21C).

C/EBPβ protein expression was verified following a 30 uM IBMX treatment in primary myoblasts as a time course from 1 to 5 days in growth medium (Figure 21D,E). C/EBPβ expression was stimulated at all time points tested, with higher variability in the response noted after 2 and 3 days of treatment, suggesting that longer treatment periods were advantageous to fully stimulate C/EBPβ expression in myoblasts. Indeed, treatment of differentiating myoblasts with IBMX at 30 uM not only inhibited myogenesis, but stimulated Pax7 expression, a C/EBPβ target gene, as well as inhibited myosin heavy chain (MyHC) expression, without impacting MyoD expression (Figure 21F and Figure S6A). Given that high C/EBPβ levels correlate with reductions in MyoD protein expression (Marchildon et al. 2012; D. Fu et al. 2015), the stable MyoD expression in the presence of IBMX was surprising and suggested that IBMX can act to maintain MyoD levels independent of its effects on C/EBPβ expression. Indeed, treatment with forskolin (an activator of adenylyl cyclase) has been shown to upregulate MyoD expression in isolated primary myoblasts (Xu et al. 2013).

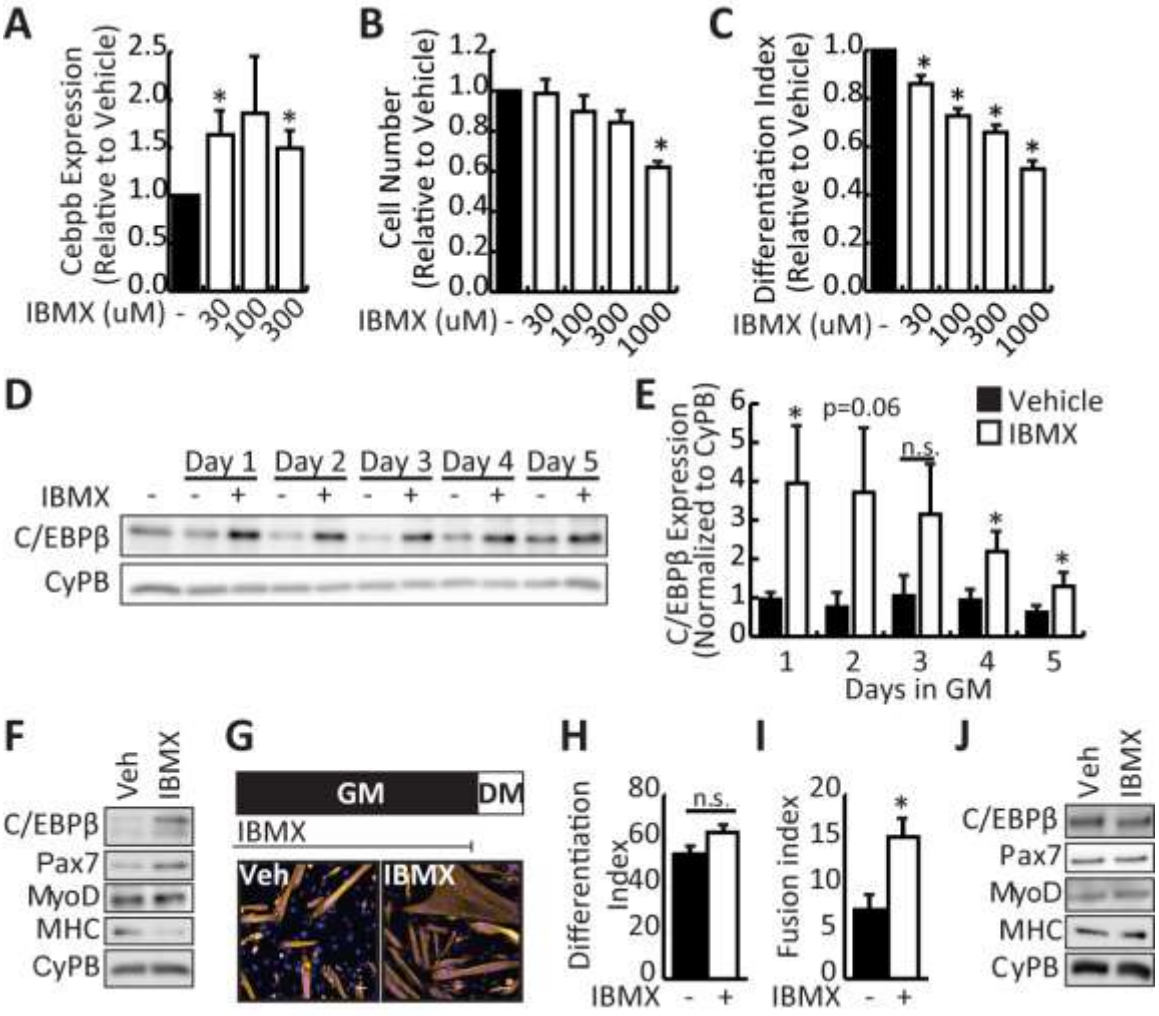

Figure 21. IBMX stimulates expression of *C/EBPβ* in myoblasts. (*A*) *Cebpb* expression in primary myoblast cultured in 0, 30, or 300 uM IBMX for 24 hours in growth medium (GM). (*B*) Cell numbers after culture of primary myoblasts in IBMX under growth conditions for 2 days. *(C)* Differentiation index (#nuclei in $MyHC^{+}$ cells/total nuclei) of primary myoblasts cultured for 2 days in differentiation medium in the absence or presence of IBMX. (*D*) Representative Western blot of C/EBPβ expression in primary myoblasts treated with 30uM IBMX for up to 5 days in GM. Cyclophilin B (CyPB) is a loading control. (*E*) Quantification of C/EBPβ expression

normalized to cyclophilin B. *(F)* Western analysis of myogenic marker expression in primary myoblasts cultured in vehicle or IBMX for 5 days under growth conditions and then induced to differentiate (DM) for 2 days in the continual absence or presence of IBMX. *(G)* Primary myoblasts were cultured in vehicle or IBMX for 5 days in GM, harvested, re-plated and induced to differentiate (DM) in the absence of IBMX (top). Cells were fixed and stained for MyHC (yellow) and DAPI (blue). Representative pictures are shown (bottom). Scale bar: 50um. $MyHC^{+}$ nuclei were counted to assess *(H)* differentiation index (as in C) and *(I)* fusion index (#nuclei in $MyHC^{+}$ cells/# myotubes) for cells treated and differentiated as in (G). *(J)* Western analysis of myogenic marker expression from myoblasts cultured as in (G). All data are presented as mean ± SEM (n=3, n.s., not significant, *$p<0.05$).

To determine if the effects of IBMX on myogenesis were reversible, primary myoblasts were cultured in growth medium in the presence of 30 uM IBMX for 5 days and re-plated at equal density for differentiation in the absence of the compound for two days. Myoblasts grown in the presence of IBMX showed effective differentiation similar to that of vehicle-treated myoblasts upon withdrawal (Figure 21G,H), but produced fibers that were significantly larger (2-fold) than those in vehicle-treated cultures (Figure 21G,I). After removal of IBMX, protein levels of C/EBPβ and Pax7 returned to levels comparable to vehicle-treated myoblasts (Figure 21J and S6B).

IBMX promotes expansion of myoblasts *in vitro*. For myoblast transplantation protocols to be successful, isolated myoblasts must be expanded in culture without losing regenerative potential. To determine whether IBMX could support the expansion of freshly isolated myoblasts in culture, cell number was assessed by crystal violet staining at different time points during growth. While numbers were equivalent between vehicle- and IBMX-treated cells after 3 days in culture, a significant increase in cell number was noted for IBMX-treated cultures on day 5 regardless of dose (Figure 22A). To determine if the growth kinetics of IBMX-treated cultures were changing, we assessed cell proliferation using BrdU incorporation and Ki67 staining. After a 6hr pulse, IBMX-treated myoblasts (30 uM) had significantly increased BrdU incorporation (42%) compared to vehicle-treated myoblasts (29%) (Fig. 22B,C) indicating a greater number of cells in S-phase during the pulse. However, the number of $Ki67^{+}$ cells did not change significantly with IBMX treatment (Figure 22D,E), suggesting that IBMX promotes expansion of myogenic precursors by reducing the transit time through the cell cycle

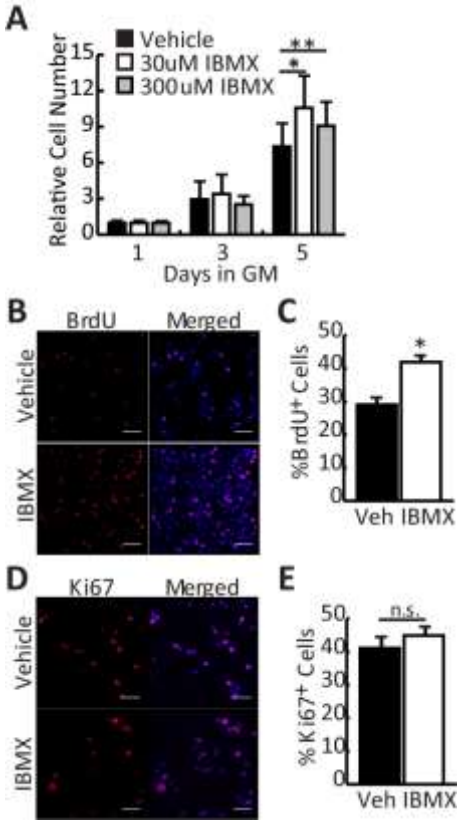

Figure 22. IBMX promotes expansion of myoblasts in vitro. (*A*) Vehicle- and IBMX-treated myoblasts (30 and 300 uM) were stained with crystal violet (absorbance at 570nm) to assess cell number at day 1, 3 and 5 of treatment. (*B*) Myoblasts in GM were cultured for 5 days with vehicle or IBMX, after which they were pulsed with BrdU for 6 hours, fixed and stained for BrdU (red) and DAPI (blue). Representative pictures are shown. Scale bar: 50uM. (*C*) $BrdU^+$ cells were counted and represented as the percentage of total nuclei. (*D*) Myoblasts were treated as in (*B*) and were fixed and stained for Ki67 (red) and DAPI (blue). Representative pictures are shown. Scale bar: 50um. (*E*) $Ki67^+$ cells were counted and represented as the percentage of total nuclei. All data are presented as mean ± SEM (n=4, n.s., not significant, **p<0.01, *p<0.05).

(BrdU$^+$ cells) without affecting the percentage of cells in the growth fraction (Ki67$^+$ cells).

Stimulation of satellite cell marker expression by IBMX is C/EBPβ-dependent. Ectopic expression of C/EBPβ potently inhibits myogenesis and promotes Pax7 expression (Marchildon et al. 2012). To examine the molecular profile of IBMX-treated myoblasts, freshly isolated myoblasts were cultured in vehicle or IBMX for 5 days in growth media at doses of 30 uM and 300 uM (Figure 23A,B). The lower IBMX dose increased Pax7 protein expression by 2.5-fold, (Figure 23A, S7A) whereas 300 uM only minimally increased Pax7 expression by approximately 1.5-fold (Figure 23B, SB). MyoD protein levels were unaffected at both doses, consistent with observations in differentiation medium (Figure 23A,B and Fig. S7). Given that the MyoD protein levels were unchanged with IBMX treatment, we examined the proportion of cells that were differentiating (Pax7$^-$/MyoD$^+$), proliferating (Pax7$^+$/MyoD$^+$) and self-renewing (Pax7$^+$/MyoD$^-$) in growth medium (Zammit et al. 2004). In vehicle-treated WT cultures, we noted that the majority of cells co-expressed Pax7 and MyoD (~70-78%). Approximately 15% of the culture expressed only MyoD while approximately 6% were positive for only Pax7 expression (Figure 23C). IBMX treatment significantly increased the proportion of reserve cells (Pax7$^+$/MyoD$^-$) in the culture to approximately 20%, while decreasing the number of differentiating cells (Pax7$^-$/MyoD$^+$) to ~5%, without affecting the double-positive population size. These results suggest that IBMX can maintain the Pax7$^+$/MyoD$^-$ state more efficiently than standard culture conditions, and thus increase the population of cells desirable for transplantation. Since Pax7 has been

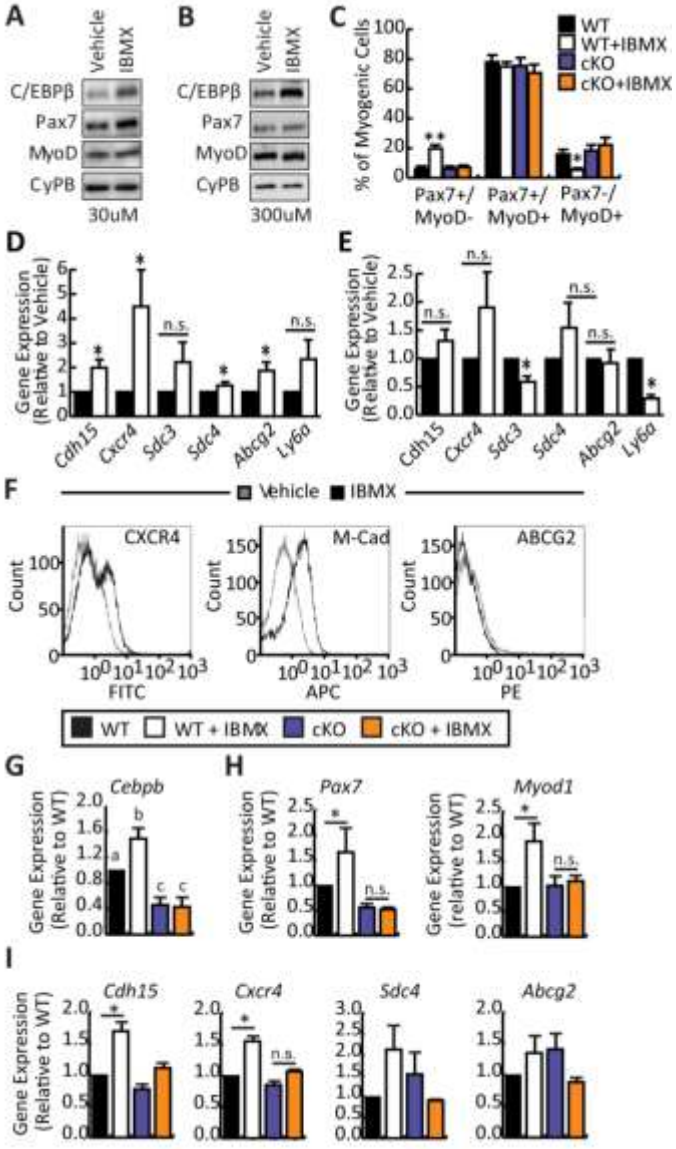

Figure 23. IBMX-mediated stimulation of satellite cell marker expression is dependent on C/EBPβ. *(A,B)* Primary myoblasts were cultured in vehicle or IBMX (30 and 300uM) for 5 days and analyzed by western blot for C/EBPβ, Pax7 and MyoD protein expression. Cyclophilin B is a loading control. *(C)* Percentage of quiescent ($Pax7^+/MyoD^-$), proliferating ($Pax7^+/MyoD^+$) and differentiating ($Pax7^-/MyoD^+$) cells as determined by immunocytochemistry in primary myoblast cultures treated for 5 days in

GM with 30 uM IBMX or vehicle. *(D)* RT-qPCR of satellite cell markers *Cdh15*, *Cxcr4*, *Sdc3*, *Sdc4*, *Abcg2*, and *Ly6a* in primary myoblasts cultured for 5 days in GM with 30 uM IBMX or vehicle. *(E)* Satellite cell marker expression in primary myoblasts cultured in GM for 5 days in the absence or presence of 300 uM IBMX. *(F)* Flow cytometry histograms of CXCR4, m-cadherin and ABCG2 expression on vehicle and IBMX-treated myoblasts. *(G) Cebpb* expression in myoblasts isolated from C/EBPβ conditional knockout (cKO) or wild-type (WT) non-Cre expressing floxed littermates treated with vehicle or 30 uM IBMX for 5 days in GM. (*H*) Myogenic markers expression (*Pax7* and *Myod1)* in myoblasts cultured as in (G). (*I*) Satellite cell marker expression (*Cdh15*, *Cxcr4*, *Sdc4*, and *Abcg2*) in myoblasts cultured as in (G). Data are presented as mean ± SEM ($n \geq 3$, n.s., not significant, $^{**}p<0.01$, $^{*}p<0.05$, means with different letters have a $p<0.05$ and means indicated with the same letter are not statistically different).

shown to be a target of C/EBPβ (Marchildon et al. 2012), we examined the effect of IBMX on the different states in C/EBPβ-null myoblasts (Figure 23C), isolated from a conditional knockout mouse (cKO, C/EBPβ$^{-/-}$Pax7$^{CreER+/-}$) in which C/EBPβ is knocked down in Pax7^{+} cells following activation of the CreER recombinase with tamoxifen. In vehicle-treated C/EBPβ-null cultures the number of differentiating (~18%), proliferating (~75%) and quiescent (~6%) cells were similar to vehicle-treated wild-type cultures. However, in cells lacking C/EBPβ, IBMX treatment did not increase the Pax7^{+}/MyoD^{-} population, suggesting that the increase in Pax7^{+} cells by IBMX is dependent on C/EBPβ expression.

Other than Pax7, expression of additional stem cell markers are also correlated with better transplantation outcomes. After a 5 day treatment with 30 uM IBMX, the expression of satellite cell and side population markers was evaluated. IBMX treatment increased *Cdh15* (2-fold), *Cxcr4* (4-fold), *Sdc4* (1.5-fold) and *Abcg2* (2-fold) expression significantly (Figure 23D). While *Sdc3* and *Ly6a* expression trended towards an increase in the presence of IBMX, this failed to reach statistical significance (Figure 23D). A higher dose of IBMX (300 uM), while able to promote C/EBPβ expression, failed to stimulate satellite cell marker expression significantly, and negatively impacted *Sdc3* and *Ly6a* expression (Figure 23E). As such, the 30 uM dose was selected for further investigation. To confirm that mRNA expression correlated with increased protein expression of these markers, CXCR4, M-Cadherin and ABCG2 expression was quantified using flow cytometry (Figure 23F). Both CXCR4 and M-cadherin expression was increased with IBMX treatment as compared to vehicle-treated control, while ABCG2 was not appreciably increased. Taken together, these results suggest that IBMX

stimulates the expression of a subset of stem cell markers in a C/EBPβ-dependent fashion and promotes a gene expression profile similar to undifferentiated myogenic progenitors.

IBMX treatment is expected to, through an increase in cellular cAMP levels, influence multiple pathways in addition to upregulating the expression of C/EBPβ. To determine if C/EBPβ is required for the upregulation of satellite cell markers in IBMX-treated myoblasts, primary myoblasts from conditional C/EBPβ knockout mice (cKO) or their wild-type (WT, C/EBPβ$^{fl/fl}$Pax7$^{CreER-/-}$) non-Cre expressing floxed littermates were treated with IBMX or vehicle in growth medium and satellite cell marker expression was examined by RT-qPCR. *Cebpb* excision was approximately 60% in cKO myoblasts and was not further stimulated by IBMX treatment (Figure 23G). *Pax7* stimulation by IBMX was dependent on the expression of C/EBPβ, as Pax7 expression was reduced in cKO myoblasts and was not further upregulated by IBMX in these cells (Figure 23H), consistent with our previous findings (Marchildon et al. 2012). IBMX also induced *Myod1* expression in WT cells, but not in C/EBPβ-deficient cells indicating that C/EBPβ is required for this effect (Figure 23H). For the stem cell markers, the induction of *Cdh15*, *Cxcr4* and *Sdc4* by IBMX was found to be C/EBPβ-dependent as their induction was lost in cKO cells (Figure 23I). IBMX-mediated stimulation of *Abcg2* expression was not dependent on C/EBPβ, and was highly variable in this genetic background (Figure 23I).

IBMX-treated myoblasts repair dystrophic muscle and occupy the host satellite cell niche with greater efficiency. Given the improved molecular profile of IBMX-treated cells, we postulated that IBMX-treated cells would engraft more efficiently into

dystrophic muscle, as has been observed for muscle stem cells (Cerletti et al. 2013; Tanaka et al. 2009). Interestingly, C/EBPβ is a potent pro-survival factor that can both directly inhibit caspases through a XEXD box as well as inhibit p53 activity (Buck et al. 2001; Yoon et al. 2007) and high Pax7 expression has been shown to support myoblast survival (F Relaix et al. 2006). Equal numbers of WT or cKO myoblasts cultured with vehicle or IBMX for 5 days under growth conditions were stained with the vital dye CSFE before transplantation into cardiotoxin (CTX)-injured tibialis anterior (TA) muscles of mdx mice. Twenty-four hours after grafting, muscle was harvested to quantify donor cell survival and proliferation (Figure 24A-C). Using immunohistochemistry, the percentage of $TUNEL^+/CSFE^+$ cells was scored, and revealed that while there was no measurable difference in apoptosis rates between vehicle-treated WT and cKO donor cells, treatment of WT cells with IBMX significantly reduced the percentage of $TUNEL^+$ cells by 75% as compared to vehicle-treated controls (Figure 24A,B). The protective effect of IBMX was lost, however, when myoblasts lacking C/EBPβ were transplanted, suggesting that the enhanced survival is mediated by C/EBPβ (Figure 24B). Despite enhanced expansion in culture, culture of isolated myoblasts with IBMX did not improve their proliferation once injected into host muscle (Figure 24C), nor was proliferation adversely affected by loss of C/EBPβ.

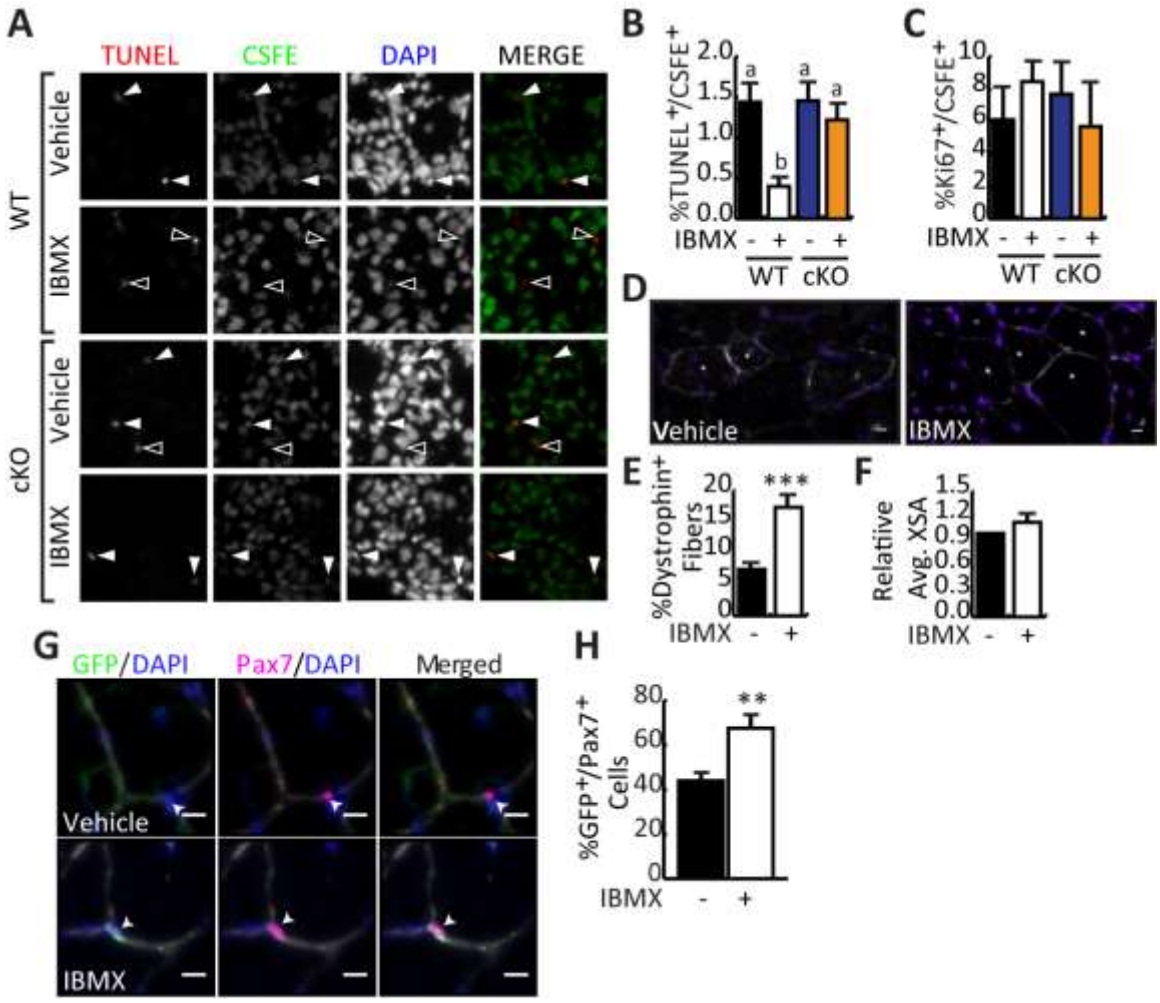

Figure 24. IBMX-treated myoblasts contribute to repair of dystrophic muscle and occupy the host satellite cell niche. *(A)* Donor myoblasts isolated from WT and cKO mice were cultured for 5 days in the absence or presence of IBMX. Equal cell numbers were stained with vital dye CSFE and transplanted into CTX-injured TA muscle of mdx mice. Twenty-four hours after transplant, TA muscles were collected to assess donor cell apoptosis and proliferation. Representative images of TUNEL and CSFE stained muscle after transplant with vehicle or IBMX-treated myoblasts. Solid arrows indicate $TUNEL^+/CSFE^+$ cells. Open arrowheads indicate $TUNEL^+$ cells that are not from the donor. DAPI staining reveals all nuclei. *(B)* Quantification of the percentage of

TUNEL$^+$/CSFE$^+$ cells from (A). *(C)* Quantification of the percentage of Ki67$^+$/CSFE$^+$ cells of stained muscles as in (A). *(D)* Myoblasts isolated from GFP mice were cultured in vehicle or IBMX for 5 days in GM. Myoblasts were then harvested and 10^5 cells were transplanted into CTX-injured TA muscles of mdx mice. Two months after transplant, TA muscles were harvested for assessment of donor cell engraftment. Dystrophin staining (white) of TA muscle of mdx mice two months after transplantation. Representative images are shown. Scale bar: 10 um. (*E*) Quantification of dystrophin$^+$ fibers in TA engrafted with vehicle- or IBMX-treated myoblasts. *(F)* Mean cross-sectional area of dystrophin$^+$ fibers, shown relative to control. (*G*) Representative images of TA sections stained with GFP (green), Pax7 (pink), Laminin (white) and DAPI (blue). Scale bar: 10um. (*H*) Quantification of GFP$^+$/Pax7$^+$ cells after transplantation. All data are presented as mean ± SEM (n=6, **p<0.01, ***p<0.001, means with different letters have a p<0.05 and means indicated with the same letter are not statistically different).

For long term study of engraftment, donor cells were isolated from GFP mice, cultured in the absence or presence of IBMX, transplanted into CTX-injured TA muscle of mdx mice and allowed to engraft for 2 months. The number of dystrophin^{+} fibers in the entire cross-section of the TA was assessed to determine the myogenic potential of transplanted myoblasts. IBMX-treated myoblasts produced more dystrophin^{+} (~17%) fibers compared to vehicle-treated myoblasts (~7%) (Figure 24D,E) suggesting that IBMX-treated myoblasts are better at repairing dystrophic muscle than vehicle-treated myoblasts. Despite enhanced fusion of IBMX-treated myoblasts in culture (Figure 21I), the cross-sectional area of dystrophin-positive fibers was not different in the muscle that received IBMX-treated donor cells as compared to vehicle-treated cells (Figure 24F). Nonetheless, quantification of sublaminar GFP^{+}/Pax7^{+} cells revealed that IBMX-treated myoblasts made up 68% of the satellite cell population found in the niche, whereas vehicle-treated myoblasts contributed only 43%, indicating that IBMX-treated myoblasts are more efficient at engrafting into the satellite cell niche (Figure 24G,H). Indeed, niche engraftment is necessary for participation of donor cells in multiple rounds of repair, and therefore long term repair. Taken together, these results indicate that IBMX-treatment of myoblasts during expansion leads to better engraftment and restoration of dystrophin expression to dystrophic muscles.

IBMX enhances myoblast migration through induction of CXCR4. CXCR4 is required for SDF-1 induced myoblast migration (Brzoska et al. 2012) and has been shown to improve myogenic progenitor extravasation and engraftment into dystrophic muscle (Perez et al. 2010). Since *Cxcr4* expression was upregulated by IBMX in a

C/EBPβ-dependent manner, we examined whether IBMX improved migration of myoblasts in vitro. Confluent monolayers of vehicle or IBMX-treated myoblasts were scratched and pictures were taken at 0h and 6h after induction to differentiate in the presence of mitomycin C, to inhibit proliferation, to determine the extent of wound closure. Myoblasts that were pre-treated with IBMX showed increased wound closure (~35%) compared to vehicle-treated myoblasts (~5%) (Figure 25A). In the absence of C/EBPβ (cKO), migration was equivalent to WT controls and was not further stimulated by IBMX (Figure 25A) suggesting that the improved migration of IBMX-treated cells requires C/EBPβ.

To determine if the enhanced migration after treatment with IBMX was mediated through CXCR4, the scratch wound assay was repeated in the presence of AMD3100, a non-peptide antagonist of CXCR4. In the absence of AMD3100, IBMX treatment stimulated wound closure when compared to vehicle-treated controls, however, this effect was lost in the presence of AMD3100, suggesting that the enhanced migration is mediated by the C/EBPβ-dependent stimulation of CXCR4 expression (Figure 25B).

Transplantation of dystrophin^{+} myoblasts can form clusters of dystrophin-expressing fibers in dystrophin-deficient muscle. Thus, to assess cell migration *in vivo*, we measured the maximum distance (in mm) between dystrophin-positive clusters in TA muscles of mdx mice transplanted with vehicle or IBMX-treated donor cells 2 months after grafting (Figure 25C). Muscles that received IBMX-treated cells had a ~40% increase in the mean cluster distance, indicating that they were better able to migrate away from the site of grafting than vehicle-treated controls.

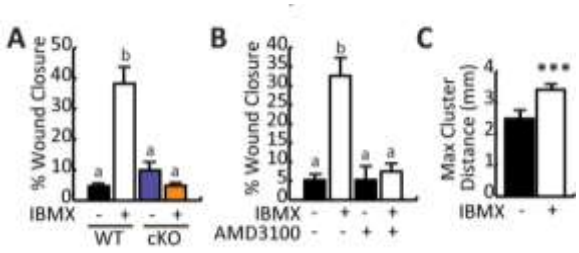

Figure 25. IBMX-enhanced cellular migration is dependent on C/EBPβ and mediated by CXCR4. *(A)* Vehicle- and IBMX-treated WT and cKO myoblasts were harvested, re-plated, treated with mitomycin C, scratched and switched to DM. Pictures were taken at 0h and 6h. Percent wound closure was calculated as follows: (width of 0h wound gap – width of 6h wound gap)/width of 0h wound gap x 100%. *(B)* Scratch wound assay on WT myoblasts pre-cultured in the presence of IBMX or vehicle as in (A) and monitored for wound closure in the presence or absence of AMD3100. *(C)* Maximum cluster distance of dystrophin^{+} fibers after 2 months after transplantation of IBMX or vehicle-treated myoblasts into CTX-injured mdx muscle. All data are presented as mean ± SEM ($n \geq 4$, ***$p < 0.001$, means with different letters are significantly different from one another and means indicated with the same letter are not statistically different).

Discussion

The use of myoblast transplantation for the treatment of muscular dystrophies has been limited by both poor engraftment and lack of significant satellite cell niche repopulation, necessary for long-term regeneration and complete restoration of dystrophin expression. While primary myoblasts are poorly suited for transplantation, other more rare populations such as the satellite side population (SP) cells and CSM4B ($CD45^-Sca\text{-}1^-Mac\text{-}1^-CXCR4^+\beta1\text{-}integrin^+$) cells engraft with greater efficiency (Tanaka et al. 2009; Cerletti et al. 2013). However, these populations are difficult to isolate in high numbers and lose their regenerative potential with in vitro expansion, precluding their therapeutic usage.

Our work has identified the bzip transcription factor C/EBPβ as an important negative regulator of myogenesis. Consistent with this, C/EBPβ expression is highest in satellite cells and decreases in concert with Pax7 during early differentiation (Marchildon et al. 2012). Since maintenance of the satellite cell undifferentiated state during myoblast culture is desirable for transplantation, and myoblasts are easy to isolate in high numbers, we used the phosphodiesterase inhibitor IBMX to stimulate the expression of satellite cell markers on myoblasts.

Contrary to what we expected, MyoD protein levels remained unchanged in the presence of high C/EBPβ found in IBMX-treated cells, an effect that may represent the equilibrium between the negative effects of C/EBPβ on MyoD protein expression and the positive effects of increased cAMP signaling (Xu et al. 2013). Despite the unchanged MyoD protein levels across the whole population, IBMX treatment did significantly reduce the $Pax7^-/MyoD^+$ population while concomitantly increasing the $Pax7^+/MyoD^-$

population, confirming that IBMX imposes a restraint on differentiation through both Pax7 and MyoD. These results suggest that the IBMX-mediated increase in MyoD expression is not homogeneous in the population, but rather restricted to a subset of the treated population. Further isolation of the MyoD$^-$ population could further enhance transplantation outcomes.

Withdrawal from IBMX restored the differentiation of treated cells allowing for efficient contribution to repair. Interestingly, cells that were pre-treated with IBMX made myotubes twice as large as vehicle-treated myoblasts. This result correlates with a previous study in which the mouse myogenic cell clone Ric10 was treated with different cAMP stimulating reagents, such as IBMX and forskolin (Mukai & Hashimoto 2008). Increased PKA-cAMP signaling after forskolin treatment increased myotube-myotube fusion resulting in extra-large myotubes, designated "myosheet", similar to that seen in IBMX-treated myoblasts in our study (Figure 21G,I) (Mukai & Hashimoto 2008). However, IBMX treatment did not produce larger dystrophin$^+$ fibers after transplantation and given that high C/EBPβ levels correlate with decreased fusion, while loss of C/EBPβ promotes hyperfusion in culture (Marchildon et al. 2012), it is unlikely that this effect of IBMX is dependent of C/EBPβ.

Clinical trials of myoblast transplantation in patients with Duchenne muscular dystrophy have been disappointing due to the rapid cell death of myoblasts within hours of transplantation (Briggs & Morgan 2013; Fan et al. 1996; Huard et al. 1994). A number of approaches have been investigated to enhance myoblast survival upon transplantation including hypoxia preconditioning, heat shock, or co-injection with small molecules (dextran sulfate), biomaterials (fibrin gel) or macrophages (Liu et al. 2012; Suzuki et al.

2000; Laumonier et al. 2013; Gerard et al. 2012; Lesault et al. 2012). Interestingly, C/EBPβ is a potent pro-survival factor (Buck et al. 2001; Yoon et al. 2007) and is upregulated in a number of tumors demonstrating its role as an important mediator of cell survival during tumorigenesis (Sterneck et al. 2006; Yoon et al. 2007). Thus, in addition to improving stem cell marker expression in myoblasts, IBMX treatment, through C/EBPβ appears to also improve transplantation outcomes through increased myoblast survival after grafting. Indeed, IBMX-treated donor cells were less apoptotic than vehicle-treated cells after transplantation into injured muscle, and this effect was dependent on C/EBPβ expression. Recently, Brg1, a component of the Swi/Snf chromatin remodelling complex, was shown to be required for maintaining viability in myoblasts through regulation of Pax7 expression (Padilla-Benavides et al. 2015). Pax7 is required for maintenance of the satellite cell pool as deletion of Pax7 triggers cell cycle abnormalities characterized by an extended G2/M phase, and a progressive loss of muscle precursors to cell death (F Relaix et al. 2006). Thus Pax7 regulation by C/EBPβ could provide a mechanism for improved survival in IBMX-treated cells (Marchildon et al. 2012). C/EBPβ is not, however, required for the development of muscle embryonically, nor the specification of $Pax7^{+}$ cells, as both of these are normal in C/EBPβ knockout mice, suggesting that C/EBPβ may be a more important regulator of Pax7 expression in the postnatal animal.

IBMX also stimulated CXCR4 mRNA and protein expression in a C/EBPβ-dependent manner. CXCR4 contributes to the migration of cells to dystrophic tissues that express high levels of SDF-1 and has been shown to be required for myoblast migration (Pescatori et al. 2007; Perez et al. 2010; Brzoska et al. 2012). CXCR4 is also a known

target of C/EBPβ in breast cancer cells (Park et al. 2013). Consistent with this, IBMX-treated myoblasts had significantly enhanced migration under differentiation conditions that was not seen in C/EBPβ-deficient myoblasts, suggesting that the increased migratory ability induced by IBMX is dependent on increased C/EBPβ expression. Further, inhibition of CXCR4 abolished the effect of IBMX on migration. In vivo, cells treated with IBMX migrated further in the injured host muscle than vehicle-treated cells, suggesting that the enhanced repair and engraftment could be due, at least in part to better movement through the injured muscle tissue. Since CXCR4 expression in muscle progenitor cells is known to increase extravasation into skeletal muscle after intravenous and intra-arterial transplantation in mdx^{5cv} mice, IBMX treatment of myoblasts may allow for improved intra-arterial delivery of graft cells (Perez et al. 2010).

Conclusion

In this study, we demonstrate that treatment with IBMX is an efficient method of expanding muscle progenitor cells, while preserving regenerative potential, before transplantation. The regulation of C/EBPβ expression by IBMX leads to the upregulation of several satellite cell markers, improves cell migration and cell survival resulting in better engraftment into dystrophic muscle. In addition to identifying C/EBPβ-dependent effects on myoblasts following IBMX treatment, it is clear that treatment with IBMX has several beneficial effects on myoblasts favoring better transplantation outcomes that are independent of C/EBPβ expression and should be further investigated. Moreover, the pharmacological reprogramming of myoblasts with IBMX reverts them to a more primitive state, but not into a cell population that has been thoroughly defined in vivo.

This hybrid profile merits further analysis to better understand the molecular mechanisms that act to preserve the undifferentiated state of muscle stem cells and their self-renewal properties. Together, the molecular profile of myoblasts treated with IBMX improved their engraftment into injured muscle and resulted in their persistence in the satellite cell niche, suggesting that long term sustained repair is possible with this approach. Our research demonstrates that phosphodiesterase inhibitor treatment improves myoblast culture conditions in such a way as to reinvigorate myoblast transplantation as a viable therapeutic approach to halt muscle wasting in DMD.

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CHAPTER SIX: GENERAL DISCUSSION

C/EBPβ has been shown to be a major regulator of mesenchymal stem cell fate, controlling differentiation of adipocytes, osteoblasts and chondrocytes (Smink & Leutz 2012). Here, we show that C/EBPβ is a negative regulator of skeletal myogenesis, promoting quiescence and self-renewal of satellite cells. We also demonstrate that pharmacological stimulation of C/EBPβ expression with the phosphodiesterase inhibitor IBMX improves cell culturing conditions prior to myoblast transplantation for diseases such as Duchenne's muscular dystrophy (DMD).

5.1. C/EBPβ Limits Terminal Differentiation and Fusion of Myoblasts

Our lab has shown that C/EBPβ regulates satellite cell quiescence, activation, early differentiation and self-renewal; however its role in terminal differentiation and fusion remains unclear. Interestingly, C/EBPβ expression increases late in differentiation (D. Fu et al. 2015) and loss of C/EBPβ results in significantly larger myotubes *in vitro* and larger fibers *in vivo* (Marchildon et al. 2012), suggesting that C/EBPβ may be playing a role in limiting cell fusion.

One possible target for C/EBPβ during myoblast fusion is Kruppel-like factor 4 (KLF4). In C2C12s, KLF4 protein levels increase after induction to differentiate and siRNA-mediated knockdown of KLF4 results in smaller myotubes compared to mock-transfected cells (Sunadome et al. 2011). Furthermore, overexpression of KLF4 in C2C12 myoblasts promotes cell fusion, suggesting that KLF4 plays an important role in myoblast fusion (Sunadome et al. 2011). Interestingly, siRNA-mediated knockdown of C/EBPβ in 3T3-L1 cells increases levels of *Klf4* transcript while overexpression of C/EBPβ decreases levels of *Klf4* transcript (Birsoy et al. 2008). Thus, C/EBPβ could limit

myoblast fusion by regulating *Klf4* expression. Preliminary work done in our lab suggests that C/EBPβ negatively regulates *Klf4* transcription in C2C12 and primary myoblasts (unpublished data).

In addition to KLF4, myoferlin can regulate myoblast fusion. While not required for initial fusion events; myoferlin is required to form large myotubes (Doherty et al. 2005; Doherty et al. 2008). From publicly available ChIP-seq data (Kent et al. 2002), we found that C/EBPβ binds to the *Myof* promoter in C2C12s 60 hours after induction to differentiate. Thus, C/EBPβ could limit myogenic fusion by inhibiting expression of myoferlin.

Indeed, while C/EBPβ-deficient satellite cells create larger myotubes *in vitro* and C/EBPβ conditional null animals have larger myofibers (Marchildon et al. 2012), we do not yet know if the myofiber hypertrophy is due to myonuclear accretion or expansion of the myonuclear domain. A better understanding of C/EBPβ's role during skeletal myogenesis, especially during myoblast fusion could be obtained by performing an unbiased assay such as RNA sequencing (RNA-seq). In this experiment, wild-type myoblasts and conditional C/EBPβ$^{-/-}$ myoblasts could be compared to identify a list of differentially expressed genes known to be involved in myoblast fusion as well as to discover novel regulators.

5.2. C/EBPβ Represses MyoD Protein Expression Post-Transcriptionally

MyoD is often referred to as the "master regulator" of skeletal muscle differentiation because its expression is sufficient to convert a large number of primary cells to skeletal myoblasts (Weintraub et al. 1989; Tapscott 2005). We show that persistent expression of

C/EBPβ inhibits MyoD protein expression; however, C/EBPβ overexpression has no effect on *Myod1* mRNA levels (Marchildon et al. 2012). Furthermore, MG132 treatment does not rescue MyoD expression, suggesting that the decreased MyoD levels are not due to protein degradation by the 26S proteasome.

Since the regulation of MyoD expression is not due to changes in *Myod1* mRNA levels or protein stability (Marchildon et al. 2012), C/EBPβ may regulate MyoD protein expression at the level of *Myod1* translation. Translation of *Myod1* mRNA has been shown to be regulated by miR-221/miR-222 in C2C12 cells (Tan et al. 2014; Cardinalli et al. 2009), miR-143 in *Siniperca chuatsi* muscle (Chen et al. 2014) and miR-203b in tilapia skeletal muscle (Yan et al. 2012). Interestingly, C/EBPβ has been shown to regulate a number of microRNAs in different systems including miR-203 in esophageal cancer cells (Li et al. 2014). In this study, they demonstrated that the inhibitory C/EBPβ isoform, C/EBPβ-LIP, can directly interact with a conserved distal regulatory element upstream of miR-203 and inhibit its expression (Li et al. 2014). Since C/EBPβ-LIP is considered a C/EBPβ-LAP antagonist (Descombes & Schibler 1991), C/EBPβ-LAP may positively regulate miR-203 expression. Indeed, knockdown of C/EBPβ in anaplastic large cell lymphoma (ALCL) cells reduced miR-203 expression and overexpression of C/EBPβ-LAP significantly increased miR-203 (Steinhilber et al. 2015). When overexpressed in chicken skeletal muscle, miR-203 inhibited cell proliferation and differentiation. Thus, C/EBPβ may be regulating translation of *Myod1* and inhibiting myogenic differentiation through upregulation of miR-203.

Translation of *Myod1* is also regulated by RNA-binding proteins (RBPs) (Apponi et al. 2011). For example, human antigen R (HuR) plays a critical role in myogenesis by

stabilizing *Myod1* transcripts (Figueroa et al. 2003; Beauchamp et al. 2010; Von Roretz et al. 2011). In proliferating myoblasts, HuR is almost exclusively nuclear, but accumulates in the cytoplasm upon differentiation. Cytoplasmic accumulation of HuR leads to stabilization of *Myod1* transcript and increased levels of MyoD protein (Beauchamp et al. 2010). Furthermore, siRNA-mediated knockdown of HuR in myoblasts decreased MyoD expression and inhibited differentiation (Van Der Giessen et al. 2003). Interestingly, HuR co-precipitates with C/EBPβ in C2C12s cultured in DM for 24 hours (unpublished data), suggesting that C/EBPβ could inhibit *Myod1* translation through this interaction. C/EBPβ contains a nuclear localization signal sequence in its bZIP domain which is required for its localization to the nucleus (Williams et al. 1997). Since C/EBPβ is normally localized to the nucleus, its interaction with HuR may sequester the HuR-*Myod1* complex in the nucleus and away from the translational machinery in the cytoplasm.

5.3. Regulators of C/EBPβ During Myogenesis

Although we have demonstrated a number of physiological functions for C/EBPβ in adult myogenesis, to fully understand how C/EBPβ exerts its biological effects, it is necessary to address the regulatory mechanisms affecting C/EBPβ. Apart from stimulation by IBMX, C/EBPβ protein levels are regulated by Mouse double minute 2 homolog (Mdm2), Transforming Growth Factor β (TGFβ) and interleukin-1β (IL-1β) (D. Fu et al. 2015; Lamarche et al. 2015; Marchildon et al. 2016). The E3 ubiquitin ligase Mdm2 ubiquinates and targets C/EBPβ for degradation by the proteasome which is

necessary for myogenic differentiation (D. Fu et al. 2015) and TGFβ inhibits adult myogenesis, at least in part, by upregulating C/EBPβ expression (Lamarche et al. 2015).

Efficient regeneration of muscle after injury involves local inflammation and cytokine production (Tidball & Villalta 2010). Since C/EBPβ is a mediator of inflammation, its expression could be regulated by cytokines produced and secreted in response to muscle injury. Indeed, expression of the proinflammatory cytokine IL-1β is elevated after cardiotoxin injury and it increases C/EBPβ expression in myoblasts (Marchildon et al. 2016). Other transcriptional regulators of C/EBPβ could be identified by performing a reverse ChIP followed by mass spectrometry (MS). To perform the reverse ChIP, nuclear extracts are subjected to DNA affinity chromatography, in which the *Cebpb* promoter region is immobilized on streptavidin magnetic beads. DNA-protein complexes eluted from the column can be resolved by SDS-PAGE, digested with trypsin and analyzed by MS (Mittler et al. 2009; Déjardin & Kingston 2009).

In addition to regulation at the level of transcription, C/EBPβ is also regulated at the level of protein isoform expression. C/EBPβ is encoded by a single exon gene, but gives rise to multiple translational isoforms called Liver-activating protein LAP*, LAP and Liver Inhibitory Protein (LIP) (Descombes & Schibler 1991). These N-terminally truncated isoforms arise from differential use of initiation codons (Descombes & Schibler 1991) and isoform ratios can be controlled by extracellular signalling such as the mammalian target of rapamycin (mTOR) signalling pathway via activation of elongation initiation factor (eIF-4E) (Calkhoven et al. 2000). In osteoclasts, inhibition of mTOR by rapamycin enhances translation of the LAP isoform and inhibits translation of the LIP isoform, thus inhibiting differentiation (Smink et al. 2009). In skeletal muscle, rapamycin

inhibits differentiation of C2C12 myoblasts (Cuenda & Cohen 1999; Erbay & Chen 2001) and skeletal muscle regeneration *in vivo* (Ge et al. 2009). Since rapamycin inhibits mTOR and mTOR reduces the LAP/LIP ratio (Calkhoven et al. 2000), rapamycin could be inhibiting myogenesis through upregulation of the LAP isoform of C/EBPβ.

C/EBPβ can also undergo a number of post-translation modifications (PTM) (i.e. phosphorylation, sumoylation and acetylation) which can affect protein stability, DNA binding, or interaction with transcription factors or coactivators (Tsukada et al. 2011). The best characterized C/EBPβ phosphorylation event occurs at Thr188 (Thr235 in humans) and results in increased transactivation activity (Pulido-Salgado et al. 2015). Interestingly, cAMP signaling and protein kinase A (PKA) have been shown to phosphorylate C/EBPβ at thr235 and ser105, respectively, to stimulate its transcriptional activity (Borland et al. 2009; Trautwein et al. 1994). Therefore, treatment of myoblasts with IBMX may be promoting C/EBPβ-dependant gene induction by increased C/EBPβ protein levels as well as promoting its transcriptional activity through phosphorylation of thr235 and ser105 (Lala-Tabbert et al. 2016).

In contrast to the predominantly stimulatory role of C/EBPβ phosphorylation, sumoylation appears to be inhibitory. Indeed, C/EBPβ is sumoylated during adipogenesis by protein inhibitor of activated STAT1 (PIAS1) resulting in increased ubiquitination and degradation of C/EBPβ (Liu et al. 2013). Acetylation of C/EBPβ can regulate association with co-regulators such as HDAC1 (Wiper-Bergeron, Salem, et al. 2007). Treatment of pre-adipocytes with glucocorticoids induces PCAF/GCN5-dependant acetylation of C/EBPβ which directly interferes with its interaction with HDAC1, thus promoting pre-adipocyte differentiation (Wiper-Bergeron, Salem, et al. 2007). Identifying the post-

translational modifications that C/EBPβ is subjected to during skeletal myogenesis and in muscle disease can further our understanding of how C/EBPβ regulates satellite cell function and provide potential therapeutic targets for disease intervention. This could be accomplished by subjecting purified C/EBPβ from whole cell extracts to MS (Gundry et al. 2009).

5.4. Misregulation of C/EBPβ in Skeletal Muscle Disorders

Although we have examined the role of C/EBPβ in satellite cells of healthy muscle, its role in dystrophic and aging muscle remains to be explored. There is, however, evidence of C/EBPβ misregulation in cancer cachexia. Recent studies from our lab have shown that cachexia can elevate C/EBPβ levels in satellite cells, protecting them from apoptosis and inhibiting their differentiation (Marchildon et al. 2015; Marchildon et al. 2016). C/EBPβ is also upregulated in muscle fibers of cachectic muscle leading to stimulation of atrophin-1 expression and fiber atrophy (Zhang et al. 2011). In our lab, we have not seen C/EBPβ expression in mature myofibers of healthy muscle, suggesting that C/EBPβ expression is only expressed in nascent myofibers and myofibers of diseased muscle.

Recently, satellite cells have been shown to play a direct contributory role in the etiology and progression of DMD (Dumont et al. 2015). In this study, the authors found that dystrophin is expressed in satellite cells of healthy muscle and that satellite cells deficient in dystrophin could not establish polarity, undergo asymmetric cell division, or enter the myogenic program, thus exacerbating disease progression (Dumont et al. 2015). Interestingly, increased numbers of satellite cells have been found in dystrophic muscle,

suggesting that dysfunctional satellite cells persist, but can't undergo myogenic differentiation (Kottlors & Kirschner 2010; Bankolé et al. 2013; Dumont et al. 2015). Since we have shown that C/EBPβ expression persists in satellite cells of cachectic muscle and that these cells do not differentiate or undergo apoptosis (same phenotype as satellite cells in DMD), it is tempting to hypothesize that C/EBPβ expression may persist in satellite cells of dystrophic muscle, contributing to the defect in function.

Sarcopenia is the age associated decline in skeletal muscle mass and function (Thompson 2009). The lower regenerative potential of aged muscle correlates with a reduction in the number of Pax7-positive satellite cells (Brack et al. 2005; Collins et al. 2007; Shefer et al. 2010). Furthermore, a large proportion of satellite cells in geriatric muscle switch from reversible quiescence to a senescent state (Sousa-Victor et al. 2014). Preliminary data from our lab suggests that C/EBPβ isoforms have different roles in skeletal muscle biology; for example, overexpression of the short isoform (LIP) in C2C12 myoblasts can induce cellular senescence (Lee 2015). Interestingly, the mTOR signaling pathway is disrupted in dystrophic muscle and rapamycin has been shown to improve the dystrophic phenotype in mdx mice (Eghtesad et al. 2011; Sakuma et al. 2014). Therefore, it would be interesting to examine whether C/EBPβ isoform expression is affected in dystrophic and sarcopenic muscle.

A number of signalling pathways have been implicated in satellite cell dysfunction in aged muscle including FGF2/Sprouty1 and TGFβ/Smad signalling (Chakkalakal et al. 2012; Carlson et al. 2008). Chakkalakal and colleagues found increased fibroblast growth factor (FGF) signalling from the aged satellite cell niche and downregulation of the FGF inhibitor Sprouty1 in satellite cells led to depletion of satellite cells due to loss of

quiescence (Chakkalakal et al. 2012; Shea et al. 2010). TGFβ signalling is also higher in old muscles leading to enhanced Smad activation which in turn blocks Notch transcriptional activity (Carlson et al. 2008). We and others have shown that Smads can inhibit C/EBPβ DNA binding activity (Zauberman et al. 2001; Choy & Derynck 2003; Marchildon et al. 2010; Dingwall et al. 2011; Lamarche et al. 2015). It is possible that the high level of TGFβ signalling in aged muscle inhibits C/EBPβ activity in satellite cells. Like Sprouty1, loss of C/EBPβ may also lead to loss of quiescence and eventual depletion of satellite cells in aged muscle.

5.5. IBMX Stimulates C/EBPβ Expression and Improves Satellite Cell Expansion in Culture

IBMX has been used to pharmacologically induce C/EBPβ expression in adipocytes for many years (Goethe et al. 2007). Here, we examined the effect of IBMX treatment in primary myoblasts under growth and differentiation conditions. As in adipocytes, IBMX increased C/EBPβ expression for up to five days in culture while inhibiting differentiation and stimulating Pax7 expression. We also found that IBMX increased expression of stem cell markers that have been shown to improve myogenic progenitor cell transplantation such as *Sdc3*, *Sdc4*, *Cxcr4*, and *Abcg2* (Lala-Tabbert et al. 2016); however, these markers were examined in a candidate-based approach and are unlikely to represent all of the genes regulated by IBMX. Examining the full molecular profile of IBMX-treated muscle progenitor cells would be beneficial in understanding the complete effect of IBMX. This could lead to identification of novel pathways that regulate myogenesis and potential therapeutic targets. For example, RNA-Seq could be performed

on vehicle- and IBMX-treated wild-type and C/EBPβ$^{-/-}$ primary myoblasts. Not only would this allow us to investigate the genes being regulated by IBMX, but it will also determine what changes are C/EBPβ-dependant. This will also be important in identifying novel C/EBPβ targets that regulate satellite cell function.

IBMX-treated myoblasts gave rise to more dystrophin-positive fibers following transplantation and were better able to reconstitute the stem cell niche (Lala-Tabbert et al. 2016). However, these experiments were performed in the mdx mouse which has a relatively mild disease which cannot accurately model the more severe phenotype seen in human DMD patients (Grounds et al. 2008). Future work should be performed in a more severe animal model such as the mdx/utrn$^{+/-}$ model because they show many signs typical of DMD such as severe disease progression, premature death, increased fibrosis and reduced muscle function (Deconinck et al. 1997; Zhou et al. 2008; Gutpell et al. 2015; McDonald et al. 2015). The mdx/utrn$^{+/-}$ model can therefore allow for systemic delivery of reprogrammed myoblasts. Indeed, as muscle function and strength are important outcomes for DMD patients, future work examining systemic delivery of cells, long-term repair and muscle function is imperative. Muscle function can be assessed using a number of non-invasive tests such as forelimb grip strength and rotarod running (Aartsma-Rus & van Putten 2014).

The fundamental purpose of part of my research is to develop better culture strategies to increase the efficiency of stem cell transplantation for the treatment of DMD. It is therefore critical that all of our findings in the mouse model be validated in human myoblasts. After validating IBMX treatment on human myoblasts *in vitro*,

xenotransplantation studies must be performed to confirm the beneficial effects of culturing human myoblasts in IBMX.

5.6. Conclusions

In this study we have established the biological role of C/EBPβ in satellite cell quiescence, activation, differentiation and self-renewal; however, the mechanism by which C/EBPβ regulates these functions remains unclear. Further insight into the mechanisms underlying C/EBPβ's function in healthy and diseased muscle will be beneficial to the development of novel therapeutic approaches for the treatment of muscle atrophies such as DMD and cancer cachexia.

REFERENCES

Aartsma-Rus, A. & van Putten, M., 2014. Assessing functional performance in the mdx mouse model. *Journal of visualized experiments : JoVE*, (85), p.e51303.

Agre, P., Johnson, P.F. & McKnight, S.L., 1989. Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. *Science*, 246(4932), pp.922–6.

Akira, S. et al., 1990. A nuclear factor for IL-6 expression (NF-IL6) is member of a C/EBP family a. *The EMBO journal*, 9(6), pp.1897–1906.

Akiyama, H. et al., 2002. The transcription factor *Sox9* has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of *Sox5* and *Sox6*. *Genes & Development*, 16, pp.2813–2828.

Allikian, M.J. & McNally, E.M., 2007. Processing and assembly of the dystrophin glycoprotein complex. *Traffic*, 8, pp.177–183.

Almada, A.E. & Wagers, A.J., 2016. Molecular circuitry of stem cell fate in skeletal muscle regeneration, ageing and disease. *Nature Reviews Molecular Cell Biology*, 17(5), pp.267–279.

Angelini, C., 2007. The role of corticosteroids in muscular dystrophy: A critical appraisal. *Muscle and Nerve*, 36(4), pp.424–435.

Angelini, C. & Peterle, E., 2012. Old and new therapeutic developments in steroid treatment in Duchenne muscular dystrophy. *Acta myologica*, 31, pp.9–15.

Apponi, L.H., Corbett, A.H. & Pavlath, G.K., 2011. RNA-binding proteins and gene regulation in myogenesis. *Trends in Pharmacological Sciences*, 32(11), pp.652–658.

Arai, F. et al., 2004. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell

quiescence in the bone marrow niche. *Cell*, 118(2), pp.149–161.

Argilés, J.M. et al., Muscle Wasting in Cancer and Ageing : Cachexia Versus Sarcopenia. , pp.9–35.

Asai, A. et al., 2007. Primary role of functional ischemia, quantitative evidence for the two-hit mechanism, and phosphodiesterase-5 inhibitor therapy in mouse muscular dystrophy. *PLoS ONE*, 2(8).

Asakura, A. et al., 2007. Increased survival of muscle stem cells lacking the MyoD gene after transplantation into regenerating skeletal muscle. *Proceedings of the National Academy of Sciences of the United States of America*, 104(42), pp.16552–7.

Asakura, A., Komaki, M. & Rudnicki, M.A., 2001. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation*, 68(4–5), pp.245–253.

Azevedo, M.F. et al., 2014. Clinical and molecular genetics of the phosphodiesterases (PDEs). *Endocrine reviews*, 35(2), pp.195–233.

Bai, L. et al., 2014. Cav-1 deletion impaired hematopoietic stem cell function. *Cell death & disease*, 5(5), p.e1140.

Baker, N. & Tuan, R.S., 2013. The less-often-traveled surface of stem cells: caveolin-1 and caveolae in stem cells, tissue repair and regeneration. *Stem Cell Research & Therapy*, 4(4), pp.1–13.

Bankolé, L.C. et al., 2013. Fibre type-specific satellite cell content in two models of muscle disease. *Histopathology*, 63(6), pp.826–832.

Beauchamp, J.R. et al., 2000. Expression of CD34 and Myf5 Defines the Majority of Quiescent Adult Skeletal Muscle Satellite Cells. *The Journal of cell biology*, 151(6),

pp.1221–1233.

Beauchamp, P. et al., 2010. The cleavage of HuR interferes with its transportin-2-mediated nuclear import and promotes muscle fiber formation. *Cell death and differentiation*, 17(10), pp.1588–1599.

Bender, A.T. & Beavo, J., 2006. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. *Pharmacological reviews*, 58(3), pp.488–520.

Bentzinger, C.F. et al., 2013. Fibronectin regulates Wnt7a signaling and satellite cell expansion. *Cell Stem Cell*, 12(1), pp.75–87.

Bentzinger, C.F. et al., 2014. Wnt7a stimulates myogenic stem cell motility and engraftment resulting in improved muscle strength. *The Journal of cell biology*, 205(1), pp.97–111.

Birsoy, K., Chen, Z. & Friedman, J., 2008. Transcriptional Regulation of Adipogenesis by KLF4. *Cell Metabolism*, 7(4), pp.339–347.

Bischoff, R., 1975. Regeneration of single skeletal muscle fibers in vitro. *The Anatomical record*, 182(2), pp.215–35.

Bjornson, C.R.R. et al., 2012. Notch signaling is necessary to maintain quiescence in adult muscle stem cells. *Stem Cells*, 30(2), pp.232–42.

Blake, D.J. et al., 2002. Function and Genetics of Dystrophin and Dystrophin-Related Proteins in Muscle. *Physiological Reviews*, 82(2), pp.291–329.

Bober, E. et al., 1991. The Muscle Regulatory Gene, Myf-6, has a biphasic pattern of expression during early mouse development. *Journal of Cell Biology*, 113(6), pp.1255–65.

Borland, G. et al., 2009. Activation of protein kinase Cα by EPAC1 is required for the

ERK- and CCAAT/enhancer-binding protein β-dependent induction of the SOCS-3 gene by cyclic AMP in COS1 cells. *Journal of Biological Chemistry*, 284(26), pp.17391–403.

Boswell-Smith, V., Spina, D. & Page, C., 2006. Phosphodiesterase inhibitors. *British Journal of Pharmacology*, 147, pp.S252-57.

Brack, A.S., 2014. Pax7 is back. *Skeletal muscle*, 4(1), p.24.

Brack, A.S., Bildsoe, H. & Hughes, S.M., 2005. Evidence that satellite cell decrement contributes to preferential decline in nuclear number from large fibres during murine age-related muscle atrophy. *Journal of cell science*, 118(Pt 20), pp.4813–4821.

Bradley, M.N., Zhou, L. & Smale, S.T., 2003. C/EBPbeta regulation in lipopolysaccharide-stimulated macrophages. *Molecular and cellular biology*, 23(14), pp.4841–58.

Braun, T. et al., 1992. Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell*, 71(3), pp.369–382.

Braun, T. & Arnold, H.H., 1995. Inactivation of Myf-6 and Myf-5 genes in mice leads to alterations in skeletal muscle development. *The EMBO journal*, 14(6), pp.1176–1186.

Briggs, D. & Morgan, J.E., 2013. Recent progress in satellite cell/myoblast engraftment -- relevance for therapy. *The FEBS journal*, 280(17), pp.4281–93.

Brzoska, E. et al., 2012. Sdf-1 (CXCL12) improves skeletal muscle regeneration via the mobilisation of Cxcr4 and CD34 expressing cells. *Biology of the cell / under the auspices of the European Cell Biology Organization*, 104(12), pp.722–37.

Buck, M. et al., 2001. C/EBPbeta Phosphorylation by RSK Creates a Functional XEXD

Caspase Inhibitory Box Critical for Cell Survival. *Molecular Cell*, 8, pp.807–816.

Buck, M. & Chojkier, M., 2003. Signal transduction in the liver: C/EBPbeta modulates cell proliferation and survival. *Hepatology (Baltimore, Md.)*, 37(4), pp.731–8.

Buckingham, M. et al., 2003. The formation of skeletal muscle: from somite to limb. *Journal of Anatomy*, 202, pp.59–68.

Buckingham, M. & Relaix, F., 2015. PAX3 and PAX7 as upstream regulators of myogenesis. *Seminars in Cell and Developmental Biology*, 44, pp.115–125.

Buckingham, M. & Vincent, S.D., 2009. Distinct and dynamic myogenic populations in the vertebrate embryo. *Current Opinion in Genetics and Development*, 19(5), pp.444–453.

Bulfield, G. et al., 1984. X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proceedings of the National Academy of Sciences of the United States of America*, 81, pp.1189–1192.

Burghes, A. et al., 1987. A cDNA clone from the Duchenne/Becker muscular dystrophy gene. *Nature*, 328, pp.434–437.

Calkhoven, C.F., Mu, C. & Leutz, A., 2000. Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes and Development*, 14, pp.1920–1932.

Cao, Z., Ume, R. & McKnight, S., 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.*, 5, pp.1538–1552.

Cardinalli, B. et al., 2009. Microrna-221 and microrna-222 modulate differentiation and maturation of skeletal muscle cells. *PLoS ONE*, 4(10).

Carlson, M.E., Hsu, M. & Conboy, I.M., 2008. Imbalance between pSmad3 and Notch induces CDK inhibitors in old muscle stem cells. *Nature*, 454(7203), pp.528–532.

Cerletti, M. et al., 2013. Highly efficient, functional engraftment of skeletal muscle stem cells in dystrophic muscles. *Cell*, 134(1), pp.37–47.

Chakkalakal, J. V et al., 2012. The aged niche disrupts muscle stem cell quiescence. *Nature*, 490(7420), pp.355–60.

Chamberlain, J.S. et al., 2007. Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 21(9), pp.2195–204.

Chargé, S.B. & Rudnicki, M., 2004. Cellular and molecular regulation of muscle regeneration. *Physiological reviews*, 84(1), pp.209–38.

Chen, J.C. & Goldhamer, D.J., 1999. Transcriptional mechanisms regulating MyoD expression in the mouse. *Cell and tissue research*, 296(1), pp.213–9.

Chen, L. et al., 2014. miR-143: A Novel Regulator of MyoD Expression in Fast and Slow Muscles of Siniperca chuatsi. *Current Molecular Medicine*, 14(3), pp.370–75.

Cheung, T.H. et al., 2012. Maintenance of muscle stem-cell quiescence by microRNA-489. *Nature*, 482(7386), pp.524–528.

Cho, M.K. & Kim, S.G., 2003. Hepatocyte Growth Factor Activates CCAAT Enhancer Binding Protein and Cell Replication Via PI3-Kinase Pathway. *Hepatology*, 37(3), pp.686–95.

Choy, L. & Derynck, R., 2003. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. *Journal of Biological Chemistry*, 278(11), pp.9609–9619.

Collins, C.A. et al., 2007. A population of myogenic stem cells that survives skeletal muscle aging. *Stem Cells*, 25(4), pp.885–894.

Collins, C.A. et al., 2005. Stem Cell Function, Self-Renewal, and Behavioral Heterogeneity of Cells from the Adult Muscle Satellite Cell Niche. *Cell*, 122(2), pp.289–301.

Conboy, I.M. & Rando, T.A., 2002. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Developmental Cell*, 3(3), pp.397–409.

Cooper, C. et al., 1995. Ig/EBP (C/EBP gamma) is a transdominant negative inhibitor of C/EBP family transcriptional activators. *Nucleic acids research*, 23(21), pp.4371–7.

Cooper, R.N. et al., 1999. In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. *Journal of cell science*, 112, pp.2895–2901.

Cornelison, D.D. et al., 2001. Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration. *Developmental biology*, 239(1), pp.79–94.

Cornelison, D.D. & Wold, B.J., 1997. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Developmental biology*, 191(2), pp.270–283.

Cornelison, D.D.W. et al., 2004. Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. *Genes and Development*, 18, pp.2231–2236.

Cornelison, D.D.W. et al., 2000. MyoD−/− Satellite Cells in Single-Fiber Culture Are

Differentiation Defective and MRF4 Deficient. *Developmental Biology*, 224(2), pp.122–137.

Cotsarelis, G., Sun, T.T. & Lavker, R.M., 1990. Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*, 61(7), pp.1329–1337.

Croniger, C. et al., 1997. Role of the isoforms of CCAAT/enhancer-binding protein in the initiation of phosphoenolpyruvate carboxykinase (GTP) gene transcription at birth. *Journal of Biological Chemistry*, 272(42), pp.26306–26312.

Cuenda, A. & Cohen, P., 1999. Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. *Journal of anatomy*, 274(7), pp.4341–4346.

Dangain, J. & Vrbova, G., 1984. Muscle development in mdx mutant mice. *Muscle and Nerve*, 7, pp.700–704.

Darlington, G.J., Ross, S.E. & Macdougald, O. a, 1998. The Role of C/EBP Genes in Adipocyte Differentiation. *Journal of Biological Chemistry*, 273(46), pp.30057–30060.

Deconinck, A.E. et al., 1997. Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. *Cell*, 90(4), pp.717–727.

Déjardin, J. & Kingston, R.E., 2009. Purification of Proteins Associated with Specific Genomic Loci. *Cell*, 136, pp.175–186.

Descombes, P. & Schibler, U., 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the sam mRNA. *Cell*, 67(3), pp.569–579.

DiMario, J.X., Uzman, A. & Strohman, R.C., 1991. Fiber regeneration is not persistent in dystrophic (MDX) mouse skeletal muscle. *Developmental biology*, 148, pp.314–21.

Dingwall, M. et al., 2011. Retinoic acid-induced Smad3 expression is required for the induction of osteoblastogenesis of mesenchymal stem cells. *Differentiation*, 82(2), pp.57–65.

Doherty, K.R. et al., 2005. Normal myoblast fusion requires myoferlin. *Development*, 132(24), pp.5565–75.

Doherty, K.R. et al., 2008. The endocytic recycling protein EHD2 interacts with myoferlin to regulate myoblast fusion. *Journal of Biological Chemistry*, 283(29), pp.20252–20260.

Dumont, N.A. et al., 2015. Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division. *Nature Medicine*, 21(12), pp.1455–1463.

Eghtesad, S. et al., 2011. Rapamycin ameliorates dystrophic phenotype of mdx mouse skeletal muscle. *Molecular Medicine*, 17, pp.917–24.

Erbay, E. & Chen, J., 2001. The Mammalian Target of Rapamycin Regulates C2C12 Myogenesis via a Kinase-independent Mechanism. *Journal of Biological Chemistry*, 276(39), pp.36079–36082.

Essayan, D., 2001. Cyclic nucleotide phosphodiesterases. *Journal of Allergy and Clinical Immunology*, 108, pp.671–80.

Fan, Y. et al., 1996. Rapid Death of Injected Myoblasts in Myoblast Transfer Therapy. *Muscle and Nerve*, 19, pp.853–860.

Figueroa, A. et al., 2003. Role of HuR in skeletal myogenesis through coordinate regulation of muscle differentiation genes. *Molecular and cellular biology*, 23(14),

pp.4991–5004.

Flanigan, K.M., 2014. Duchenne and becker muscular dystrophies. *Neurologic Clinics*, 32, pp.671–688.

Fortier, M. et al., 2013. Sphingosine-1-phosphate receptor 3 influences cell cycle progression in muscle satellite cells. *Developmental Biology*, 382(2), pp.504–516.

Friedman, A.D., 2007. Transcriptional control of granulocyte and monocyte development. *Oncogene*, 26(47), pp.6816–28.

Frontera, W.R. & Ochala, J., 2015. Skeletal muscle: a brief review of structure and function. *Calcified tissue international*, 96(3), pp.183–195.

Fu, D. et al., 2015. Mdm2 Promotes Myogenesis through the Ubiquitination and Degradation of CCAAT/Enhancer Binding Protein β. *Journal of Biological Chemistry*, 290(16), pp.1–18.

Fu, X. et al., 2015. Combination of inflammation-related cytokines promotes long-term muscle stem cell expansion. *Cell research*, 25, pp.655–73.

Fuchs, E., Tumbar, T. & Guasch, G., 2004. Socializing with the neighbors: Stem cells and their niche. *Cell*, 116(6), pp.769–778.

Füchtbauer, E.M. & Westphal, H., 1992. MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. *Developmental dynamics*, 193, pp.34–39.

Fukada, S. et al., 2011. Hesr1 and Hesr3 are essential to generate undifferentiated quiescent satellite cells and to maintain satellite cell numbers. *Development*, 138(21), pp.4609–4619.

Fukada, S. et al., 2007. Molecular signature of quiescent satellite cells in adult skeletal muscle. *Stem Cells*, 25(10), pp.2448–59.

Gao, Q.Q. & McNally, E.M., 2015. The Dystrophin Complex: Structure, Function, and Implications for Therapy. *Comprehensive Physiology*, 5, pp.1223–1239.

Gayraud-Morel, B. et al., 2007. A role for the myogenic determination gene Myf5 in adult regenerative myogenesis. *Developmental Biology*, 312(1), pp.13–28.

Ge, Y. et al., 2009. mTOR regulates skeletal muscle regeneration in vivo through kinase-dependent and kinase-independent mechanisms. *Am J Physiol Cell Physiol*, 297(3), pp.1434–1444.

Gerard, C. et al., 2012. Fibrin Gel Improves the Survival of Transplanted Myoblasts. *Cell transplantation*, 21, pp.127–137.

Van Der Giessen, K. et al., 2003. RNAi-mediated HuR Depletion Leads to the Inhibition of Muscle Cell Differentiation. *Journal of Biological Chemistry*, 278(47), pp.47119–47128.

Giresi, P.G. et al., 2005. Identification of a molecular signature of sarcopenia. *Physiological genomics*, 21(2), pp.253–63.

Gnocchi, V.F. et al., 2009. Further characterisation of the molecular signature of quiescent and activated mouse muscle satellite cells. *PloS one*, 4(4), p.e5205.

Goethe, R., Basler, T. & Phi-van, L., 2007. Regulation of C/EBPbeta mRNA expression and C/EBPbeta promoter activity by protein kinases A and C in a myelomonocytic cell line (HD11). *Inflammation research*, 56(7), pp.274–81.

Goetsch, K.P. & Niesler, C.U., 2011. Optimization of the scratch assay for in vitro skeletal muscle wound healing analysis. *Analytical Biochemistry*, 411(1), pp.158–160.

Greenbaum, L.E. et al., 1998. CCAAT enhancer- binding protein beta is required for

normal hepatocyte proliferation in mice after partial hepatectomy. *The Journal of clinical investigation*, 102(5), pp.996–1007.

Grimm, S. & Rosen, J., 2003. The role of C/EBPbeta in mammary gland development and breast cancer. *Neoplasia*, 8, pp.191–204.

Gros, J. et al., 2005. A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature*, 435(7044), pp.954–958.

Gross, J.G. & Morgan, J.E., 1999. Muscle precursor cells injected into irradiated mdx mouse muscle persist after serial injury. *Muscle and Nerve*, 22, pp.174–185.

Grounds, M. et al., 1992. Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell and tissue research*, 267, pp.99–104.

Grounds, M.D. et al., 2008. Towards developing standard operating procedures for pre-clinical testing in the mdx mouse model of Duchenne muscular dystrophy. *Neurobiology of Disease*, 31, pp.1–19.

Gundry, R.L. et al., 2009. Preparation of proteins and peptides for mass spectrometry analysis in a bottom-up proteomics workflow. *Current protocols in molecular biology*, 88, pp.342–355.

Günther, S. et al., 2013. Myf5-Positive Satellite Cells Contribute to Pax7-Dependent Long-Term Maintenance of Adult Muscle Stem Cells. *Cell Stem Cell*, 13, pp.590–601.

Gutierrez, S. et al., 2002. CCAAT/enhancer-binding proteins (C/EBP) beta and delta activate osteocalcin gene transcription and synergize with Runx2 at the C/EBP element to regulate bone-specific expression. *Journal of Biological Chemistry*, 277(2), pp.1316–1323.

Gutpell, K.M., Hrinivich, W.T. & Hoffman, L.M., 2015. Skeletal Muscle Fibrosis in the mdx/utrn+/- Mouse Validates Its Suitability as a Murine Model of Duchenne Muscular Dystrophy. *Plos One*, 10, p.e0117306.

Guttridge, D.C. et al., 2000. NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. *Science*, 289, pp.2363–2366.

Haldar, M. et al., 2008. Two cell lineages, myf5 and myf5-independent, participate in mouse skeletal myogenesis. *Developmental cell*, 14(3), pp.437–445.

Halevy, O. et al., 2004. Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. *Developmental Dynamics*, 231(3), pp.489–502.

Hasty, P. et al., 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature*, 364(6437), pp.501–506.

Head, B.P. et al., 2010. Loss of caveolin-1 accelerates neurodegeneration and aging. *PLoS ONE*, 5(12), p.e15697.

Heslop, L. et al., 2001. Transplanted primary neonatal myoblasts can give rise to functional satellite cells as identified using the Myf5nlacZl+ mouse. *Gene therapy*, 8, pp.778–783.

Hinterberger, T.J. et al., 1991. Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Developmental Biology*, 147, pp.144–156.

Hirata, M. et al., 2009. C/EBPbeta Promotes transition from proliferation to hypertrophic differentiation of chondrocytes through transactivation of p57. *PloS one*, 4(2), p.e4543.

Hoffman, E.P., Brown, R.H. & Kunkel, L.M., 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell*, 51, pp.919–28.

Hu, J. et al., 2001. ERK1 and ERK2 activate CCAAAT/enhancer-binding protein-β-dependent gene transcription in response to interferon-γ. *Journal of Biological Chemistry*, 276(1), pp.287–297.

Huard, J. et al., 1994. Gene Transfer into Skeletal Muscles by Isogenic Myoblasts. *Human Gene Therapy*, 5, pp.949–958.

Jasmin, J.F. et al., 2009. Genetic ablation of caveolin-1 increases neural stem cell proliferation in the subventricular zone (SVZ) of the adult mouse brain. *Cell Cycle*, 8(23), pp.3978–3983.

Johnson, P.F., 2005. Molecular stop signs : regulation of cell-cycle arrest by C/EBP transcription factors. *Journal of cell science*, 118(12), pp.2545–2555.

Jones, N.C. et al., 2001. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. *Journal of Cellular Physiology*, 186(1), pp.104–115.

Kassar-Duchossoy, L. et al., 2004. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature*, 431, pp.466–471.

Kassar-duchossoy, L. et al., 2005. Pax3 / Pax7 mark a novel population of primitive myogenic cells during development. *Genes & Development*, 19, pp.1426–1431.

Kent, W. et al., 2002. The human genome browser at UCSC. *Genome Research*, 12(6), pp.996–1006.

Kitzmann, M. et al., 1998. The muscle regulatory factors MyoD and myf-5 undergo distinct cell cycle-specific expression in muscle cells. *The Journal of cell biology*,

142(6), pp.1447–59.

Knapp, J.R. et al., 2006. Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size. *Development*, 133, pp.601–610.

Kobayashi, Y.M. et al., 2008. Sarcolemma-localized nNOS is required to maintain activity after mild exercise. *Nature*, 456(7221), pp.511–515.

Komori, T., 2002. Runx2, a multifunctional transcription factor in skeletal development. *Journal of Cellular Biochemistry*, 87, pp.1–8.

Kottlors, M. & Kirschner, J., 2010. Elevated satellite cell number in Duchenne muscular dystrophy. *Cell and Tissue Research*, 340(3), pp.541–548.

Kuang, S. et al., 2007. Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. *Cell*, 129(5), pp.999–1010.

Kuang, S. et al., 2006. Distinct roles for Pax7 and Pax3 in adult regenerative myogenesis. *Journal of Cell Biology*, 172(1), pp.103–113.

Kuang, S. & Rudnicki, M.A., 2008. The emerging biology of satellite cells and their therapeutic potential. *Trends in Molecular Medicine*, 14(2), pp.82–91.

Kunkel, L.M. et al., 1986. Analysis of deletions in DNA from patients with Becker and Duchenne muscular dystrophy. *Nature*, 322, pp.73–77.

Lagirand-cantaloube, J. et al., 2009. Inhibition of Atrogin-1 / MAFbx Mediated MyoD Proteolysis Prevents Skeletal Muscle Atrophy In Vivo. *PloS one*, 4(3), p.e4973.

Lala-Tabbert, N., Fu, D. & Wiper-Bergeron, N., 2016. Induction of C/EBPβ Expression with the Phosphodiesterase Inhibitor Isobutylmethylxanthine Improves Myoblast Engraftment into Dystrophic Muscle. *Stem Cells Translational Medicine*, 5(4), pp.500–10.

LaMarca, H.L.H. et al., 2010. CCAAT/Enhancer Binding Protein Beta Regulates Stem Cell Activity and Specifies Luminal Cell Fate in the Mammary Gland. *Stem cells*, 28(3), pp.535–544.

Lamarche, É. et al., 2015. Retinoic acid promotes myogenesis in myoblasts by antagonizing transforming growth factor-beta signaling via C/EBPβ. *Skeletal Muscle*, 5(1), p.8.

Landschulz, W.H., Johnson, P.F. & Mcknight, S.L., 1989. The DNA Binding Domain of the Rat Liver Nuclear Protein C/EBP Is Bipartite. *Science*, 3246, pp.1681–8.

Lang, D. et al., 2007. PAX genes: Roles in development, pathophysiology, and cancer. *Biochemical Pharmacology*, 73, pp.1–14.

Lang, K.C. et al., 2009. Simultaneous overexpression of Oct4 and Nanog abrogates terminal myogenesis. *American journal of physiology. Cell physiology*, 297(1), pp.C43-54.

Laumonier, T. et al., 2013. Low molecular weight dextran sulfate binds to human myoblasts and improves their survival after transplantation in mice. *Cell transplantation*, 22(7), pp.1213–26.

Lechner, S. et al., 2013. Role of C/EBPβ-LAP and C/EBPβ-LIP in early adipogenic differentiation of human white adipose-derived progenitors and at later stages in immature adipocytes. *Differentiation; research in biological diversity*, 85(1–2), pp.20–31.

Lee, H., 2015. *The regulation of satellite cell function and myogenesis by isoforms of C/EBPβ*.

Lepper, C., Conway, S.J. & Fan, C.-M., 2009. Adult satellite cells and embryonic muscle

progenitors have distinct genetic requirements. *Nature*, 460(7255), pp.627–31.

Lepper, C. & Fan, C.M., 2010. Inducible lineage tracing of Pax7-descendant cells reveals embryonic origin of adult satellite cells. *Genesis*, 48, pp.424–436.

Lepper, C., Partridge, T.A. & Fan, C.M., 2011. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development*, 138(17), pp.3639–3646.

Lesault, P.-F. et al., 2012. Macrophages improve survival, proliferation and migration of engrafted myogenic precursor cells into MDX skeletal muscle. *PloS one*, 7(10), p.e46698.

Li, J. et al., 2014. EGF-induced C/EBPβ participates in EMT by decreasing the expression of miR-203 in esophageal squamous cell carcinoma cells. *Journal of cell science*, 127, pp.3735–44.

Li, J. et al., 2005. Loss of caveolin-1 causes the hyper-proliferation of intestinal crypt stem cells, with increased sensitivity to whole body γ-radiation. *Cell Cycle*, 4(12), pp.1817–1825.

Li, L. & Clevers, H., 2010. Coexistence of quiescent and active adult stem cells in mammals. *Science (New York, N.Y.)*, 327(5965), pp.542–5.

Li, Y. & Reid, M.B., 2000. NF- κB mediates the protein loss induced by TNF- α in differentiated skeletal muscle myotubes. *American journal of physiology*, 279, pp.R1165–R1170.

Liu, S. et al., 1999. Hypoglycemia and impaired hepatic glucose production in mice with a deletion of the C/EBPbeta gene. *The Journal of clinical investigation*, 103(2), pp.207–13.

Liu, W. et al., 2012. Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. *Development*, 139(16), pp.2857–2865.

Liu, Y. et al., 2010. Cooperation between myogenic regulatory factors and SIX family transcription factors is important for myoblast differentiation. *Nucleic Acids Research*, 38(20), pp.6857–6871.

Liu, Y.Y.Y.Y. et al., 2013. Protein Inhibitor of Activated STAT 1 (PIAS1) Is Identified as the SUMO E3 Ligase of CCAAT/Enhancer-Binding Protein β (C/EBPβ) during Adipogenesis. *Molecular and cellular biology*, 33(22), pp.4606–17.

von Maltzahn, J. et al., 2013. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. *Proceedings of the National Academy of Sciences*, 110(41), pp.16474–16479.

Marchildon, F. et al., 2012. CCAAT/enhancer binding protein beta is expressed in satellite cells and controls myogenesis. *Stem cells*, 30(12), pp.2619–30.

Marchildon, F. et al., 2016. CCAAT/enhancer binding protein beta protects muscle satellite cells from apoptosis after injury and in cancer cachexia. *Cell death & disease*, 7, p.e2109.

Marchildon, F. et al., 2015. Expression of CCAAT/enhancer binding protein beta in muscle satellite cells inhibits myogenesis in cancer cachexia. *PLoS ONE*, 10(12), pp.1–16.

Marchildon, F. et al., 2010. Transcription factor Smad3 is required for the inhibition of adipogenesis by retinoic acid. *Journal of Biological Chemistry*, 285(17), pp.13274–13284.

Maurice, D.H. et al., 2014. Advances in targeting cyclic nucleotide phosphodiesterases.

Nature reviews. Drug discovery, 13(4), pp.290–314.

Mauro, A., 1961. Satellite Cell of Skeletal Muscle Fibers. *The Journal of Cell Biology*, 9(2), pp.493–495.

McCarthy, J.J. et al., 2011. Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. *Development*, 138, pp.3657–3666.

McCullagh, K.J. a & Perlingeiro, R.C.R., 2014. Coaxing stem cells for skeletal muscle repair. *Advanced drug delivery reviews*.

McDonald, A.A. et al., 2015. Disease course in mdx:utrophin+/- mice: comparison of three mouse models of Duchenne muscular dystrophy. *Physiological reports*, 3(4), p.e12391.

McFarlane, C. et al., 2008. Myostatin signals through Pax7 to regulate satellite cell self-renewal. *Experimental cell research*, 314, pp.317–29.

McKinnell, I.W. et al., 2008. Pax7 activates myogenic genes by recruitment of a histone methyltransferase complex. *Nature Cell Biology*, 10(1), pp.77–84.

Meadows, E. et al., 2008. Myogenin regulates a distinct genetic program in adult muscle stem cells. *Developmental Biology*, 322(2), pp.406–414.

Meadows, E., Flynn, J.M. & Klein, W.H., 2011. Myogenin regulates exercise capacity but is dispensable for skeletal muscle regeneration in adult mdx mice. *PLoS ONE*, 6(1).

Megeney, L. et al., 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes & Development*, 10, pp.1173–1183.

Megeney, L.A. & Rudnicki, M.A., 1995. Determination versus differentation and the MyoD family of transcription factors. *Biochem Cell Biol*, 73, pp.723–732.

Mendell, J.R. et al., 2012. Evidence-based path to newborn screening for duchenne muscular dystrophy. *Annals of Neurology*, 71, pp.304–313.

Mendell, J.R. et al., 1995. Myoblast transfer in the treatment of Duchenne's muscular dystrophy. *The New England journal of medicine*, 333, pp.832–838.

Miller, R.G. et al., 1997. Myoblast implantation in Duchenne muscular dystrophy: The San Francisco study. *Muscle and Nerve*, 20, pp.469–478.

Millward, C.A. et al., 2007. Mice with a deletion in the gene for CCAAT/enhancer-binding protein beta are protected against diet-induced obesity. *Diabetes*, 56, pp.161–167.

Mittler, G., Butter, F. & Mann, M., 2009. A SILAC-based DNA protein interaction screen that identifies candidate binding proteins to functional DNA elements A SILAC-based DNA protein interaction screen that identifies candidate binding proteins to functional DNA elements. *Genome Research*, 19, pp.284–293.

Monaco, A.P. et al., 1988. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics*, 2(1), pp.90–95.

Montarras, D. et al., 2000. Cultured myf5 null and myoD null muscle precursor cells display distinct growth defects. *Biology of the Cell*, 92, pp.565–572.

Montarras, D., Morgan, J., Collins, C., Relaix, F., et al., 2005. Direct Isolation of Satellite Cells for Skeletal Muscle Regeneration. *Science*, 309(5743), pp.2064–2067.

Montarras, D., Morgan, J., Collins, C., Relaix, F., et al., 2005. Direct isolation of satellite cells for skeletal muscle regeneration. *Science*, 309(5743), pp.2064–2067.

Motohashi, N. & Asakura, A., 2014. Muscle satellite cell heterogeneity and self-renewal. *Frontiers in cell and developmental biology*, 2, pp.1–11.

Mourikis, P. et al., 2012. A Critical Requirement for Notch Signaling in Maintenance of the Quiescent Skeletal Muscle Stem Cell State. *Stem Cells*, 30(2), pp.243–252.

Mukai, A. & Hashimoto, N., 2008. Localized cyclic AMP-dependent protein kinase activity is required for myogenic cell fusion. *Experimental cell research*, 314(2), pp.387–97.

Murphy, M. & Kardon, G., 2011. *Origin of vertebrate limb muscle: The role of progenitor and myoblast populations*,

Murphy, M.M. et al., 2011. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development*, 138, pp.3625–3637.

Nabeshima, Y. et al., 1993. Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature Letters*, 365, pp.532–35.

Nakajima, T. et al., 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Pnas*, 90(6), pp.2207–11.

Nakashima, K. et al., 2002. The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. *Cell*, 108(1), pp.17–29.

Nieder, C. et al., 2005. The role of pentoxifylline as a modifier of radiation therapy. *Cancer treatment reviews*, 31(6), pp.448–55.

Niehof, M., Manns, M.P. & Trautwein, C., 1997. CREB controls LAP/C/EBP beta transcription. *Molecular and cellular biology*, 17(7), pp.3600–3613.

Niehof, M., Manns, M.P. & Trautwein, C., 1997. CREB Controls LAP/C/EBPbeta Transcription. *Molecular and cellular biology*, 17(7), pp.3600–3613.

Nishijo, K. et al., 2009. Biomarker system for studying muscle, stem cells, and cancer in

vivo. *FASEB journal*, 23(8), pp.2681–90.

Okazaki, K. et al., 2002. CCAAT/enhancer-binding proteins beta and delta mediate the repression of gene transcription of cartilage-derived retinoic acid-sensitive protein induced by interleukin-1beta. *Journal of Biological Chemistry*, 277(35), pp.31526–31533.

Olguin, H.C. et al., 2007. Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. *Journal of Cell Biology*, 177(5), pp.769–779.

Olguin, H.C. & Olwin, B.B., 2004. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. *Developmental biology*, 275(2), pp.375–88.

Olson, E.. et al., 1996. Know Your Neighbors: Three Phenotypes in Null Mutants of the Myogenic bHLH Gene MRF4. *Cell*, 85(1), pp.1–4.

Omori, K. & Kotera, J., 2007. Overview of PDEs and their regulation. *Circulation Research*, 100(3), pp.309–327.

Orford, K.W. & Scadden, D.T., 2008. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet*, 9(2), pp.115–128.

Osada, S. et al., 1996. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *Journal of Biological Chemistry*, 271(7), pp.3891–3896.

Ott, M.O. et al., 1991. Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. *Development*, 111(4), pp.1097–1107.

Oustanina, S., Hause, G. & Braun, T., 2004. Pax7 directs postnatal renewal and

propagation of myogenic satellite cells but not their specification. *Embo J*, 23(16), pp.3430–3439.

Padilla-Benavides, T., Nasipak, B.T. & Imbalzano, A.N., 2015. Brg1 Controls the Expression of Pax7 to Promote Viability and Proliferation of Mouse Primary Myoblasts. *Journal of cellular physiology*, 230(12), pp.29990–7.

Pallafacchina, G. et al., 2010. An adult tissue-specific stem cell in its niche: A gene profiling analysis of in vivo quiescent and activated muscle satellite cells. *Stem Cell Research*, 4(2), pp.77–91.

Papadimitriou, J. et al., 1990. The Process of New Plasmalemma Formation in Focallyinjured Skeletal Muscle Fibers. *Journal of Structural Biology*, 103(2), pp.124–134.

Park, B.-H. et al., 2013. An isoform of C/EBPβ, LIP, regulates expression of the chemokine receptor CXCR4 and modulates breast cancer cell migration. *The Journal of biological chemistry*, 288(40), pp.28656–67.

Parker, M.H. et al., 2012. Activation of notch signaling during ex vivo expansion maintains donor muscle cell engraftment. *Stem Cells*, 30(10), pp.2212–2220.

Pastoret, C. & Sebille, A., 1995. Age-related differences in regeneration of dystrophic (mdx) and normal muscle in the mouse. *Muscle and Nerve*, 18(10), pp.1147–1154.

Pastoret, C. & Sebille, A., 1995. Mdx Mice Show Progressive Weakness and Muscle Deterioration With Age. *Journal of the Neurological Sciences*, 129, pp.97–105.

Pasut, A., Jones, A.E. & Rudnicki, M. a, 2013. Isolation and culture of individual myofibers and their satellite cells from adult skeletal muscle. *Journal of visualized experiments : JoVE*, (73), p.e50074.

Patapoutian, A. et al., 1995. Disruption of the mouse MRF4 gene identifies multiple waves of myogenesis in the myotome. *Development*, 121, pp.3347–58.

Penner, G. et al., 2011. C/EBP DNA-binding activity is upregulated by a glucocorticoid-dependent mechanism in septic muscle. *American journal of physiology. Regulatory, integrative and comparative physiology*, 282, pp.R439-44.

Perez, A.L. et al., 2010. CXCR4 enhances engraftment of muscle progenitor cells. *Muscle and Nerve*, 40(4), pp.562–572.

Pescatori, M. et al., 2007. Gene expression profiling in the early phases of DMD: a constant molecular signature characterizes DMD muscle from early postnatal life throughout disease progression. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 21(4), pp.1210–26.

Petrof, B.J., 2002. Molecular pathophysiology of myofiber injury in deficiencies of the dystrophin-glycoprotein complex. *American journal of physical medicine & rehabilitation*, 81, pp.S162-74.

Pisconti, A. et al., 2010. Syndecan-3 and Notch cooperate in regulating adult myogenesis. *Journal of Cell Biology*, 190(3), pp.427–441.

Piwien-Pilipuk, G., MacDougald, O. & Schwartz, J., 2002. Dual regulation of phosphorylation and dephosphorylation of C/EBPbeta modulate its transcriptional activation and DNA binding in response to growth hormone. *Journal of Biological Chemistry*, 277(46), pp.44557–44565.

Poli, V., Mancini, F.P. & Cortese, R., 1990. IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell*, 63, pp.643–653.

Price, F., Kuroda, K. & Rudnicki, M., 2007. Stem cell based therapies to treat muscular dystrophy. *Biochimica et biophysica acta*, 1772(2), pp.272–83.

Pulido-Salgado, M., Vidal-Taboada, J.M. & Saura, J., 2015. *C/EBPβ and C/EBPδ transcription factors: Basic biology and roles in the CNS*,

Rahimi, R. et al., 2010. A review of the herbal phosphodiesterase inhibitors; future perspective of new drugs. *Cytokine*, 49, pp.123–9.

Rahimov, F. & Kunkel, L.M., 2013. The cell biology of disease: cellular and molecular mechanisms underlying muscular dystrophy. *The Journal of cell biology*, 201(4), pp.499–510.

Ramji, D. & Foka, P., 2002. CCAAT/enhancer-binding proteins : structure , function and regulation. *Biochemical Journal*, 365, pp.561–575.

Rando, T.A., 2001. The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. *Muscle & nerve*, 24, pp.1575–1594.

Ratajczak, M. et al., 2003. Expression of functional CXCR4 by muscle satellite cells and secretion of SDF-1 by muscle-derived fibroblasts is associated with the presence of both muscle progenitors in bone marrow and hematopoietic stem/progenitor cells in muscle. *Stem Cells*, 21, pp.363–371.

Ray PN, Belfall B, Duff C, Logan C, Vanora K, Thompson MW, Sylvester JL, Gorski JL, S.R. and W.R., 1985. Cloning of the Breakpoint of an X;21 Translocation Associated with Duchenne Muscular Dystrophy. *Nature*, 318, pp.672–675.

Razani, B. et al., 2001. Caveolin-1 Null Mice Are Viable but Show Evidence of Hyperproliferative and Vascular Abnormalities. *Journal of Biological Chemistry*,

276(41), pp.38121–38138.

Relaix, F. et al., 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature*, 435(7044), pp.948–53.

Relaix, F. et al., 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *Journal of Cell Biology*, 172(1), pp.91–102.

Relaix, F. et al., 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol*, 172(1), pp.91–102.

Rendell, M. & Bamisedun, O., 1992. Skin Blood Flow and Current Perception in Pentoxifylline-Treated Diabetic Neuropathy. *Angiology*, 43(10), pp.843–851.

Robertson, T. et al., 1990. Fusion between myogenic cells in Vivo: An ultrastructural study in regenerating murine skeletal muscle. *Journal of Structural Biology*, 105, pp.170–182.

Robertson, T., Papadimitriou, J. & Grounds, M., 1993. Fusion of myogenic cells to the newly sealed region of damaged myofibres in skeletal muscle regeneration. *Neuropathology and applied neurobiology*, 19(4), pp.350–358.

Robinson, G.W. et al., 1998. The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes & development*, 12(12), pp.1907–16.

Von Roretz, C. et al., 2011. HuR and myogenesis: Being in the right place at the right time. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1813, pp.1663–1667.

Rosen, E.D. et al., 2000. Transcriptional regulation of adipogenesis Transcriptional regulation of adipogenesis. *Genes & Development*, 14, pp.1293–1307.

Rudnicki, M.A. et al., 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell*, 71, pp.383–390.

Rudnicki, M.A. et al., 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell*, 75(7), pp.1351–1359.

Ruffell, D. et al., 2009. A CREB-C/EBPbeta cascade induces M2 macrophage- specific gene expression and promotes muscle injury repair. *Proceedings of the National Academy of Sciences*, 106(41), pp.17475–80.

Ryder-Cook, A.S. et al., 1988. Localization of the mdx mutation within the mouse dystrophin gene. *The EMBO journal*, 7(10), pp.3017–21.

Sabourin, L. et al., 1999. Reduced differentiation potential of primary MyoD-/-myogenic cells derived from adult skeletal muscle. *Cell*, 144(4), pp.631–643.

Sacco, A. et al., 2008. Self-renewal and expansion of single transplanted muscle stem cells. *Nature*, 456, pp.502–506.

Sakuma, K., Aoi, W. & Yamaguchi, A., 2014. The intriguing regulators of muscle mass in sarcopenia and muscular dystrophy. *Frontiers in Aging Neuroscience*, 6(AUG), pp.1–17.

Salotti, J. et al., 2015. An Arf-Egr-C/EBPβ Pathway Linked to Ras-Induced Senescence and Cancer. *Molecular and Cellular Biology*, 35(5), pp.866–83.

Sambasivan, R. et al., 2011. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. *Development*, 138, pp.3647–3656.

Schaaf, G. et al., 2012. Ex-vivo Expansion of Muscle-Regenerative Cells for the Treatment of Muscle Disorders. *Journal of Stem Cell Research & Therapy*, S11,

pp.1–15.

Schienda, J. et al., 2006. Somitic origin of limb muscle satellite and side population cells. *Proceedings of the National Academy of Sciences of the United States of America*, 103(4), pp.945–950.

Schmalbruch, H. & Hellhammer, U., 1976. The number of satellite cells in normal human muscle. *The Anatomical record*, 185(3), pp.279–87.

Schrauwen, I. et al., 2015. A frame-shift mutation in CAV1 is associated with a severe neonatal progeroid and lipodystrophy syndrome. *PLoS ONE*, 10(7), p.e0131797.

Schultz, E., Gibson, M.C. & Champion, T., 1978. Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. *Journal of Experimental Zoology*, 206(3), pp.451–6.

Schultz, E., Jaryszak, D.L. & Valliere, C.R., 1985. Response of satellite cells to focal skeletal muscle injury. *Muscle & nerve*, 8, pp.217–222.

Screpanti, I. et al., 1995. Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. *The EMBO journal*, 14(9), pp.1932–1941.

Seale, P. et al., 2004. Pax7 Is Necessary and Sufficient for the Myogenic Specification of CD45+: Sca1+ Stem Cells from Injured Muscle. *PLoS biology*, 2(5), pp.664–672.

Seale, P. et al., 2000. Pax7 Is Required for the Specification of Myogenic Satellite Cells. *Cell*, 102, pp.777–786.

Sebastian, T. & Johnson, P.F., 2006. Stop and Go: Anti-Proliferative and Mitogenic Functions of the Transcription Factor C/EBPbeta. *Cell Cycle*, 5, pp.953–957.

Shea, K.L. et al., 2010. Sprouty1 Regulates Reversible Quiescence of a Self-Renewing Adult Muscle Stem Cell Pool during Regeneration. *Cell Stem Cell*, 6, pp.117–129.

Shefer, G. et al., 2010. Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. *PLoS ONE*, 5(10), p.e13307.

Shefer, G., Wleklinski-Lee, M. & Yablonka-Reuveni, Z., 2004. Skeletal muscle satellite cells can spontaneously enter an alternative mesenchymal pathway. *Journal of cell science*, 117(Pt 22), pp.5393–404.

Shen, B., Chang, C. & Lee, H., 1997. Transcriptional induction of the agp/ebp (c/ebp beta) gene by hepatocyte growth factor. *DNA Cell Biology*, 16, pp.703–11.

Sherwood, R.I. et al., 2004. Isolation of adult mouse myogenic progenitors: Functional heterogeneity of cells within and engrafting skeletal muscle. *Cell*, 119(4), pp.543–554.

Sicinski, P. et al., 2016. The Molecular Basis of Muscular Dystrophy in the mdx Mouse : A Point Mutation. *Science*, 244(4912), pp.1578–1580.

Siegel, A.L., Kuhlmann, P.K. & Cornelison, D., 2011. Muscle satellite cell proliferation and association: new insights from myofiber time-lapse imaging. *Skeletal muscle*, 1(1), p.7.

Sienkiewicz, D. et al., 2015. Duchenne muscular dystrophy: Current cell therapies. *Therapeutic Advances in Neurological Disorders*, 8(4), pp.166–177.

Skuk, D. et al., 2004. Dystrophin expression in myofibers of Duchenne muscular dystrophy patients following intramuscular injections of normal myogenic cells. *Molecular Therapy*, 9(3), pp.475–482.

Smink, J.J. et al., 2009. Transcription factor C/EBPbeta isoform ratio regulates osteoclastogenesis through MafB. *The EMBO journal*, 28, pp.1769–81.

Smink, J.J. & Leutz, A., 2012. Instruction of mesenchymal cell fate by the transcription

factor C/EBPβ. *Gene*, 497, pp.10–17.

Smith, C.K., Janney, M.J. & Allen, R.E., 1994. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. *Journal of cellular physiology*, 159, pp.379–385.

Sotgia, F. et al., 2005. Caveolin-1-deficient mice have an increased mammary stem cell population with upregulation of Wnt/beta-catenin signaling. *Cell Cycle*, 4(12), pp.1808–1816.

Sousa-Victor, P. et al., 2014. Geriatric muscle stem cells switch reversible quiescence into senescence. *Nature*, 506(7488), pp.316–21.

Steinhilber, J. et al., 2015. Next-generation sequencing identifies deregulation of micrornas involved in both innate and adaptive immune response in ALK+ ALCL. *PLoS ONE*, 10(2), pp.1–20.

Sterneck, E. et al., 2006. Conditional ablation of C/EBPβ demonstrates its keratinocyte-specific requirement for cell survival and mouse skin tumorigenesis. *Oncogene*, 25(8), pp.1272–1276.

Sterneck, E., Tessarollo, L. & Johnson, P.F., 1997. An essential role for C/EBPβ in female reproduction. *Genes Dev*, 11, pp.2153–2162.

Summerbell, D., Halai, C. & Rigby, P.W.J., 2002. Expression of the myogenic regulatory factor Mrf4 precedes or is contemporaneous with that of Myf5 in the somitic bud. *Mechanisms of Development*, 117, pp.331–335.

Sunadome, K. et al., 2011. ERK5 Regulates Muscle Cell Fusion through Klf Transcription Factors. *Developmental Cell*, 20, pp.192–205.

Suzuki, K. et al., 2000. Heat Shock Treatment Enhances Graft Cell Survival in Skeletal

Myoblast Transplantation to the Heart. *Circulation*, 102, pp.216–221.

Tajbakhsh, S., 2009. Skeletal muscle stem cells in developmental versus regenerative myogenesis. *Journal of Internal Medicine*, 266(4), pp.372–389.

Tajbakhsh, S., Rocancourt, D. & Buckingham, M., 1996. Muscle progenitor cells failing to respond to positional cues adopt non-myogenic fates in myf-5 null mice. *Nature*, 384(6606), pp.266–70.

Tan, S.-B. et al., 2014. Small Molecule Inhibitor of Myogenic microRNAs Leads to a Discovery of miR-221/222-myoD-myomiRs Regulatory Pathway. *Chemistry & Biology*, 21, pp.1265–1270.

Tanaka, K.K. et al., 2009. Syndecan-4-expressing muscle progenitor cells in the SP engraft as satellite cells during muscle regeneration. *Cell stem cell*, 4(3), pp.217–25.

Tanaka, T. et al., 1997. Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. *The EMBO journal*, 16(24), pp.7432–43.

Tang, J.G. & Koeffler, H.P., 2001. Structural and functional studies of CCAAT/enhancer-binding protein epsilon. *Journal of Biological Chemistry*, 276(21), pp.17739–17746.

Tang, Q.-Q., Otto, T.C. & Lane, M.D., 2003. CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 100(3), pp.850–855.

Tapscott, S., 2005. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development*, 132(12), pp.2685–2695.

Tedesco, F.S. et al., 2010. Review series Repairing skeletal muscle : regenerative potential of skeletal muscle stem cells. *Journal of Clinical Investigation*, 120(1),

pp.11–19.

Thompson, L. V., 2009. Age-related muscle dysfunction. *Experimental Gerontology*, 44, pp.106–111.

Tidball, J.G. & Villalta, S.A., 2010. Regulatory interactions between muscle and the immune system during muscle regeneration. *American journal of physiology*, 298(5), pp.R1173-87.

Tilley, S., 2011. Methylxanthines in Asthma. *Handbook of Experimental Pharmacology*, 200, pp.439–456.

Tintignac, L.A. et al., 2005. Degradation of MyoD mediated by the SCF (MAFbx) ubiquitin ligase. *Journal of Biological Chemistry*, 280(4), pp.2847–2856.

Tominaga, H. et al., 2008. CCAAT/Enhancer-binding Protein beta Promotes Osteoblast Differentiation by Enhancing Runx2 Activity with ATF4. *Molecular Biology of the Cell*, 19, pp.5373–86.

Trautwein, C. et al., 1994. Protein kinase A and C site-specific phosphorylations of LAP (NF-IL6) modulate its binding affinity to DNA recognition elements. *Journal of Clinical Investigation*, 93, pp.2554–2561.

Troy, A. et al., 2012. Coordination of satellite cell activation and self-renewal by Par-complex-dependent asymmetric activation of p38α/β MAPK. *Cell stem cell*, 11(4), pp.541–53.

Tsukada, J. et al., 2011. The CCAAT/enhancer (C/EBP) family of basic-leucine zipper (bZIP) transcription factors is a multifaceted highly-regulated system for gene regulation. *Cytokine*, 54, pp.6–19.

Urciuolo, A. et al., 2013. Collagen VI regulates satellite cell self-renewal and muscle

regeneration. *Nature communications*, 4(May), p.1964.

Ustanina, S. et al., 2007. The myogenic factor Myf5 supports efficient skeletal muscle regeneration by enabling transient myoblast amplification. *Stem cells*, 25, pp.2006–16.

Venuti, J.M. et al., 1995. Myogenin is required for late but not early aspects of myogenesis during mouse development. *Journal of Cell Biology*, 128(4), pp.563–576.

Volonte, D., Liu, Y. & Galbiati, F., 2005. The modulation of caveolin-1 expression controls satellite cell activation during muscle repair. *The FASEB journal*, 19(2), pp.237–239.

Wang, L. et al., 2000. Increased insulin receptor substrate-1 and enhanced skeletal muscle insulin sensitivity in mice lacking CCAAT/enhancer-binding protein Beta. *Journal of Biological Chemistry*, 275(19), pp.14173–14181.

Wang, Y.X. & Rudnicki, M.A., 2012. Satellite cells, the engines of muscle repair. *Nature Reviews*, 13(2), pp.127–133.

Wang, Z. et al., 2014. An in vitro culture system that supports robust expansion and maintenance of in vivo engraftment capabilities for myogenic progenitor cells from adult mice. *BioResearch open access*, 3(3), pp.79–87.

Weintraub, H. et al., 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. *Proceedings of the National Academy of Sciences of the United States of America*, 86, pp.5434–8.

Wen, Y. et al., 2012. Constitutive Notch Activation Upregulates Pax7 and Promotes the Self-Renewal of Skeletal Muscle Satellite Cells. *Molecular and Cellular Biology*,

32(12), pp.2300–2311.

Wernig, A. et al., 2004. M-cadherin is a Reliable Marker of Quiescent Satellite Cells in Mouse Skeletal Muscle. *Basic and Applied Myology*, 14(3), pp.161–168.

Wethmar, K., Smink, J.J. & Leutz, A., 2010. Upstream open reading frames: Molecular switches in (patho)physiology. *BioEssays*, 32, pp.885–893.

White, R.B. et al., 2010. Dynamics of muscle fibre growth during postnatal mouse development. *BMC developmental biology*, 10, p.21.

Williams, S. et al., 1995. CRP2 (C/EBP beta) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. *The EMBO journal*, 14(13), pp.3170–3183.

Williams, S., Angerer, N. & Johnson, P., 1997. C/EBP proteins contain nuclear localization signals imbedded in their basic regions. *Gene Expression*, 6(6), pp.371–85.

Williamson, E. a et al., 1998. Identification of transcriptional activation and repression domains in human CCAAT/enhancer-binding protein epsilon. *The Journal of biological chemistry*, 273(24), pp.14796–14804.

Wilschut, K., Ling, V. & Bernstein, H., 2012. Concise Review : Stem Cell Therapy for Muscular Dystrophies. *Stem Cells Translational Medicine*, 1, pp.833–842.

Wiper-Bergeron, N., Salem, H.A., et al., 2007. Glucocorticoid-stimulated preadipocyte differentiation is mediated through acetylation of C/EBPβ by GCN5. *Proceedings of the National Academy of Sciences*, 104(8), pp.2703–2708.

Wiper-Bergeron, N. et al., 2003. Stimulation of preadipocyte differentiation by steroid through targeting of an HDAC1 complex. *EMBO Journal*, 22(9), pp.2135–2145.

Wiper-Bergeron, N., St-Louis, C. & Lee, J.M., 2007. CCAAT/Enhancer binding protein beta abrogates retinoic acid-induced osteoblast differentiation via repression of Runx2 transcription. *Molecular endocrinology*, 21(9), pp.2124–2135.

Xu, C. et al., 2013. A zebrafish embryo culture system defines factors that promote vertebrate myogenesis across species. *Cell*, 155(4), pp.909–21.

Yablonka-Reuveni, Z. et al., 1999. The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. *Developmental biology*, 210, pp.440–455.

Yablonka-Reuveni, Z. & Rivera, A., 1994. Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Developmental biology*, 164, pp.588–603.

Yan, B. et al., 2012. miR-203b: a novel regulator of MyoD expression in tilapia skeletal muscle. *Journal of Experimental Biology*, 216, pp.447–451.

Yang, H. et al., 2005. Expression and activity of C/EBPb and d are upregulated by dexamethasone in skeletal muscle. *Journal of Cellular Physiology*, 204(1), pp.219–226.

Yeh, W.C. et al., 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes and Development*, 9, pp.168–181.

Yennek, S. et al., 2014. Cell adhesion geometry regulates non-random DNA segregation and asymmetric cell fates in mouse skeletal muscle stem cells. *Cell Reports*, 7(4), pp.961–970.

Yin, H., Price, F. & Rudnicki, M., 2013. Satellite cells and the muscle stem cell niche.

Physiological reviews, 93(1), pp.23–67.

Yin, M. et al., 1996. Tumor necrosis factor alpha promotes nuclear localization of cytokine-inducible CCAAT/enhancer binding protein isoforms in hepatocytes. *J Biol Chem*, 271(30), pp.17974–17978.

Yiu, E.M. & Kornberg, A.J., 2015. Duchenne muscular dystrophy. *Journal of Paediatrics and Child Health*, 51, pp.759–764.

Yokota, T. et al., 2006. Expansion of revertant fibers in dystrophic mdx muscles reflects activity of muscle precursor cells and serves as an index of muscle regeneration. *Journal of cell science*, 119, pp.2679–2687.

Yoon, K. et al., 2007. Decreased survival of C/EBPβ-deficient keratinocytes is due to aberrant regulation of p53 levels and function. *Oncogene*, 26(3), pp.360–7.

Yoshida, N. et al., 1998. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates "reserve cells". *Journal of cell science*, 111, pp.769–779.

Zammit, P.S. et al., 2004. Muscle satellite cells adopt divergent fates: A mechanism for self-renewal? *Journal of Cell Biology*, 166(3), pp.347–357.

Zammit, P.S. et al., 2006. Pax7 and myogenic progression in skeletal muscle satellite cells. *Journal of cell science*, 119, pp.1824–1832.

Zanotti, S. et al., 2009. Misexpression of CCAAT/enhancer binding protein beta causes osteopenia. *Journal of Endocrinology*, 201, pp.263–274.

Zauberman, A., Lapter, S. & Zipori, D., 2001. Smad Proteins Suppress CCAAT/Enhancer-binding Protein (C/EBP) b- and STAT3-mediated Transcriptional Activation of the Haptoglobin Promoter. *Journal of Biological*

Chemistry, 276(27), pp.24719–24725.

Zhang, G., Jin, B. & Li, Y.-P., 2011. C/EBPβ mediates tumour-induced ubiquitin ligase atrogin1/MAFbx upregulation and muscle wasting. *The EMBO Journal*, 30, pp.4323–4335.

Zhang, J.W. et al., 2004. Role of CREB in Transcriptional Regulation of CCAAT/Enhancer-binding Protein beta Gene during Adipogenesis. *Journal of Biological Chemistry*, 279(6), pp.4471–4478.

Zhang, J.-W. et al., 2004. Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. *Proceedings of the National Academy of Sciences*, 101(1), pp.43–47.

Zhang, K., Sha, J. & Harter, M.L., 2010. Activation of Cdc6 by MyoD is associated with the expansion of quiescent myogenic satellite cells. *Journal of Cell Biology*, 188(1), pp.39–48.

Zhang, W., Behringer, R. & Olson, E., 1995. Inactivation of the myogenic bHLH gene MRF4 results in up-regulation of myogenin and rib anomalies. *Genes and Development*, 9, pp.1388–1399.

Zhou, L. et al., 2008. Haploinsufficiency of utrophin gene worsens skeletal muscle inflammation and fibrosis in mdx mice. *Journal of the Neurological Sciences*, 264, pp.106–111.

Zhu, S. et al., 1999. C/EBPbeta modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. *Molecular and cellular biology*, 19(10), pp.7181–7190.

APPENDIX ONE: SUPPLEMENTAL FIGURES

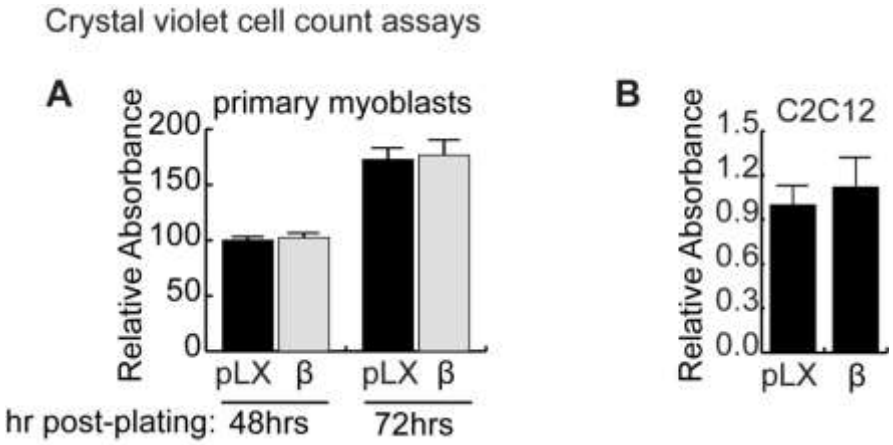

Figure S1. Overexpression of C/EBPβ in myoblasts does not affect cell growth. (A) Crystal violet assay to determine cell number of satellite cell cultures retrovirally transduced to express C/EBPβ or with empty vector (pLX) and cultured for 48 hours in growth medium. Error bars are the SEM, n=3. **(B)** Crystal violet assay to determine cell number of C2C12 cells retrovirally transduced to express C/EBPβ or with empty vector (pLX) and cultured for 48 hours in growth medium. Error bars are the SEM, n=4.

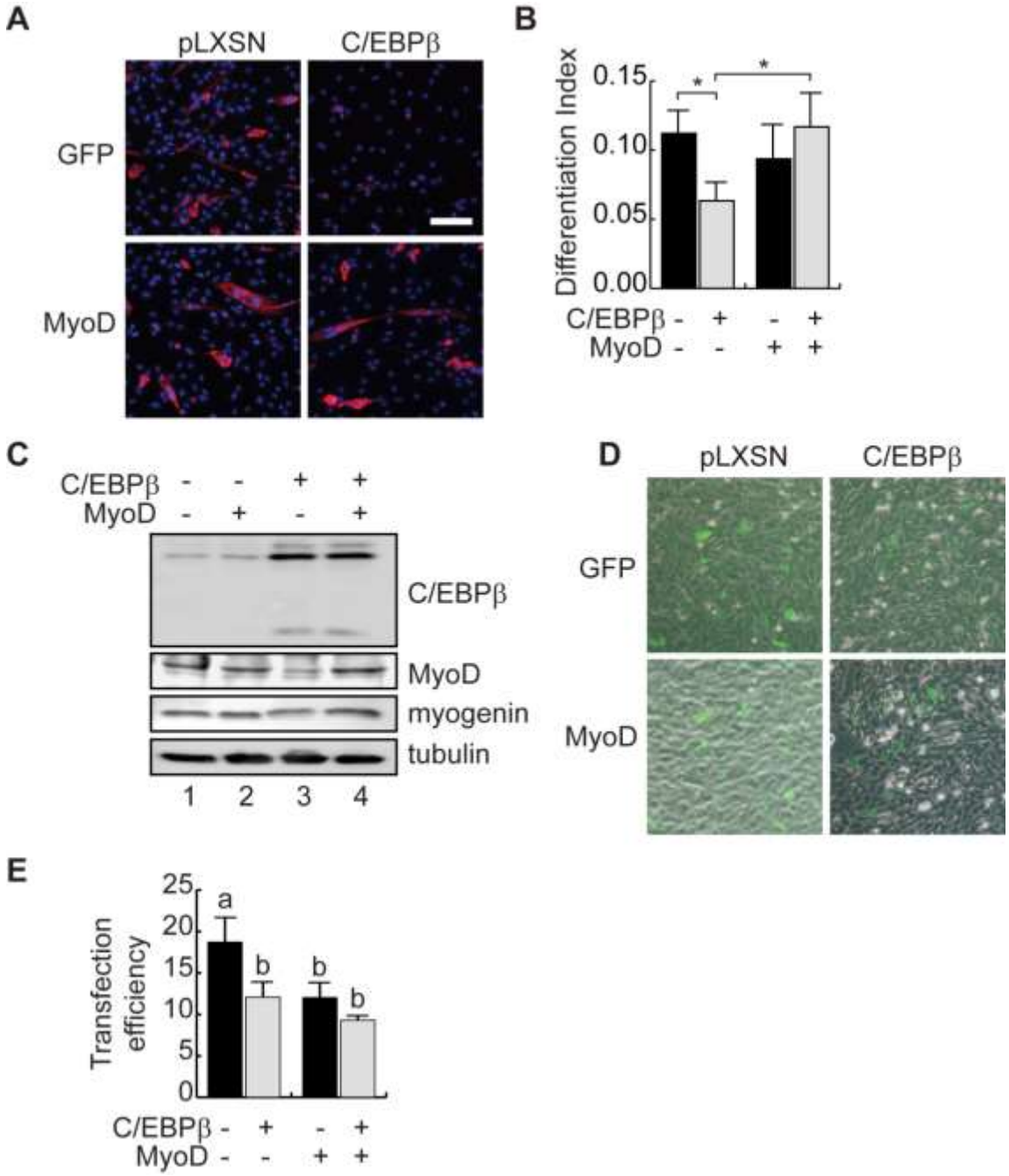

Figure S2. Restoration of MyoD protein expression rescues the differentiation defect in C/EBPβ-overexpressing C2C12 cells. (A) Indirect immunostaining for MHC expression in C2C12 cells retrovirally transduced to express C/EBPβ or with empty vector (pLXSN), and transiently transfected to express GFP and MyoD as indicated. Cells were differentiated in low serum conditions for 4 days prior to immunostaining.

DAPI staining reveals nuclei. Scale bar = 50μm. **(B)** Differentiation index of cultures treated as in (A). *$p<0.05$, n=3. Error bars are the standard deviation. **(C)** Western analysis of C/EBPβ, MyoD and myogenin expression in C2C12 cultures transfected and differentiated as in (A) B-tubulin is used as a loading control. **(D)** Images of GFP expression, merged with phase contrast images, to determine transfection efficiency. **(E)** Transfection efficiencies, defined as the percentage of GFP+ cells in the culture 24 hours after transfection, for each transfection condition. One way ANOVA analysis revealed a significant decrease ($p<0.05$) between conditions labeled a and b.

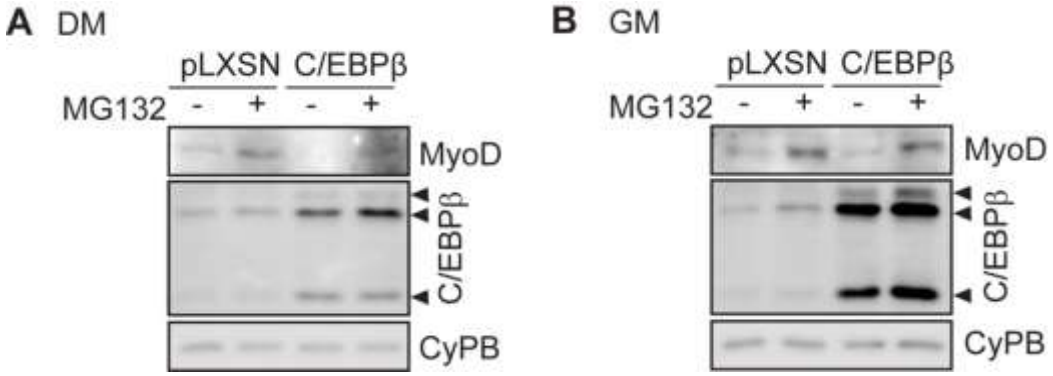

Figure S3. Loss of MyoD expression is partially rescued by the inhibition of the proteasome. (A) Western analysis of C2C12 myoblasts retrovirally transduced to express C/EBPβ or with empty vector (pLXSN) and cultured in 2% horse serum for 36 hours. Two hours prior to harvest, cells were treated with 50 μM MG132. Cyclophilin B is used as a loading control. **(B)** Western analysis of C2C12 myoblasts retrovirally transduced to express C/EBPβ or with empty vector (pLXSN) and cultured in growth medium pretreated with MG132 for 2 hours prior to harvest.

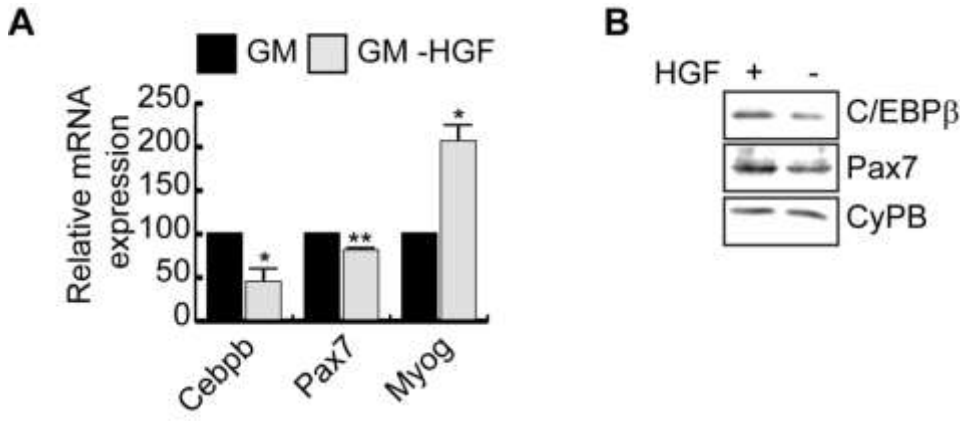

Figure S4. Withdrawal of HGF from growth medium reduces *Cebpb* expression and promotes differentiation. (A) RT-qPCR analysis of *Cebpb*, *Pax7* and *Myog* expression in WT satellite cell cultures grown in complete growth medium or growth medium lacking HGF for 48 hours. *p<0.05 **p<0.01, n=4 **(B)** Western analysis of C/EBPβ and Pax7 expression in SCs cultured and treated as in (A).

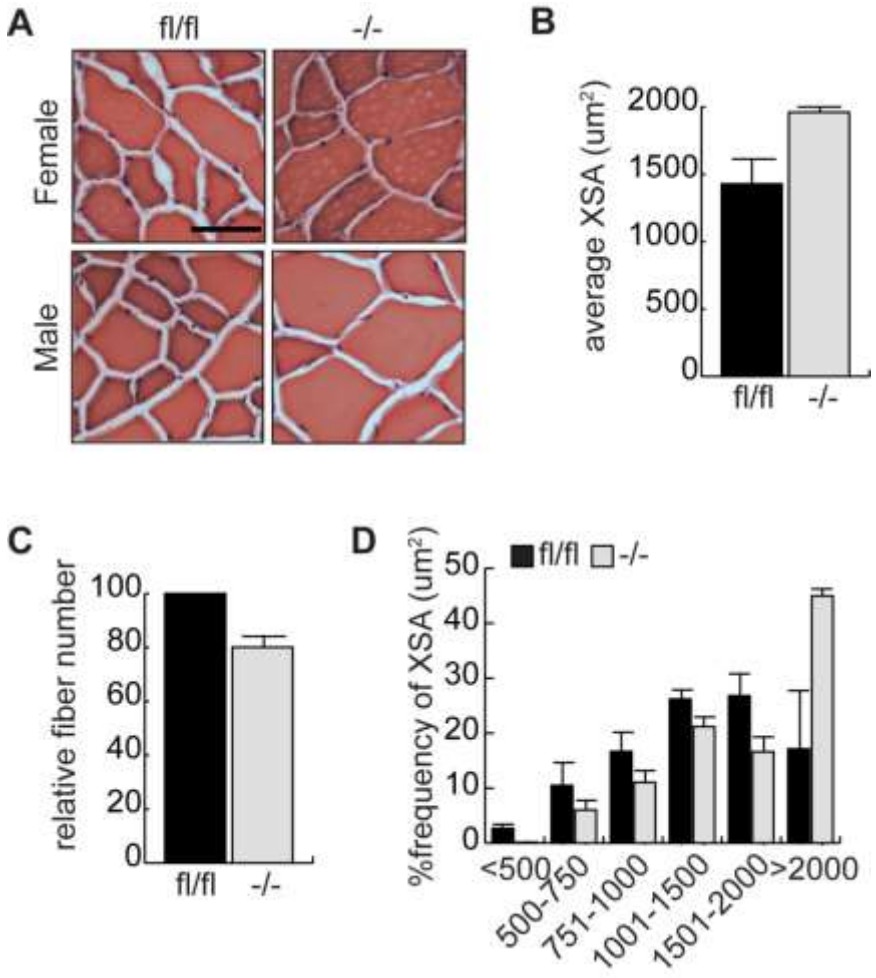

Figure S5. Fiber hypertrophy increased at day P56 in conditional knockout mice. **(A)** Representative bright field images of tibialis anterior cross sections from control *Cebpb*$^{fl/fl}$ and conditional null *Cebpb*$^{-/-}$*Pax7*$^{Cre/+}$ animals at postnatal day 56 stained with hematoxylin and eosin. Scale bar = 100 μm. **(B)** Average cross-sectional areas of muscle fibers from control and conditional null animals. For each group n=2 animals (1 male and 1 female). Error bars are the standard deviation. **(C)** Relative fiber number in the tibialis anterior muscles of control and conditional null mice (n=2 for each group). Control animals are set arbitrarily at 100. **(D)** Frequency distribution of fiber size in control and conditional null mice. Error bars are the standard deviation.

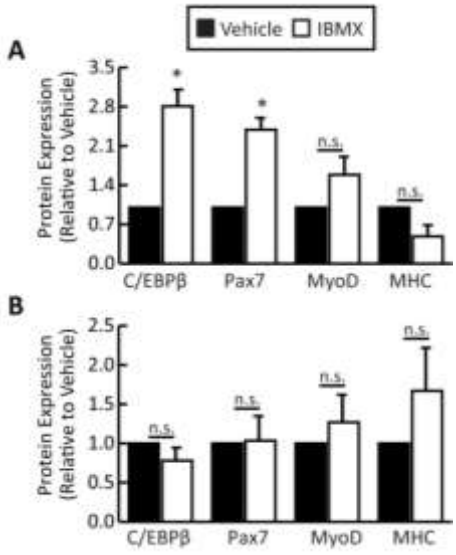

Figure S6. C/EBPβ and myogenic marker expression in primary myoblasts treated for 5 days in growth medium *(A)* and differentiated in the absence of IBMX *(B)*. Data are presented as mean ± SEM (n=3, *$p<0.05$, n.s = not significant).

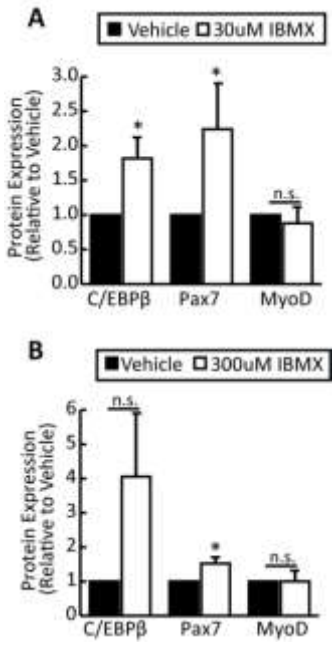

Figure S7. Quantification of C/EBPβ, Pax7 and MyoD protein expression in primary myoblasts treated with *(A)* 30 uM IBMX or *(B)* 300 uM IBMX as indicated in growth medium for 5 days, normalized to cyclophilin B expression. (n=3, *$p<0.05$, n.s = not significant).

APPENDIX TWO: SUPPLEMENTAL TABLE

Table S1. Real-time quantitative PCR primers used in this study.

Primers	Sequences (5' to 3')	Product Size (bp)
Cdh15-F	GGTACAGGGTGCTGGGTTC	96
Cdh15-R	CAATGGGTTCTGCTCTGCTC	
Cxcr4-F	TCCAGACCCCACTTCTTCAG	124
Cxcr4-R	AGTGACCCTCTGAGGCGTTT	
Sdc3-F	GGTGGTCAGAAGGGACTCTG	101
Sdc3-R	AGCCTGATGTTGCTGAGAGG	
Sdc4-F	GGGAGGGCTCCAGAGAAGTA	97
Sdc4-R	GAGGCTTCCCCTTGGTCC	
Abcg2-F	TCTAGCAACGAAGACTTGCCT	108
Abcg2-R	CCGGAAAACAGTTGAGAAAGA	
Ly6a-F	GGCAGATGGGTAAGCAAAGA	108
Ly6a-R	CAATTACCTGCCCCTACCCT	
Cebpb-F	TCGAACCCGCGGACTGCAAG	134
Cebpb-R	CGACGACGACGTGGACAGGC	
Pax7-F	GACGACGAGGAAGGAGACAA	124
Pax7-R	CGGGTTCTGATTCCACATCT	
Myod1-F	TGGCATGATGGATTACAGCG	146
Myod1-R	CCACTATGCTGGACAGGCAGT	
18s-F	CGCCGCTAGAGGTGAAATC	150
18s-R	CCAGTCGGCATCGTTTATGG	