The role of CCAAT/Enhancer Binding Protein beta (C/EBPβ) in skeletal muscle satellite cells after injury and in cancer cachexia

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ABSTRACT

CCAAT/Enhancer Binding Proteins are a family of six bZIP transcription factors. C/EBPβ, the second member cloned, has been implicated in adipogenesis and osteogenesis, but the role of C/EBPβ in myogenesis remained undetermined. In adults, muscle-resident stem cells, called satellite cells (SCs), have the greatest propensity to regenerate the skeletal muscle. We found that C/EBPβ is expressed in SCs, and its expression progressively declines upon differentiation. Forcing the expression of C/EBPβ in myoblasts enhanced the expression of the SC marker Pax7, and repressed MyoD and the myogenic genes expression, resulting in the inhibition of myogenesis. Using a SC-specific conditional knockout (cKO) mouse model, we found that cKO myoblasts have decreased expression of Pax7, and we identified Pax7 as a direct target of C/EBPβ action. *In vivo*, excision of C/EBPβ resulted in muscle hypertrophy at the juvenile age, and adult cKO animals had enhanced muscle regeneration following $BaCl_2$ muscle injury. Moreover, the number of Pax7+ cells in cKO animals decreased following $BaCl_2$ injury. Upon performing a second injury into cKO animals, we demonstrate a decreased muscle fiber size and an exacerbation of the percentage number of SCs. While cKO animals repaired well a $BaCl_2$ injury, regeneration failed in cKO animals following cardiotoxin (CTX) injury. We demonstrate that IL-1β expression is enhanced in muscle after CTX injury when compared to $BaCl_2$, and we found that IL-1β can stimulate the expression of C/EBPβ in myoblasts. Ectopic C/EBPβ expression can protect myoblasts from apoptosis when triggered with thapsigargin, whereas cKO myoblasts are more sensitive to apoptosis. Using cancer cachexia as a model of

chronic inflammation, we found that the expression of C/EBPβ is stimulated in the SCs of cachectic animals, and this correlated with a decrease in regenerative capacity. The severity of muscle wasting was not improved in cKO animals, but rather cKO SCs were lost to apoptosis. Together, this study establishes a protective role for C/EBPβ in muscle SCs in conditions of inflammation.

RÉSUMÉ

« CCAAT/Enhancer Binding Proteins » est une famille de six facteurs de transcription bZIP. C/EBPβ, le second membre cloné, est impliqué dans l'adipogénèse et l'ostéogénèse mais le rôle de C/EBPβ lors de la myogenèse est inconnu. Chez l'adulte, les cellules souches du muscle squelettique appelées cellules satellites (SCs), ont un rôle essentiel pour régénérer le muscle. Nous avons observé que C/EBPβ était exprimé dans les SCs, alors son expression diminue progressivement pendant la myogenèse. L'expression ectopique de C/EBPβ dans les myoblastes stimule Pax7, et réprime MyoD ainsi que des gènes myogéniques, donc bloquant la myogenèse. En créant une souris « knockout » conditionnelle (cKO), nous avons caractérisé le rôle de C/EBPβ dans les SCs. L'expression *in vitro* de Pax7 est diminuée dans les myoblastes cKO, suggérant que Pax7 est une cible directe de C/EBPβ. *In vivo*, l'excision de C/EBPβ engendre une hypertrophie juvénile, et les souris cKO adultes régénèrent mieux une blessure au $BaCl_2$. De plus, le nombre de SCs diminue chez la souris cKO suite à une blessure au $BaCl_2$. Ainsi, nous avons observé une diminution de l'aire des fibres musculaires chez la souris cKO ainsi qu'une exacerbation du nombre de SCs suite à une seconde blessure. Bien que la régénération musculaire soit accrue chez la souris cKO, la régénération a échouée lors d'une blessure avec de la cardiotoxine (CTX). Nous démontrons que l'expression de IL-1β est augmentée suite à une blessure CTX, et aussi que IL-1β stimule l'expression de C/EBPβ. C/EBPβ bloque l'apoptose des myoblastes provoquée par la thapsigargine, alors que les myoblastes cKO ont une sensibilité accrue à l'apoptose. En utilisant la cachexie cancéreuse comme modèle

d'une condition inflammatoire chronique, nous démontrons que l'expression de C/EBPβ est stimulée dans les SCs des souris cachectiques. Cette stimulation de C/EBPβ est corrélée avec une diminution de la régénération musculaire. Par contre, lorsque nous avons injecté une tumeur à la souris cKO, les SCs moururent par apoptose. En somme, cette recherche supporte un rôle de C/EBPβ en tant que médiateur de l'inflammation pour la protection des SCs.

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	messenger ribonucleic acid
C/EBP	CCAAT/Enhancer Binding Protein
bZIP	basic leucine zipper
SC	Satellite cell
IL-1	Interleukin 1
IL-6	Interleukin 6
TNF	Tumor necrosis factor
bFGF	basic fibroblast growth factor
HGF	Hepatocyte growth factor
BrdU	5'-bromo-2'-deoxyuridine
NF-IL6	Nuclear factor for interleukin 6
TPG	Thapsigargin
MRF	Muscle regulatory factor
bHLH	Basic region helix-loop-helix

Myf5	Myogenic factor 5
MyoD	Myogenic determination factor 1
Myog	Myogenin
Pax	Paired-box factor
MyHC	Myosin heavy chain
WT	Wild type
KO	Knockout
cKO	Conditional knockout
TA	Tibialis anterior
CTX	Cardiotoxin

DEDICATION

To all those that were crazy enough to think they can change the world

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CHAPTER 1: INTRODUCTION

Myogenesis is the cellular differentiation process of skeletal muscle formation

Animals have three types of muscle tissue: smooth, cardiac and skeletal. The adult human has approximately 600 different muscles and given its abundance, skeletal muscle is the heaviest organ in mammals (at least 40% of the weight of an adult is muscle tissue). Skeletal muscles have the physiological role of transforming chemical energy into mechanical force.

In the vertebrate developing embryo, the skeletal muscle originates from the mesoderm germ layer (Shi and Garry, 2006). Segments of the mesoderm are transformed to make somites, and regions of the somites further mature into a specialized embryonic structure that is called the myotome from which limb skeletal muscle cells found in the adult originate (Shi and Garry, 2006). Stem cells found in the myotome become myoblasts and differentiate in skeletal muscle cells (myocyte or myofiber) through a cellular differentiation process termed myogenesis. In developing mouse limbs, multinucleated myofibers are first observed around E10.5 (Hutcheson et al., 2009; Miller, 1991). Myogenesis occurs via transcriptional actions, and these changes are achieved by a family of transcription factors that direct the skeletal muscle phenotype: the myogenic regulatory factors (MRFs).

MRFs are transcription factors found in the large family of basic helix-loop-helix (bHLH) genes. The basic and HLH domains are required for DNA-binding and for dimerization respectively. The MRF family is composed of four genes: myogenic determination factor 1 (MyoD), Myogenic factor 5 (Myf5), Myogenic Regulatory Factor 4 (MRF4 or Myf6) and Myogenin (Myog or Myf4) governing myogenesis

(Braun and Gautel, 2011). Importantly, the roles of MRFs in both embryonic and adult myogenesis overlap. Thus, many of the roles of MRFs for adult regeneration were established from observations made at the embryonic stage.

MRFs have the unique property to make muscle cells and MRFs have both specific and redundant roles for the formation of the mature skeletal muscle (Molkentin and Olson, 1996). Some MRFs are required for the specification or determination (Myf5 and MyoD) of the myogenic lineage, while others are required for the terminal differentiation (Myog and MRF4) of myoblasts into multinucleated skeletal muscle fibers (Olson, 1990). Importantly, MRFs can autoregulate each other's expression and often, the stimulation of one factor is sufficient to promote the expression of the remaining of the family (Olson, 1990; Thayer et al., 1989). The expression of a single MRF gene is sufficient to convert non-muscle cells into a functioning skeletal muscle cell (Davis et al., 1987; Yutzey et al., 1990). Moreover, deletion of all MRF genes in mice depletes the animal of all skeletal muscle, making MRFs both sufficient and necessary for myogenesis.

Satellite cells are skeletal muscle stem cells found in postnatal muscle

One of the most phenomenal properties of the adult skeletal muscle is its capacity to regenerate (Bischoff, 1975). Following an injury, skeletal muscle can be rebuilt to full functionality within a few weeks. In mice, the tibialis anterior muscle can be injured fifty times with only a minor decrease in regenerative capacity (Luz et al., 2002). Rats have also been showed to repair efficiently up to 24 serial injuries

(Sadeh et al., 1985). Myonuclei of adult skeletal muscle have irreversibly exited the cell cycle and therefore cannot proliferate; a constraint for regeneration (Bischoff, 1986; Moss and Leblond, 1970; Schultz et al., 1978). Muscle regeneration is accomplished by a muscle-resident adult stem cell population named satellite cells (SCs) (Bischoff, 1986; Sambasivan et al., 2011; Snow, 1977). Electron microscopy examination of *Xenopus leavis* revealed that mononuclear cells were associated with its muscle fibers producing “wedges” on the basal lamina. These cells were hypothesized to be “dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate embryonic myogenesis” (Mauro, 1961). Indeed, SCs are adult stem cells located on the muscle sarcolemma under the basal lamina.

SCs are mitotically quiescent but they can re-enter the cell cycle after weight-bearing exercise or after a muscle injury. Cultures of intact muscle myofibers demonstrated that SCs are capable of proliferation and have the ability to fuse to form myofibers (Bischoff, 1975, 1986; Konigsberg et al., 1975). As quiescent cells, SCs have low metabolic rates, a thin cytoplasm with a nucleus composed of heterochromatin, and low turnover rates in adults because muscle cells are long-lived and myofibers are infrequently damaged and repaired (Freter et al., 2010; Sambasivan, 2015). Estimates suggest that a muscle nucleus turns over every 15 years in humans (Spalding et al., 2005). Upstream of the expression of MRF in the myogenic transcriptional cascade are the Pax transcription factors, whose role is critical for making skeletal muscle as well as SCs (Tajbakhsh, 2009).

In adults, all quiescent SCs express Pax7 (Kuang and Rudnicki, 2008; Nishijo et al., 2009; Seale et al., 2000). All skeletal muscles examined so far have SCs,

although with variable abundance, forming between 2% to 7% of muscle nuclei depending on age and muscle type (Cornelison and Wold, 1997; Kuang et al., 2007; Megeney et al., 1996). Today, estimates favor the idea that SCs are the most abundant adult stem cell population in mammals (Bentzinger et al., 2012).

SCs can undergo adult myogenesis and regenerate skeletal muscle

In adults, all SCs maintain the expression of Pax7 and when injury occurs, they can recapitulate the cellular and molecular steps of embryonic myogenesis to build new muscle fibers (Figure 1) (Seale et al., 2000). The molecular mechanisms that govern SC activation are poorly described, though it has been established that HGF release from the surrounding connective tissue is sufficient for SCs activation, although the requirement of HGF has recently been challenged (Cornelison and Wold, 1997; Tatsumi and Allen, 2004; Tatsumi et al., 1998; Webster and Fan, 2013; Wozniak and Anderson, 2007). FGF has also been shown to promote the activation of SCs while blocking their differentiation (Allen and Boxhorn, 1989; Cornelison et al., 2001; Jones et al., 2005; Li et al., 1992).

Upon activation, SCs stimulate the expression of Myf5 and MyoD, which triggers the myogenic transcriptional cascade (Cooper et al., 1999; Cornelison and Wold, 1997; Kassar-Duchossoy et al., 2005; Murphy and Kardon, 2011; Rudnicki et al., 2008). The stimulation of MyoD is dependent on the expression of Pax3 or Pax7

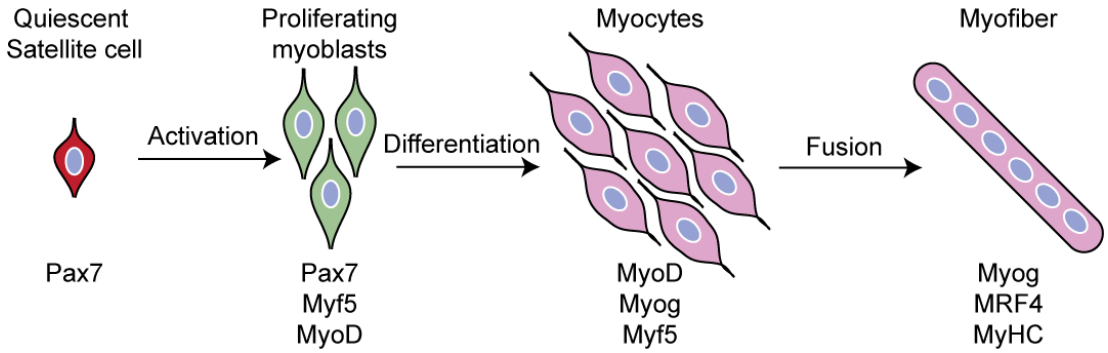

Figure 1 The hierarchical transcriptional cascade of adult myogenesis.

SCs are quiescent stem cells found on every muscle. They have a high expression of Pax7. Following injury, SCs enter the cell cycle, proliferate as myoblasts, and the expression of Myf5, MyoD and Pax3 is promoted. Upon differentiation, Myog expression is stimulated and Pax7, Myf5 and MyoD expression decreases. Multiple myocytes eventually fuse with each other creating a syncytium called a myofiber that expresses MRF4 and Myog. MyHC is indicated as a phenotypical molecular marker. Figure adapted from (Le Grand and Rudnicki, 2007; Zammit et al., 2006a).

(Relaix et al., 2006). Muscle myoblasts therefore proliferate as Pax3+/Pax7+/Myf5+/MyoD+ myoblasts although the expression of Pax3 in SCs is muscle-dependent (Bentzinger et al., 2012; Conboy and Rando, 2002; Relaix et al., 2006). When mitogen levels decrease, myoblasts progressively differentiate as myocytes where they promote the expression of myogenin (Myog) (Rudnicki et al., 2008). The stimulation of Myog expression is associated with a decrease in the expression of Pax7, Myf5 and to a lesser extent MyoD. Concomitantly, Myog promotes the expression of skeletal muscle specific genes such as MyHC. Expression of Myog also marks cell-cycle exit and terminal differentiation. Following cell-cycle exit, myocytes align and fuse with one another (Wakelam, 1985). While MRF4 expression is strong in mature myofibers and has a role in the maintenance of the differentiated skeletal muscle phenotype, the expression of the other MRFs decreases in mature myofibers and Pax7 expression is absent in the multinucleated cell. Together, the best accepted model views Pax3 and Pax7 as myogenic stem cells factors, Myf5 and MyoD as myogenic commitment factors, and Myog and MRF4 as terminal differentiation transcription factors (Figure 1) (Braun and Gautel, 2011).

Definition and functions of Pax7 and MRFs

Pax7

Pax7 is a transcription factor of 55kDa and is a member of the Pax family of transcription factors (Buckingham and Relaix, 2007). Each member of the Pax family has a conserved paired-box domain as well as a homeodomain; two common

DNA-binding motifs (Buckingham and Relaix, 2007; Sabourin and Rudnicki, 2000). The name pax was given from the sequence similarity with *Drosophila melanogaster* gene *paired*; Pax and *paired* are orthologues (Buckingham and Relaix, 2007; Tajbakhsh et al., 1997). In mice, nine Pax genes are found, but only Pax3 and Pax7 have a role in myogenesis (Table 1) (Buckingham and Relaix, 2007). Pax3 and Pax7 act to specify mesenchymal stem cells towards a myogenic determination via activation of the MRFs (Buckingham and Relaix, 2007; Relaix et al., 2005). All skeletal muscle stem cells express either Pax3 or Pax7 and most precursors express both (Relaix et al., 2006; Relaix et al., 2005). Pax3 is expressed in multipotent stem cells of the somite and those stem cells can differentiate into skeletal muscle (Bober et al., 1994; Molkentin and Olson, 1996; Williams and Ordahl, 1994). Breeding of a $Pax3^{Cre}$ transgenic mouse with a Z/EG lineage tracing reporter animal indicates that 95% of limb muscles are GFP+ (Schienda et al., 2006). This indicates that most limb muscle cells originate from a stem cell previously expressing Pax3, while few muscle fibers originate from a Pax3- stem cell.

Pax3/7+ embryonic stem cells are multipotent stem cells because they can also generate dermis and adipose tissue cells (Hutcheson et al., 2009; Lepper and Fan, 2010). Pax3+ stem cells are more likely to commit to myogenesis while Pax3/7 double positive stem cells remain in a proliferating stage (Relaix et al., 2005). Indeed, all Pax3+ stem cells eventually promote the expression of Myf5 and/or

Table 1 Summary of the most important skeletal muscle phenotypes of Pax null mouse at fetal and adult stage.

	Pax3$^{-/-}$	**Pax7$^{-/-}$**	**Pax3$^{-/-}$: Pax7$^{-/-}$**	**Pax3$^{-/-}$: Myf5$^{-/-}$: MRF4$^{-/-}$**
Embryonic	No limbs muscles. Failure of myoblasts migration. Dispensable for myogenesis. Lethal	No phenotype	No limbs muscles. Loss of muscle stem cells by apoptosis. Lethal	No muscle formation. No MyoD expression.
Neonatal	Lethal	Failure of post-natal muscle growth. SCs apoptosis.	Lethal	Lethal
Adult	Lethal	Failure at regeneration. No SCs. Lethal	Lethal	Lethal
References	(Goulding et al., 1993; Lepper et al., 2009; Relaix et al., 2004)	(Lepper et al., 2009; Relaix et al., 2006; Seale et al., 2000; von Maltzahn et al., 2014)	(Kassar-Duchossoy et al., 2005; Relaix et al., 2006; Relaix et al., 2005)	(Tajbakhsh et al., 1997)

Table adapted from (Murphy and Kardon, 2011).

MyoD and commit to myogenesis (Relaix et al., 2005). Following expression of the MRFs, Pax3 expression decreases. In newborn muscles, Pax3 expression is low but Pax7 expression persists in the adult (Mansouri et al., 1996). Together, this prompted the hypothesis that Pax3 is important for the embryonic stem cells commitment to myogenesis while Pax7 may be more important in a postnatal stage.

The $Pax7^{-/-}$ mouse is produced at expected mendelian ratios (Mansouri et al., 1996). However, although Pax7 null offspring appear normal at birth, they have severe growth retardation and all $Pax7^{-/-}$ pups die within three weeks (Table 1) (Kuang et al., 2006; Mansouri et al., 1996; Seale et al., 2000). This suggests that Pax7 is dispensable for myogenesis but is required in the post-natal period. This finding is consistent with previous observations that fetal myoblasts are required for post-natal muscle growth (White et al., 2010). At birth, the skeletal muscle interstitium is rich in muscle stem cells, making up to 30% of nuclei, an indication of their importance for post-natal muscle growth. In fact, newborn $Pax7^{-/-}$ muscle fibers have 50% fewer nuclei, suggesting that Pax7 expression is required for hyperplasia of muscle cells (Rudnicki et al., 2008). Moreover, given that $Pax7^{-/-}$ animals undergo essentially normal embryonic myogenesis and that the expression pattern of MRFs was comparable to WT littermates, this suggest that Pax7 is dispensable for myogenesis (Mansouri et al., 1996; Oustanina et al., 2004). Conditional knockout of Pax7 in an adult mouse context further established a requirement for Pax7 in regeneration, as this muscle failed to regenerate (von Maltzahn et al., 2014). However, homozygous *splotch* mice ($Pax3^{sp/sp}$), carrying a naturally occurring mutation in the *Pax3* gene, do not undergo limb muscle myogenesis (Franz et al.,

1993; Goulding et al., 1993). This suggests that Pax3 and Pax7 have distinct function in myogenesis and it also suggests that Pax7 cannot compensate for loss of Pax3, or vice versa (Relaix et al., 2006).

In vitro analysis of $Pax7^{-/-}$ SCs indicated that while no differences were noted in Ki67 staining (a marker of cell in proliferation), a decrease in cell number was observed (Relaix et al., 2006). While apoptosis wasn't significantly increased in $Pax7^{-/-}$ SCs as compared to WT, there was an increase in the number of SCs positive for cyclin A, a marker of S/G2 phase, suggesting that $Pax7^{-/-}$ SCs have a cell-cycle defect (Relaix et al., 2006).

While apoptosis wasn't a feature of $Pax7^{-/-}$ SCs *in vitro*, it was a significant phenotype of $Pax7^{-/-}$ mice *in vivo*, with cells positive for cleaved caspase-3 was a prominent feature in the growing limbs of $Pax7^{-/-}$ mice (Oustanina et al., 2004; Relaix et al., 2006). WT SCs infected with a dominant-negative Pax7 also had increased cellular apoptosis, although dominant-negative Pax3 had no effects on apoptosis (Relaix et al., 2006). This is an indication that Pax7 has anti-apoptotic roles in SCs at the post-natal stage, a function that Pax3 cannot fulfill.

Myf5

Myf5 was discovered in 1988 as a cDNA sequence that could convert fibroblasts into myoblasts cells (Pinney et al., 1988). Myf5 has a bHLH DNA-binding motif common to MRFs (Braun et al., 1990). In mice, Myf5 is a 28kDa protein and as all MRFs, it has to dimerize with another bHLH protein to increase its affinity for DNA and for full transcriptional activity (Winter et al., 1992).

Measurements of Myf5 expression in tissue culture models of skeletal muscle suggest a high expression in proliferating myoblasts and lower expression in differentiated myotubes (Braun et al., 1989). In adult mouse, Myf5 mRNA expression is detected in most SCs, but its protein product is undetectable (Crist et al., 2012; Tajbakhsh, 2009). Pax3 was shown to be an important transcription factor for the stimulation of Myf5 in the embryo, and Pax7 was also shown to the important for Myf5 expression in the adult (Bajard et al., 2006; Maroto et al., 1997; McKinnell et al., 2008).

$Myf5^{-/-}$ animals die at birth without obvious skeletal muscle malformation (Table 2) (Braun et al., 1992). Upon MyoD stimulation in the embryo, myogenesis proceeds normally in Myf5 null embryos (Braun et al., 1992). This suggests that Myf5 expression is dispensable for myogenesis and also that MyoD expression is sufficient for myogenesis in $Myf5^{-/-}$ animals. Lethality in $Myf5^{-/-}$ pups originates from defective rib cage formation preventing inflation of the lungs (Braun et al., 1992). Another Myf5 null allele where LacZ was inserted in place of the Myf5 coding region ($Myf5^{nLacz/nLacz}$) revealed that without Myf5 expression, stem cells maintain a multipotent phenotype, becoming committed to alternative mesenchymal lineages in the absence of Myf5 protein (Tajbakhsh et al., 1996).

Table 2 Summary of the most obvious phenotypes of MRFs null mouse models in the embryo, newborn and adult.

	Myf5$^{-/-}$	**MyoD$^{-/-}$**	**MRF4$^{-/-}$**	**Myog$^{-/-}$**	**Myf5$^{-/-}$: MyoD$^{-/-}$**	**Myf5$^{-/-}$: MRF4$^{-/-}$**	**MyoD$^{-/-}$: MRF4$^{-/-}$**	**Myf5$^{-/-}$: MyoD$^{-/-}$: MRF4$^{-/-}$**	**MyoD$^{-/-}$: Myog$^{-/-}$: MRF4$^{-/-}$**
Embryo	Delay in muscle formation. Dispensable	Delay in myogenesis. Dispensable.	No phenotype. Dispensable.	Normal myoblasts numbers. No myofibers.	Delay in myogenesis. Few muscle fibers.	No phenotype.	Normal myoblasts numbers. No myofibers. Identical to Myog$^{-/-}$	No myogenesis. Alternative commitment and persistence of stem cells.	Increase muscle progenitors. No myofibers
Neonatal	No phenotype	No phenotype	No phenotype	Few myofibers. Lethal.	No myoblasts. Few myofibers. Lethal.	Ribs defects. Lethal.	Few myofibers. Lethal.	Lethal	No myofibers. Lethal
Adult	Delay in regeneration. Myopathy. Muscle hypertrophy. Fat and connective accumulation.	Impaired regeneration. Increase myogenic stem cell numbers.	No phenotype	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal
Reference	(Braun et al., 1992; Gayraud-Morel et al., 2007; Kassar-Duchossoy et al., 2004)	(Gayraud-Morel et al., 2007; Megeney et al., 1996; Rudnicki et al., 1992; Yablonka-Reuveni et al., 1999)	(Kassar-Duchossoy et al., 2004)	(Hasty et al., 1993; Knapp et al., 2006; Nabeshima et al., 1993)	(Kassar-Duchossoy et al., 2004)	(Braun and Arnold, 1995; Kassar-Duchossoy et al., 2004; Olson et al., 1996)	(Rawls et al., 1998)	(Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993)	(Valdez et al., 2000)

Table adapted from (Murphy and Kardon, 2011).

This suggest that Myf5 expression is required for the myogenic commitment of mesenchymal stem cells (Tajbakhsh et al., 1996). Furthermore, although Myf5 can auto-regulate its promoter, given that LacZ is expressed in the absence of a Myf5 protein in this mouse model, it is unlikely that Myf5 is required for maintenance of its expression (Molkentin and Olson, 1996). Additionally, Myf5 is not sufficient for myogenesis because animals with null mutation of the three other MRFs ($MyoD^{-/-}$: $MRF4^{-/-}$: $Myog^{-/-}$) do not make skeletal muscle tissue (Valdez et al., 2000).

To evaluate the role of Myf5 in an adult context, mice with another Myf5 null allele that bypass perinatal lethality (see MRF4 below) were generated. Although Myf5 null newborns are seemingly normal, adult Myf5 null muscles have an increase in fibrosis and the number of regenerated myofibers, an indication of a progressive myopathy (Gayraud-Morel et al., 2007; Ustanina et al., 2007).

MyoD

MyoD was the first MRF cloned (Davis et al., 1987). Mouse MyoD is a 34kDa protein and like Myf5, MyoD can convert a wide variety of non-muscle cell types into skeletal muscle cell (Buckingham, 1992; Davis et al., 1990; Weintraub et al., 1989). The $MyoD^{-/-}$ mouse is viable, fertile, and without obvious malformation of the skeletal muscle (Table 2) (Rudnicki et al., 1992). These mice have increased expression of Myf5, suggesting that Myf5 might compensate for MyoD function (Rudnicki et al., 1992).

Indeed, the $Myf5^{-/-}$: $MyoD^{-/-}$ double mutant mouse dies at birth without skeletal muscle tissue (Rudnicki et al., 1993). This supports the hypothesis that either Myf5

or MyoD is required for myogenesis and that both genes are redundant in function in the embryo (Rudnicki et al., 1993). Moreover, embryonic myoblasts were absent from this mouse model, but Pax3/7+ stem cells in the somites are present and committed to non-myogenic fate, supporting the notion that Myf5 and MyoD are myogenic commitment factors (Kablar et al., 1997; Rudnicki et al., 1993; Tajbakhsh et al., 1996). However, a new Myf5 null allele ($Myf5^{loxp/loxp}$) where MRF4 expression isn't reduced display myogenesis in $Myf5^{loxp/loxp}$: $MyoD^{-/-}$ mice suggesting that MRF4 and Myog are sufficient for embryonic myogenesis, albeit at a decreased efficiency (Kassar-Duchossoy et al., 2004).

In adults, MyoD expression is restricted to skeletal muscle tissue and MyoD expression is high in proliferating myoblasts and decreases in differentiated myofibers (Braun et al., 1989; Buckingham, 1992). Upon muscle injury, Pax3 or Pax7 in SCs promotes the expression of MyoD and Myf5 within hours, and most activated SCs co-express both genes (Cornelison et al., 2000; Kitzmann et al., 1998; Zammit et al., 2002). MyoD null mice have delayed regeneration, with decreased myogenin expression and absence of MRF4 expression (Cornelison et al., 2000; Megeney et al., 1996; Sabourin et al., 1999; White et al., 2000). Furthermore, the muscles of $MyoD^{-/-}$:mdx mutant mice are filled with connective tissue when compared to mdx alone. $MyoD^{-/-}$:mdx mutant have an exacerbated pathology and decreased survival while mdx mice have a normal lifetime. Given that SCs promote both muscle homeostasis and survival of the mdx mouse, this suggests that MyoD is important for SCs biology in a model of chronic muscle regeneration. Also, $MyoD^{-/-}$ mice have an increased number of myoblasts

suggesting that loss of MyoD expression promotes the maintenance of a muscle stem cell phenotype (Megeney et al., 1996; Yablonka-Reuveni et al., 1999).

In vitro, while Myf5$^{-/-}$ myoblasts differentiate into myofibers normally, MyoD$^{-/-}$ myoblasts have a decreased differentiation potential (Gayraud-Morel et al., 2007; Sabourin et al., 1999). Indeed, MyoD$^{-/-}$ myoblasts have an increase propensity to self-renew (Macharia et al., 2010; Sabourin et al., 1999). In addition, it takes longer on average for a MyoD$^{-/-}$ SC to enter the cell cycle and to begin proliferating following muscle injury (Macharia et al., 2010). Together, this suggests that expression of MyoD is required for regeneration in adults, a contrast to Myf5 expression, and that Myf5 and MyoD have non-redundant functions in both embryos and adult models (Rudnicki et al., 2008). MyoD's role is not restricted to SCs as the muscle contractile properties of MyoD$^{-/-}$ mice are also abnormal when compared to WT animals, generating reduced force following nerve stimulation when compared to WT littermates (Macharia et al., 2010).

The MyoD promoter is long and complex as multiple enhancer elements and a proximal promoter regulate its spatiotemporal expression (Tapscott et al., 1992). The proximal promoter of MyoD spans from -275bp to +1 and contains putative sites for Sp1, CCAAT and a classic TATA-box, although this promoter region alone cannot potentiate full *MyoD* expression (Tapscott et al., 1992). Instead, a chromatinized distal enhancer region located from -5.4kb to -4.6kb is required to cooperate with the basal promoter (Tapscott et al., 1992). The distal enhancer region has predicted E-boxes, suggesting that MRFs are both binding and stimulating transcription from this region (Tapscott et al., 1992). Although these two

regions were strong activators of *Myod1* transcription in mouse myoblasts cells, they were inactive in fibroblasts cells, suggesting that a transcription factor absent from fibroblasts binds to and potentiates the *MyoD* promoter regions (Tapscott et al., 1992). However, co-transfection of MyoD cDNA in fibroblasts cells could stimulate a full activity of this promoter, suggesting that MyoD, or another factor expressed by MyoD, could activate its promoter in non-muscle cells (Tapscott et al., 1992). For full spatial expression of MyoD during embryogenesis, a second downstream enhancer located at -22kb to -18kb is required (Goldhamer et al., 1995).

MRF4 was shown to activate the endogenous MyoD promoter in C3H10T1/2 cells, but only when cells were cultured in low serum conditions (Rhodes and Konieczny, 1989). *In vitro*, forced expression of Myf5 also resulted in induction of MyoD and MyoD expression is regulated by Pax3 in the embryo (Tajbakhsh et al., 1997; Weintraub et al., 1991). Wnt (Wnt7a) can also stimulate the expression of MyoD (Tajbakhsh et al., 1998). Interestingly, the stimulatory effects of Wnt7a on both MyoD and Myf5 expression were dependent on the activity of cAMP response-element binding protein (CREB) (Chen et al., 2005). Indeed, CREB null mouse mutants ($CREB^{-/-}$) have decreased myogenesis and are born at lower frequency than expected mendelian ratio (Chen et al., 2005). MyoD target genes include myosin light chain and desmin, structural proteins of the sarcomere as well as muscle creatine kinase and p21(WAF1/CIP1) (Halevy et al., 1995; Lassar et al., 1989; Wheeler et al., 1999).

MRF4

The protein product of MRF4 has a size of 27kDa (Rhodes and Konieczny, 1989). MRF4 expression is biphasic in the embryo and among the four MRFs, it is the one with the strongest expression in adult skeletal muscle (Bober et al., 1991; Braun et al., 1992; Buckingham, 1992; Hinterberger et al., 1991; Rhodes and Konieczny, 1989). In a tissue culture model of myogenesis, MRF4 is expressed at later stages of muscle cell differentiation, when cells are mature, suggesting a role in the maintenance of the myofiber phenotype (Figure 1) (Buckingham, 1992; Gayraud-Morel et al., 2007).

The role of MRF4 in myogenesis took longer to be establish because three null mouse models had divergent phenotypes (Olson et al., 1996). This divergence occurred because of the genomic proximity of MRF4 and Myf5 (about 9kb) and classic targeting methods modified cis-acting elements, thus targeting both genes instead of only MRF4 (Kassar-Duchossoy et al., 2004; Olson et al., 1996). In other words, disruption of the MRF4 locus negatively impact Myf5 expression, and vice-versa. MRF4$^{-/-}$ mice where Myf5 expression is normal were generated at mendelian ratios and were viable and fertile (Table 2) (Kassar-Duchossoy et al., 2004). No significant skeletal muscle defects were noted in MRF4$^{-/-}$ mice and body weight was normal, suggesting that MRF4 expression is dispensable for myogenesis (Kassar-Duchossoy et al., 2004). Myog was strongly upregulated in the muscles of MRF4, suggesting that Myog compensates the loss of MRF4 to maintain skeletal muscle homeostasis. However, MRF4 is expressed in all muscle cells and the ubiquitous expression of MRF4 in myogenesis was further demonstrated by using a MRF4 cell

lineage ablation strategy (MRF4^{Cre}: Rosa26^{DTA}), where deletion of MRF4+ cells decreased myogenesis *in utero* while adult skeletal muscle is progressively lost, resulting in lethality (Haldar et al., 2008).

Interestingly, mice with double MyoD$^{-/-}$: MRF4$^{-/-}$ mutations phenocopied Myog$^{-/-}$ animals with severe loss of myofibers and perinatal lethality, suggesting that MyoD and MRF4 have some redundant myogenic functions (Rawls et al., 1998). Myog is expressed in MyoD$^{-/-}$: MRF4$^{-/-}$ double mutant, yet because no muscles are created, this suggests that Myog alone cannot perform myogenesis *in vivo* (Rawls et al., 1998).

Myog

Myog (myogenin or Myf4) is a 25kDa protein that was first cloned from early differentiating rat myoblasts (Wright et al., 1989). In tissue culture models of myogenesis, Myog is barely detectable in proliferating myoblasts, but its expression is rapidly upregulated upon induction to differentiate by serum starvation (Figure 1) (Wright et al., 1989). Following MRF4 stimulation in mature myofiber, Myog expression progressively declines (Megeney et al., 1996). Thus, Myog acts to promote the cellular transition from myoblast to myocyte.

Mice homozygous for a null mutation in Myog survive embryogenesis, but die at birth with severely decreased skeletal muscle (Table 2) (Hasty et al., 1993; Nabeshima et al., 1993). Instead of densely packed skeletal muscle fibers seen in WT animals at birth, the same area was filled with mononuclear muscle stem cells of which only a few expressed skeletal muscle myosin (Hasty et al., 1993). These

mononuclear cells express MyoD but fail to express MRF4 (Hasty et al., 1993). This suggests that Myog is not required for the induction of MyoD, and also it suggests that Myog stimulates the expression of MRF4. Furthermore, muscle stem cells are present in Myog$^{-/-}$ mouse, indicating that Myog is dispensable for stem cells myogenic commitment (Hasty et al., 1993). Thus, MyoD cannot compensate for the loss of Myog and Myog is the only MRF whose individual expression is absolutely required to make skeletal muscle *in vivo* (Hasty et al., 1993). However, Myog is not sufficient for myogenesis because triple mutant Myf5$^{-/-}$: MyoD$^{-/-}$: MRF4$^{-/-}$ have a loss of both myoblasts and myofibers (Kassar-Duchossoy et al., 2004). Nonetheless, when Myog is inserted in the Myf5 locus, myogenesis can occur normally, suggesting that Myog can functionally replace Myf5 (Wang et al., 1996).

Establishment and heterogeneity of skeletal muscle SCs

Stem cells in the somite area in the embryo commit to a muscle stem cell progenitor lineage by the expression of Pax3 and Pax7 (Figure 2) (Bentzinger et al., 2012; Gros et al., 2005; Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). A population of Pax3/7+ stem cells proliferates extensively in the embryo and is distinct from the myoblasts that induce MyoD expression to commit to myogenesis (Relaix et al., 2005). Using lineage-tracing experiments, multipotent Pax3/7+ stem cells have been demonstrated to be the progenitors of

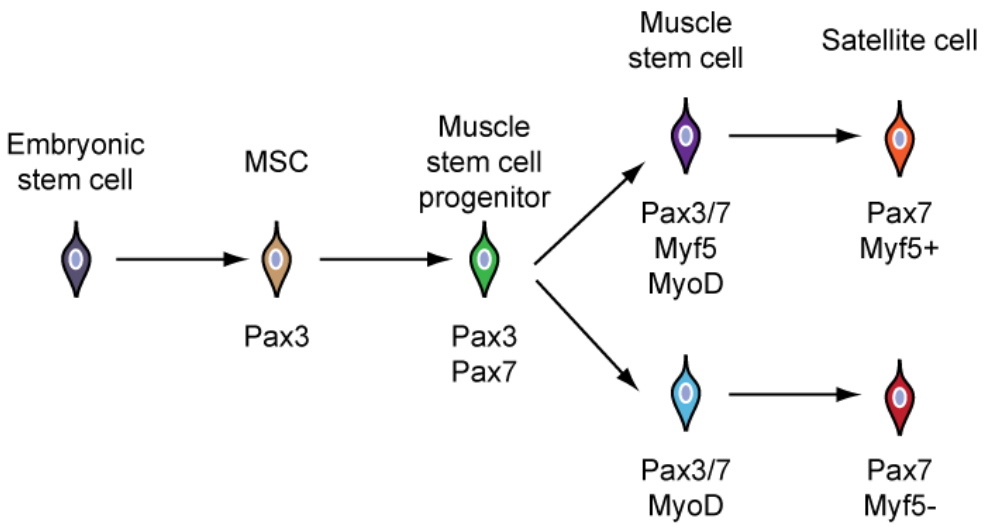

Figure 2 Embryonic development of adult SCs.

Most adult skeletal muscle SCs originate from a mesenchymal stem cell (MSC) that expresses Pax3 and all SCs have a history of Pax3 expression. MSC further commit to become a muscle stem cell progenitor via stimulation of Pax7 expression. Pax3 and Pax7 are required for satellite cell specification. Muscle stem cell progenitors commit to a muscle stem cell stage via expression of MyoD. All SCs have a history of MyoD expression (Kanisicak et al., 2009). While the vast majority of muscle stem cell also stimulates Myf5 (>90%), a small population of muscle stem cell found in adult have never expressed Myf5 (<10%) (Kuang et al., 2007). Following MyoD and Myf5 induction in muscle stem cell, Pax3 expression decreases but Pax7 remains elevated. Sometime between fetal commitment and the adult stage, SCs decrease MyoD expression such that MyoD is not expressed in adult SCs. In normal adults, all SCs maintain Pax7 expression and the vast majority also express Myf5.

adult SCs (Kassar-Duchossoy et al., 2005; Lepper and Fan, 2010; Relaix et al., 2005). Using a $Pax3^{Cre}$ transgenic mouse and a Z/EG reporter line, most Pax7+ SCs were GFP+, indicating that most SCs in the adult have expressed Pax3 in their history (Schienda et al., 2006). Using a $Pax7^{CreER}$ transgenic mouse with a $Rosa26^{Stop\text{-}\beta gal}$ reporter, labeling of Pax7+ stem cells at E9.5 or E11.5 indicated the presence of βgal+ SCs in sublaminal muscle location of newborns and of adults (Lepper and Fan, 2010). This indicated that Pax7+ stem cells were the progenitors of adult SCs.

The roles of Pax3 and Pax7 were further established by using conventional transgenic animals, where Pax3 was shown to be necessary for embryonic SCs development while Pax7 is required for post-natal SCs function and muscle regeneration (Kassar-Duchossoy et al., 2005; Lepper and Fan, 2010; Relaix et al., 2005). While stem cells of $Pax3^{sp/sp}$ do not migrate in the budding limbs and later apoptose, in $Pax7^{-/-}$ mouse, SCs are lost by apoptosis but only in the post-natal period, suggesting that Pax7 has anti-apoptotic functions in SCs (Table 1) (Relaix et al., 2006; Relaix et al., 2005). This suggests that Pax7 is required for the maintenance of the SC population in an adult context (Seale et al., 2000; von Maltzahn et al., 2014). This also suggests that Pax3 cannot compensate for the loss of Pax7 about the protection of SCs in the post-natal period. In adults, a large of number of factors and cell-surface proteins have been described (Table 3).

Table 3 Skeletal muscle SCs molecular markers identified in *homo sapiens* or in *mus musculus*.

Gene name	Q	A	Function
Pax7	+	+	Transcription factor required for SCs specification.
Pax3	+/-	-	Transcription factor required for myogenesis.
Integrin α7	+	+	Myoblasts fusion and migration.
Integrin β1	+	+	Myoblasts fusion, sarcomere physiology.
M-cadherin	+	+	Myoblasts fusion, cell-cycle exit. Dispensable for regeneration.
CXCR4	+	+	Migration of SCs
Myf5	+	+	Commitment of stem cells to myogenesis.
MyoD	-	+	Differentiation into skeletal myofibers. Required for regeneration.
CD34	+	+	Unknown.
Nestin	+	-	Cytoskeleton intermediate filament.
Msx1	+	-	Homeobox transcription factor repressor of MyoD.
C-MET	+	+	Embryonic myoblasts migration, myoblasts proliferation.
FGFR1	+	+	Increases myoblasts proliferation, inhibits differentiation.
Notch3	+	+	Represses skeletal muscle hypertrophy following injury.
Sprouty1	+	-	Promotes SCs self-renewal following injury
C/EBPβ	+	+	Promotes SCs self-renewal following injury. Repress myocyte fusion.
Syndecan4	+	+	Required for adult SC function.

Abbreviations Q: quiescent satellite cell, A: activated satellite cell, + : Present, - : Absent, +/- : Tissue specific expression. Table adapted from (Fukada et al., 2007; Kuang and Rudnicki, 2008).

Perhaps the most convincing evidence that Pax7+ SCs are required for regeneration comes from a cell lineage ablation strategy using a $Pax7^{DTR}$ transgenic animal, a finding corroborated independently by three groups (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). In $Pax7^{DTR}$, all Pax7+ cells express the diphtheria toxin receptor. Simultaneous muscle injury and intramuscular injection of diphtheria toxin into $Pax7^{DTR}$ mice stimulated myofiber loss, immune cell infiltration, inhibition of muscle regeneration, and scar tissue with fatty infiltration (Sambasivan et al., 2011). Similar findings were documented by using different transgenic animal ($Pax7^{CreER}$: $Rosa26^{eGFP\text{-}DTA}$) to deplete Pax7+ SCs (Lepper et al., 2011). Transplantation of SCs-ablated myofibers of $Pax7^{CreER}$: $Rosa26^{eGFP\text{-}DTA}$ into a nude injured host muscle also failed to regenerate new myofibers, demonstrating the muscle-intrinsic nature of the regenerative defect (Lepper et al., 2011). Finally, injection of WT Pax7+ SCs into the injured muscle of SC-depleted $Pax7^{DTR}$ mice increased up to 400-fold the number of regenerated myofibers (Sambasivan et al., 2011). This demonstrates that Pax7+ SCs are both sufficient and required for regeneration.

Mechanisms of SCs self-renewal

A key property of adult SCs is their ability to self-renew. Self-renewal of SCs refers to the replenishment of this stem cell population at the end of each bout of regeneration.

Symmetrical versus asymmetrical SCs self-renewal

SCs have been described to adopt both symmetric and asymmetric cell divisions (Figure 3). Asymmetrical SCs divisions produce a linear cell expansion while symmetrical cell division is associated with exponential mitosis. SCs can self-renew via asymmetrical cell division thus generating two different cells. In particular, Pax7+/Myf5- SCs can divide in an apical-basal fashion to produce two daughter cells: Pax7+/Myf5- that remains in the SC niche, and Pax7+/Myf5+ that commits to myogenesis and can proliferate via planar cell divisions (Figure 3A,B) (Kuang et al., 2007). Activated Pax7+/Myf5+ SCs can stimulate MyoD to orchestrate myogenesis (Figure 3C) (Kuang et al., 2007). Pax7+/Myf5- cells can also undergo a symmetrical cell division in a planar orientation, generating equal daughter cells, either Pax7+/Myf5- to replenish the uncommitted SCs population, or Pax7+/Myf5+ to expend the population of committed SCs (Figure 3B). Pax7+/Myf5- cells have the ability to perform asymmetrical cell divisions in this model, whereas Pax7+/Myf5+ exclusively perform symmetrical cell divisions (Kuang et al., 2007). Moreover, FACS isolation and transplantation of Pax7+/Myf5- SCs show increased regenerative and self-renewal capacity when compared to Pax7+/Myf5+ SCs, supporting the idea of increased "stemness" of the Pax7+/Myf5- population (Kuang et al., 2007).

SCs can also self-renew via cellular fate divergence in a stochastic model. A few Pax7+/Myf5+/MyoD+ SCs proliferating as myoblasts can decrease MyoD expression and return to a quiescent state, thus generating a new SC, while the

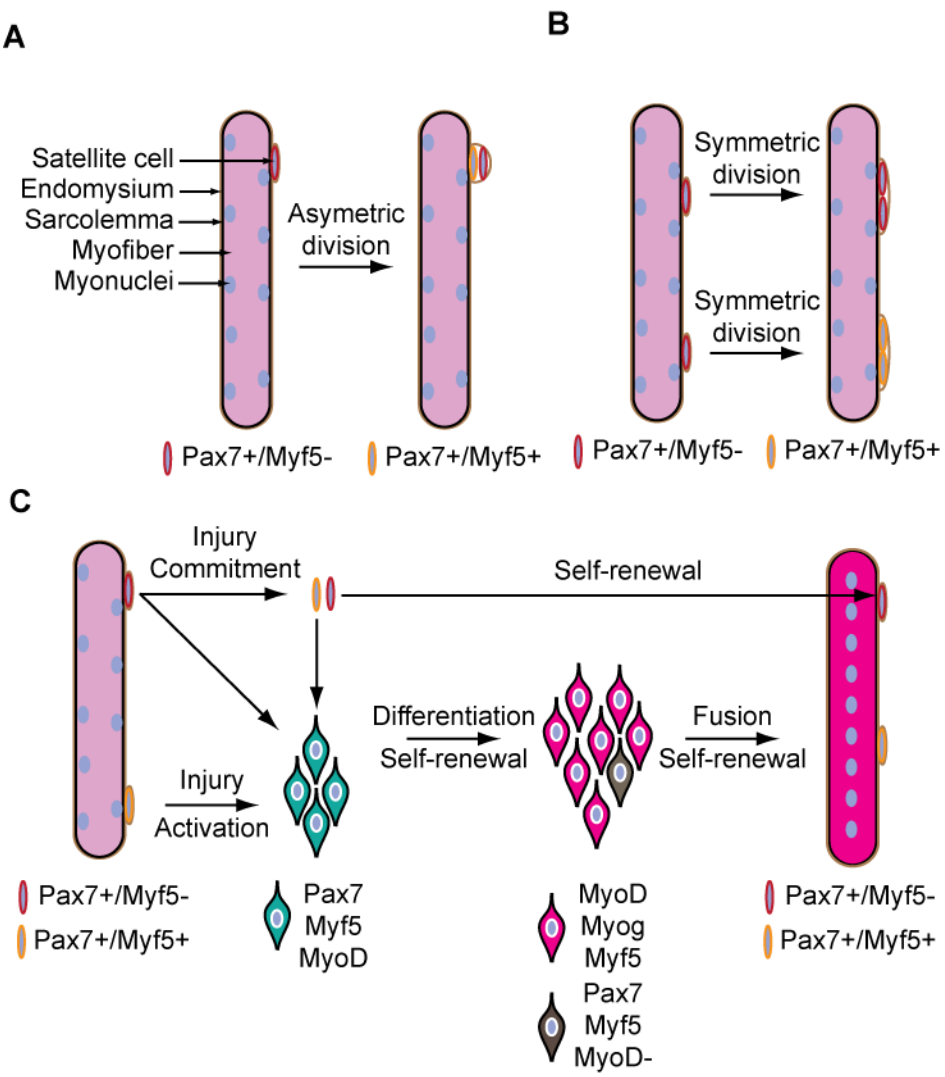

Figure 3 Mechanisms of SCs self-renewal.

(A) A Pax7+/Myf5- SC can divide asymmetrically, on an axis parallel to the fiber, to generate a committed Pax7+/Myf5+ myoblast and an identical Pax7+/Myf5- muscle SC. **(B)** Pax7+/Myf5- SCs can divide symmetrically on the plane axis to expand the muscle stem cell population by generating two Pax7+/Myf5- daughter satellite stem cell, or Pax7+/Myf5- SCs can divide symmetrically to generate two committed Pax7+/Myf5+ muscle SCs. **(C)** After injury and during regeneration, Pax7+/Myf5- muscle SCs can asymmetrically divide as in (A) where the Pax7+/Myf5- SC can

return to quiescence, while the Pax7+/Myf5+ proliferate as myoblast to regenerate the muscle. A Pax7+/Myf5- SC can also directly commit to and proliferate as myoblast. A minor proportion of proliferating Pax7+/Myf5+/MyoD+ myoblasts can escape differentiation and instead return to the SC quiescent state by decreasing MyoD expression, thus generating a new Pax7+/Myf5+ SC.

remaining MyoD+ myoblasts decrease Pax7 and upregulate myogenin to differentiate (Figure 3C) (Yoshida et al., 1998; Zammit et al., 2004). In *ex vivo* myofiber cultures, activated Pax7+/MyoD+ cells can asymmetrically segregate MyoD, where Pax7+/MyoD- SC return to quiescence (Zammit et al., 2004). Myoblasts changing fate and returning to quiescence via downregulation of MyoD expression is also documented in tissue culture system, and we call these non-differentiating cells reserve cells (Kitzmann et al., 1998; Yoshida et al., 1998). It remains unclear whether all activated SCs stimulate MyoD expression or whether a few activated SCs do not express MyoD during regeneration (Cooper et al., 1999; Kuang et al., 2008).

Evidence supports the hypothesis that MyoD expression restricts SC self-renewal. Indeed, Pax7+ SCs numbers increase by 4-fold on the injured myofiber of $MyoD^{-/-}$ mice, concomitant with a decrease number of Myog+ differentiating cells (Macharia et al., 2010). $MyoD^{-/-}$ SCs also lose their sensitivity to mitogens and continue to proliferate in serum-starved media while WT SCs are growth arrested (Macharia et al., 2010). This suggests that repression of MyoD expression promotes a partial quiescent SC phenotype.

The satellite cell niche is required for self-renewal

The SCs niche is the local anatomical environment that promotes the quiescence and maintenance of SCs (Kuang et al., 2008; Sambasivan and Tajbakhsh, 2007). The SC niche is asymmetrical by nature as SC contact both the sarcolemma and the basal lamina (Tajbakhsh, 2009). In mutant $Myf5^{GFP\text{-}P/GFP\text{-}P}$: $MyoD^{-/-}$ mice where no skeletal muscle develops, multipotent stem cells do not

adopt the sublaminal anatomical location and instead are lost by apoptosis (Kassar-Duchossoy et al., 2005). This is a proof of principle that the skeletal muscle niche is instructive in the maintenance Pax7 expression in SCs and preserves this stem cell population. The niche is the source of many extra-cellular signals that maintain and control SCs, including Wnt/β-catenin, Notch and sphingolipid (Brack et al., 2009; Conboy et al., 2003; Nagata et al., 2006). When SCs are removed from the niche, they progressively lose their stem cell properties as they differentiate into myofibers (Bentzinger et al., 2012).

In vitro and *in vivo* models of myogenesis

Myogenesis as it occurs at the embryonic development can be recapitulated in many *in vitro* and *in vivo* models. Importantly, for the most part, the embryonic transcriptional cascade is conserved when it comes to adult muscle regeneration. To study adult regeneration and myogenesis, a number of models have been developed and are commonly used.

Primary myoblasts and C2C12

The transcriptional cascade that promotes the differentiation of quiescent SCs into multinucleated myofibers can be modeled using simple tissue culture models (Figure 1). Immortalized myoblasts C2C12 cells, or primary myoblasts isolated from minced skeletal muscles, can be maintained as undifferentiated cells in proliferation in a medium with a high serum concentration, typically 10-20% of fetal bovine serum. However, primary myoblasts requires the addition of FGF and HGF in order

to be maintained in an undifferentiated and proliferative state. C2C12 are a myoblast cell line originally isolated from the crushed regenerating muscles of a mouse (Blau et al., 1983; Yaffe and Saxel, 1977). Both C2C12 and primary myoblasts, upon reaching 80-90% confluency, can be induced to differentiation by serum starvation by decreasing the serum nature and concentration to 2% horse serum. The appearance of MyHC+ muscle syncytium is observed after a few days (2 to 5 days for primary cells and C2C12 respectively) in culture.

C2C12 are commonly used in basic research and they model myogenesis effectively. However, C2C12 do not always recapitulate the biological relevance of primary myoblasts or *in vivo* regeneration. Indeed, C2C12 are tetraploid with an average of 80 chromosomes (Chang et al., 2007). In addition, the tumor suppressor gene p19ARF is deleted in C2C12, making C2C12 particularly resistant to growth-arrest (Pajcini et al., 2010). P19ARF antagonizes Mdm2 resulting in the stabilization of p53 and cell cycle arrest (Weber et al., 2000). On the other hand, primary myoblasts require more effort to culture but they have the advantage of being highly biologically relevant because they represent the heterogeneity of stem cell population as seen *in vivo*.

Physical methods

To study muscle regeneration *in vivo*, a muscle is injured to trigger SC-based myogenesis. Although crushing or laceration may be the most intuitive method to promote a muscle injury, these methods are not common practice for the study of muscle regeneration. These physical methods of injury suffer from low

reproducibility and decrease the development of scar tissue (Vignaud et al., 2007). These methods also injure the connective tissue of skeletal muscle, as well as the associated vasculature.

CTX muscle injury

Maybe the most commonly used model to study adult skeletal muscle regeneration is through intra-muscular injection of cardiotoxin (CTX). CTX is a small peptide isolated from the venom of snakes. When injected in a normal skeletal muscle (10^{-5}M), CTX induces the rapid necrosis and degeneration of skeletal muscle tissue (Couteaux et al., 1988). CTX creates pores in the sarcolemma and cause depolarization and degradation of the muscle cell (Forouhar et al., 2003; Harris, 2003). CTX does not destroy SCs, the basal lamina, blood vessels or nerves (Harris, 2003; Lefaucheur and Sebille, 1995). Cells of the immune system are rapidly recruited to the site of injury and they act to promote muscle debris clearance and activation of SCs (Lefaucheur and Sebille, 1995; Tidball et al., 2014). CTX is the model of choice because the injury to the muscle is highly reproducible. Following regeneration, the muscle histology presents muscle fibers with centrally located nuclei whereas the nucleus of an un-injured muscle fiber is located at the periphery. Fibers with central nuclei can persist as long as two months after the injury (Couteaux et al., 1988).

Barium chloride

Injection of barium chloride is also used to trigger adult skeletal muscle regeneration. Usually injected intra-muscularly at a concentration of 1.2%, $BaCl_2$ causes a rapid muscle degeneration 24 hrs post-injury, but spares both the muscle

fiber endomysium and SCs as seen with CTX injury (Caldwell et al., 1990). The mechanism of actions of $BaCl_2$ on muscle degeneration/regeneration remains poorly defined. Ba^{2+} can enter the sarcoplasm and this results in increased K+ intracellular concentration (Schott and McArdle, 1974). Given the inability of calcium chloride to rescue the detrimental effects of barium chloride, it is unlikely that a simple divalent cation competition is involved in the mechanism of actions of $BaCl_2$ (Caldwell et al., 1990; Schott and McArdle, 1974). Injection of barium chloride in the skeletal muscle causes paralysis, and barium blocks muscle contraction *in vitro* (Blaineau et al., 1993; Schott and McArdle, 1974). Barium can accumulate in mitochondria, inhibit the release of calcium exocytosis from vesicles, and can also block neutrophil chemotactic factors (Blaineau et al., 1993; Simchowitz et al., 1990). Although the mechanism of action of CTX differs from $BaCl_2$, they both result in muscle fiber necrosis and they both trigger muscle regeneration (Pichavant and Pavlath, 2014).

Murine genetic models of adult regeneration also have been created. The best-documented model is the C57BL/10ScSn Dmd ^{mdx} /J (*mdx*), a mouse model of Duchenne muscular dystrophy. The *mdx* mouse has a spontaneous nonsense mutation in the DMD gene causing muscle fiber degeneration and chronic activation of muscle regeneration (Chandrasekharan and Martin, 2011).

Cancer is a significant cause of mortality in North America

In Canada in 2011, approximately 72,000 individuals died of cancer, representing 30% of all deaths and making cancer the leading cause of mortality in the country. The most conservative estimates suggests that an excessive weight loss and muscle wasting directly cause 20% of deaths in cancer patients, a paraneoplastic syndrome known as cancer cachexia (Bruera, 1997; Warren, 1932). As much as 80% of all cancer patients will have a degree of weight loss or cachexia in advance phases of their disease (Bruera, 1997; Bruera and Sweeney, 2000; von Haehling and Anker, 2010). Cachexia correlates with frailty, morbidity and poor outcome.

Aetiology and pathophysiology of cancer cachexia

The cause of cancer cachexia is postulated to be multifactorial (Argiles et al., 2005; Argiles et al., 2014). Three groups of factors have a described role in the development of cachexia: digestive (decreased appetite, anorexia), humoral (inflammatory cytokines such as TNFα, IL-6, IL-1β, CRP, LIF, complement proteins, and IFNγ) and tumor-derived (inflammatory cytokines, but also proteolysis-inducing factor, hormonal mediators and lipid mobilization factor) (Matthys et al., 1991; Oliff et al., 1987; Strassmann et al., 1992; Tuca et al., 2013).

Mechanisms of skeletal muscle wasting in cancer cachexia

In cancer cachexia syndrome, there is evidence that both catabolism and anabolism pathways are disregulated and this results in muscle wasting, but the

role of muscle regeneration remains poorly defined in this condition (Glass, 2010; Johns et al., 2013; Pessina et al., 2010; Rennie et al., 1983). There is strong evidence that protein synthesis is decreased and protein breakdown is increased in the skeletal muscle in cancer cachexia (Argiles et al., 2005).

MAFbx & MURF1

In cancer cachexia, there is a correlation between the degree of weight loss and expression of genes involved in the ATP-dependent proteasome pathway in experimental mouse models of cancer cachexia (Khal et al., 2005). Transcriptome analysis of rats with cancer cachexia indicated that a number of genes involved in the ATP-dependent ubiquitin proteasome pathway were upregulated (Lecker et al., 2004). Of these genes, two were found to be muscle-specific, atrogin-1 (MAFbx) and MURF1, two E3 ubiquitin ligases (Zhao et al., 2007). Ectopic expression of atrogin-1 was sufficient to promote myotube atrophy, and loss of atrogin-1 in mice prevented skeletal muscle atrophy (Zhao et al., 2007). Furthermore, MURF1 was shown to specifically target thick myofilaments for proteolysis in glucocorticoid-induced mouse models of atrophy (Clarke et al., 2007; Cohen et al., 2009; Fielitz et al., 2007).

In vitro, atrogin-1 was shown to bind to and to promote the degradation of eIF3-f, a translational initiator factor (Lagirand-Cantaloube et al., 2008). Loss of eIF3 promotes atrophy, while knockdown of atrogin-1 *in vitro* prevents the degradation of eIF3-f in models of starvation, suggesting that stimulation of atrogin-1 can block protein synthesis and translation in cachexia (Lagirand-Cantaloube et al., 2008). In addition, atrogin-1 can ubiquitynate and promote the degradation of

MyoD *in vitro*, and this prevents the differentiation of myoblasts and the expression of muscle structural genes (Acharyya et al., 2004; Lagirand-Cantaloube et al., 2009; Tintignac et al., 2005).

The proteasome pathway does not exclusively mediate proteolysis of myofibrillar proteins in cachexia. Calpains have also been implicated in proteolysis in cachexia. In septic rats, treatment with a calpain inhibitor decreased the rate of proteolysis, as was muscle atrophy in dexamethasone-treated myotubes expressing constitutively active calpain-inhibitor (Fareed et al., 2006).

TNFα

TNFα is also named cachectin. In animal models, injection of cells transformed to express high levels of TNFα results in severe weight loss and cachexia, and direct infusion of TNFα in rats has been shown to decrease protein synthesis in skeletal muscle, an effect that was even amplified by co-infusion of IL-1 (Flores et al., 1989; Mahony and Tisdale, 1988; Oliff et al., 1987). Treatment of cachectic rats with antibodies targeting TNFα decreased skeletal muscle protein breakdown when compared to a second group of rats injected with a non-specific antibody (Costelli et al., 1993). Moreover, in mouse model where the TNFr1 cell surface receptor was knocked out, the negative actions of TNFα on skeletal muscle wasting were significantly decreased in tumor-bearing animals (Llovera et al., 1998). This supplementary finding supports the involvement of TNFα and its downstream signal transduction for skeletal muscle wasting. Interestingly, TNFα can destabilize the *MyoD* transcript, while MyoD protein can be targeted to the proteasome by atrogin-

1, potentially decreasing a regenerative response in cancer cachexia (Guttridge et al., 2000).

IL-6

IL-6 is another candidate secreted factor promoting cancer cachexia and a significant number of human tumors produce IL-6 (Fearon et al., 2012). IL-6 concentration positively correlated with both cachexia and mortality in a cohort of cachectic patients with prostate cancer and mouse models of cachexia (Baltgalvis et al., 2008; Kuroda et al., 2007; Moses et al., 2009; Scott et al., 1996; Strassmann et al., 1992). Further, the IL-6 receptor antagonist suramin inhibited muscle wasting and weight loss in tumor-bearing mice while loss of IL-6 in the $Apc^{min/+}$ model ($Apc^{min/+}$: IL-$6^{-/-}$) rescued muscle mass (Baltgalvis et al., 2008; Strassmann et al., 1992). Despite a role for IL-6 in the development of muscle wasting, overexpression of IL-6 alone does not cause wasting, suggesting that IL-6 is not sufficient to cause cachexia (Baltgalvis et al., 2008; Yasumoto et al., 1995).

IL-1β

IL-1β can activate the NF-κB pathway, a role also shared by TNFα. The activated NF-κB pathway can decrease muscle regeneration by inhibiting myogenesis and therefore blocking a regenerative anabolic response in cancer cachexia (Guttridge et al., 2000). IL-1β activated NF-κB can also promote the expression of E3 ligases atrogin-1 and MURF-1 (Glass, 2010). IL-1β can also activate the p38-MAPK pathway, which is important for the stimulation of atrogin-1 (Baldassare et al., 1999).

Animal models of cancer cachexia

Many animal models of cancer cachexia have been established and they can be divided into two categories: tumor graft and genetic models. Tumor graft models involve the culture of murine cancer cells and injection into either a nude mouse or a syngeneic recipient. Tumor graft models promote the rapid development of cachexia, but they do not recapitulate the slow and progressive wasting since in human cancer patients. Classic tumor graft models include the Lewis Lung Carcinoma (LLC), colon-26 and MAC-16 models.

Genetic models involve most often a single germline gene mutation promoting the spontaneous appearance of neoplasms. In contrast with tumor graft models, genetic cachexia models are slow to develop, often taking months before significant wasting is measurable, but also better represents the human pathology. The most common genetic models of cancer cachexia are the $APC^{+/min}$ and the inhibin-$\alpha^{-/-}$.

The bZIP domain is a biochemical feature of the C/EBPs

CCAAT/Enhancer Binding proteins (C/EBP) are a family of bZIP transcription factors. Orthologues of the C/EBP family have been found in *Drosophilia melanogaster*, *Xenopus laevis*, *Gallus gallus*, and in higher mammals such as *Rattus norvegicus*, *Mus musculus*, *Mesocricetus auratus* and *Homo sapiens* (Akira et al., 1990; Fornace et al., 1989; Katz et al., 1993; Kousteni et al., 1998; Landschulz et al., 1988; Poli et al., 1990; Rorth and Montell, 1992). The primary

polypeptide structure of all members of this family of genes can be divided in two regions: the N-terminus (location of trans-activation and/or repressor domains) whose homology between members is low, and the C-terminus with a high level of conservation (Figure 4) (Lekstrom-Himes and Xanthopoulos, 1998). This later region harbors a highly conserved basic region and a leucine rich region, collectively commonly called a bZIP domain (Agre et al., 1989). This bZIP domain is a requirement for both DNA-binding and dimerization with other C/EBP members (Agre et al., 1989). Dimerization is a requirement for DNA binding by C/EBP proteins, but direct contact to DNA is made by a short region composed of basic amino acids, upstream of the leucine zipper, that has been termed the “basic” region (Agre et al., 1989; Landschulz et al., 1989). C/EBPs can gain access to the nucleus because they have a nuclear localization signal sequence located in the bZIP domain (Williams et al., 1997). The classic DNA-binding sequence of C/EBPs, 5'-ATTGCGCAAT-3', is a symmetric dyad sequence, though significant variability is tolerated (Agre et al., 1989). Despite their high carboxyl-end homology, C/EBPs are strongly divergent at their N-terminus. The amino-end of C/EBPs is the location of trans-activation and/or repressor domains depending on the member. Indeed, some C/EBPs are potent repressors of transcriptional activity and others are potent activators of transcription.

The C/EBP family is composed of six genes members named α to ζ according to their order of discovery (Figure 4) (Cao et al., 1991). C/EBPα was first identified

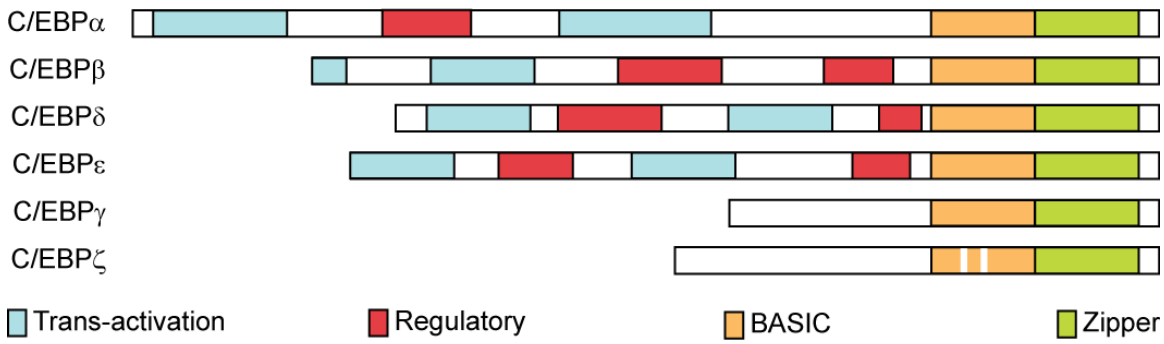

Figure 4 CCAAT/Enhancer Binding Proteins is a family of six bZIP genes.

The primary polypeptide structure of the six C/EBPs genes found in *mus musculus*. C/EBPs are name α to ζ from their discovery date. Each member has a highly conserved basic and leucine zipper regions. The only exception is C/EBPζ that has two missense mutations preventing C/EBPζ to bind DNA but remains competent for dimerization. The leucine zipper domain is required for dimerization, a necessity of DNA-binding. Trans-activation and regulatory regions are more divergent between C/EBP members. Figure adapted from (Johnson, 2005; Tsukada et al., 2011).

from *Rattus norvegicus* hepatocytes as a heat-stable protein that can bind to two types of DNA promoter sequences: the proximal promoter sequence 5'-CCAAT-3' common to murine virus promoters, and secondly to the viral enhancer regions of SV40, polyoma and also to the Moloney murine sarcoma virus (Graves et al., 1986; Johnson et al., 1987). CCAAT box is the most frequent *cis*-regulatory promoter element supporting its strong importance in stimulating the transcriptional activity (McKnight, 2001).

C/EBPβ is a mediator of inflammation and an inhibitor of apoptosis

C/EBPβ promotes the stimulation of pro-inflammatory cytokines

C/EBPβ is the second member of the C/EBP family identified, cloned independently by four groups in *Homo sapiens*, *Rattus norvegius* and *Mus musculus* (Akira et al., 1990; Chang et al., 1990; Descombes et al., 1990; Poli et al., 1990). C/EBPβ is expressed in liver, white and brown adipose tissue, lungs, myeloid cells, leukocytes, placenta, skin, spleen, intestine, kidneys, bone marrow, skeletal muscle and heart (Birkenmeier et al., 1989; Cao et al., 1991; Graves et al., 1986; Katz et al., 1993; McKnight, 2001; Scott et al., 1992). C/EBPβ expression is high in proliferating cells (McKnight, 2001; Ramji and Foka, 2002; Sebastian and Johnson, 2006). Except from C/EBPγ whose expression is ubiquitous in mammals, C/EBPβ is the member with the widest expression and hence C/EBPβ has pleiotropic functions. A review of the phenotypes of the C/EBPβ null mouse is presented in appendix (Table 4).

Work from Akira *et al.* found that C/EBPβ is a gene that is stimulated by LPS, IL-1β and IL-6 (Akira et al., 1990). Indeed, injection of LPS in mice stimulates

C/EBPβ in lungs, liver, adipose tissue, spleen and kidneys (Akira et al., 1990; Alam et al., 1992). LPS treatment also stimulated the expression of IL-6 in all tissues where C/EBPβ was expressed, suggesting that IL-6 could be a target gene of C/EBPβ (Akira et al., 1990). Furthermore, treatment of hepatocytes with IL-6 (or IFNγ) triggered the expression of a high number of C/EBPβ-dependent genes (Cantwell et al., 1998; Poli et al., 1990). In addition to stimulating C/EBPβ following cytokine treatments, IL-6 can also increase C/EBPβ DNA-binding affinity on the albumin, haptoglobin, CRP and hemopexin promoters (Oliviero and Cortese, 1989; Poli and Cortese, 1989; Poli et al., 1990). This suggests that C/EBPβ could mediate the transcriptional effects of IL-6 signaling. For these reasons, C/EBPβ was initially named both NF-IL6 and IL6-DBP.

IL-1β signal transduction can stimulate the production of cAMP, which through stimulation of PKA and CREB can induce C/EBPβ expression and phosphorylation (Gonzalez and Montminy, 1989; Niehof et al., 1997; Shirakawa et al., 1988). Moreover, IL-1β can stimulate p38 MAPK activity that is also known to stimulate C/EBPβ activity (Raingeaud et al., 1995; Trautwein et al., 1993).

Binding sites for C/EBPβ have been found in a number of cytokine promoters including: G-CSF, IL-8, TNFα, TNFr1, HGF, IL-12, IL-6, IL-1β and CRP, α1-acid glycoprotein, angiotensin, serum amyloid A and albumin (Akira et al., 1990; Bristol et al., 2009; Chang et al., 1990; Descombes et al., 1990; Drouet et al., 1991; Lekstrom-Himes and Xanthopoulos, 1998; Matsusaka et al., 1993; Plevy et al., 1997; Ramji and Foka, 2002). The function of C/EBPβ as a nuclear protein binding to IL-6 response elements in a number of promoters of inflammatory cytokines and

acute phase response genes makes C/EBPβ an important transcription factor that can transduce and mediate inflammatory signals (Poli et al., 1990). Indeed, C/EBPβ null animals are immune-compromised. In addition to being a transcription factor important for the immune system, C/EBPβ mediates the transcription of many other genes.

C/EBPβ inhibit caspases and antagonize p53 to promote tumorigenesis

C/EBPβ null animals cannot regenerate their liver following a partial hepatectomy. In this model, C/EBPβ null hepatocytes die by apoptosis. The anti-apoptotic function of C/EBPβ was mapped to a single phosphorylation event. In fact, phosphorylation of C/EBPβ by RSK was shown to be a critical post-translational modification for hepatocytes survival (Buck et al., 2001). Moreover, using a phosphorylation-mutant RSK, or an upstream MEKK phosphorylation-mutant, or an IκB phosphorylation-mutant (anti-apoptotic NF-κB member activated by MEKK), they all stimulated apoptosis of hepatocytes in this model, but were all rescued with a constitutive phosphorylated C/EBPβ at threonine 217 (Buck et al., 2001). This suggested that pThr217-C/EBPβ can promote survival in the absence of active MEKK, RSK or NF-κB (Buck et al., 2001). The K-phosphoThr217-V-D protein motif found in C/EBPβ was found to associate and to block the activation of procaspases-1 and 8 (Buck et al., 2001). Only in a serum free condition, which triggers the activation of procaspase 9, was the constitutively phosphorylated C/EBPβ mutant unable to rescue apoptosis of hepatocytes (Buck et al., 2001). This anti-apoptotic role of phosphorylated C/EBPβ is likely conserved throughout evolution as threonine 217 is conserved in mouse, bovine, rats and humans (Buck et al., 1999).

A skin epidermis conditional knockout model of C/EBPβ also reported an increased apoptosis of keratinocytes following tumorigenic treatments, and this was reported to be dependent on aberrant stimulation of tumor suppressor p53 activity and expression (Sterneck et al., 2006; Yoon et al., 2007). The basal levels of p53 are increased in C/EBPβ$^{-/-}$ keratinocytes and greatly stimulated following skin cancer chemical treatment, suggesting that C/EBPβ acts as a general inhibitor of p53 (Yoon et al., 2007). Although C/EBPβ can inhibit p53 activity *in vitro* and *in vivo*, C/EBPβ did not bind to the *trp53* promoter, and loss of C/EBPβ did not increase p53 mRNA levels, suggesting that the regulation of p53 by C/EBPβ is post-translationally regulated (Ewing et al., 2008).

C/EBPβ promotes skeletal muscle fiber atrophy in models of cancer cachexia

To date, only a small number of publications have investigated the role of C/EBPβ in muscle (Greenbaum et al., 1998; Screpanti et al., 1995; Tanaka et al., 1995). The first report on the function of C/EBPβ in skeletal muscle described an increased sensitivity to insulin via an increased expression and activity of the insulin receptor (Wang et al., 2000). In a rat model of sepsis, glucocorticoids were showed to stimulate the expression of C/EBPβ in skeletal muscle while sepsis increased the DNA-binding affinity of C/EBPβ (Penner et al., 2002). *In vitro*, glucocorticoids also potentiated the transcriptional activity of C/EBPβ in skeletal muscle (Yang et al., 2005). Moreover, glucocorticoid treatment of proliferating myoblasts stimulated the expression of atrogin-1 and MURF-1, and the induction of both genes were decreased with transfection of a siRNA for C/EBPβ (Gonnella et al., 2011). Similar

finding were also found in myofibers treated with glucocorticoids, but only the expression of atrogin-1 was rescued when a siRNA for C/EBPβ was introduced (Gonnella et al., 2011). However, forcing the expression of C/EBPβ in myoblasts and myotubes did not stimulate the expression of atrogin-1 and MURF-1, suggesting that the C/EBPβ is not sufficient to stimulate expression of these two genes (Gonnella et al., 2011).

These *in vitro* roles of C/EBPβ on muscle atrophy were also corroborated *in vivo* on animals with cancer cachexia (Zhang et al., 2011). Treatment of myofibers with conditioned media form the LLC tumor resulted in increase expression of C/EBPβ in myonuclei and stimulation of atrogin-1 expression, with concomitant loss of MyHC and muscle atrophy (Zhang et al., 2011). Grafting of the LLC tumor into C/EBPβ$^{-/-}$ animals prevented the skeletal muscle stimulation of atrogin-1 and prevented relative muscle weight loss (Zhang et al., 2011). This suggests that loss of C/EBPβ can prevent muscle atrophy in cancer cachexia, though the complete C/EBPβ$^{-/-}$ animal is immunocompromised, and thus is unlikely to have normal responses to tumor grafting (Poli, 1998; Tanaka et al., 1995).

In addition to fiber-intrinsic roles, C/EBPβ expression in cells of the immune system is also important for normal muscle regeneration (Heredia et al., 2013; Tidball et al., 2014; Tidball and Villalta, 2010). C/EBPβ expression in M2-type macrophages is required for efficient muscle regeneration and mice carrying mutations in the two CREB-binding sites on *Cebpb* proximal promoter did not regenerate efficiently ten days after injury (Ruffell et al., 2009). The role of C/EBPβ in macrophages has also been suggested to be important for muscle strength as a

correlation between strength and *Cebpb* blood expression is linked to muscle strength in 698 participants (Harries et al., 2011).

EXPERIMENTAL RATIONALE

We and others have shown that C/EBPβ is a potent promoter of adipogenesis (Marchildon et al., 2010; Rosen et al., 2002; Tanaka et al., 1997; Wiper-Bergeron et al., 2003). In addition, we have demonstrated that C/EBPβ represses the commitment of mesenchymal stem cells to osteoblastogenesis by regulating the expression of RunX2 (Dingwall et al., 2011; Wiper-Bergeron et al., 2007b). The work of others has also shown that C/EBPβ could promote chondrogenesis (Wang and Sul, 2009), but the role of C/EBPβ in myogenesis was previously unknown.

Given that C/EBPβ promoted adipogenesis while repressing the development of bone, **we hypothesized that C/EBPβ would inhibit myogenesis, and thus the formation of lean tissue**. Having established a role in muscle satellite cells, **we predicted that the regulation of C/EBPβ expression by inflammatory cytokine signaling, as in cancer cachexia, would impinge on normal muscle function**.

CHAPTER 2: CCAAT/ENHANCER BINDING PROTEIN BETA IS EXPRESSED IN SCS AND CONTROLS MYOGENESIS.

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CCAAT/Enhancer Binding Protein beta is expressed in SCs and controls myogenesis.

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

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

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

ABSTRACT

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; Charge and 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 and 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 and 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-κB can regulate *Myod1* mRNA expression through increased turnover (Guttridge et al., 2000; Li and 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 and 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 and Chojkier, 2003; Friedman, 2007; Grimm and Rosen, 2003; Rosen et al., 2000; Sebastian and 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 et al., 2007a; Wiper-Bergeron et al., 2007b; 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/EBPβ 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/EBPβ 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 pEGFP plasmid 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 was 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 carboxyl 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).

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

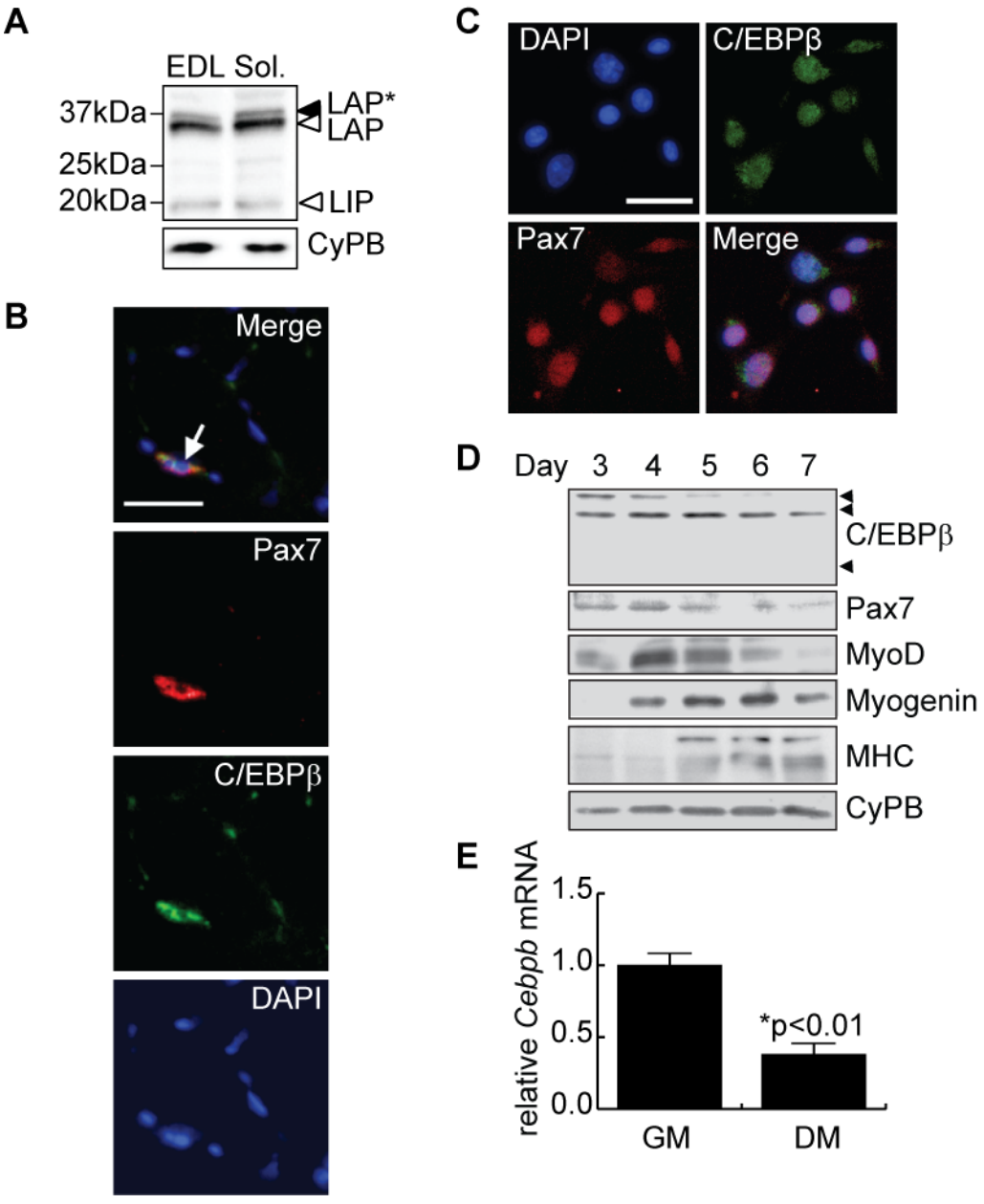

Figure 5 C/EBPβ is expressed in muscle SCs.

(A) Western analysis of C/EBPβ expression in female 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. Myoblasts were harvested from female C57BL/6 mice aged 4-6 weeks old. 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. Myoblasts were harvested from female C57BL/6 mice aged 4-6 weeks old. The expected migrations 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.

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 6B). Given that C/EBPβ has been implicated in the regulation of cell proliferation in other systems, and that

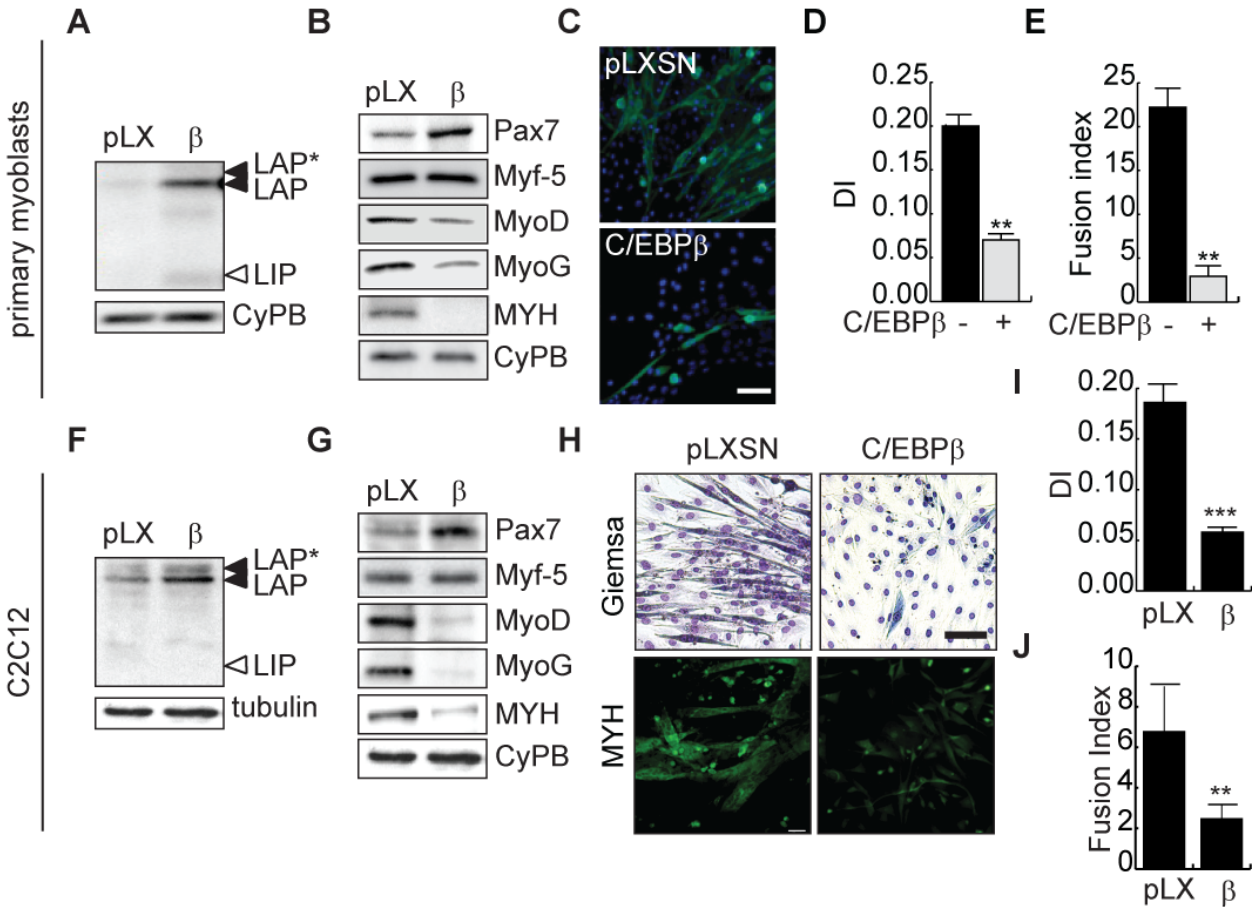

Figure 6 C/EBPβ inhibits the differentiation of myoblasts.

(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.

reduced cell proliferation could impact the efficiency of differentiation and fusion, we measured cell number in proliferating cultures using crystal violet (Figure 7A) (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

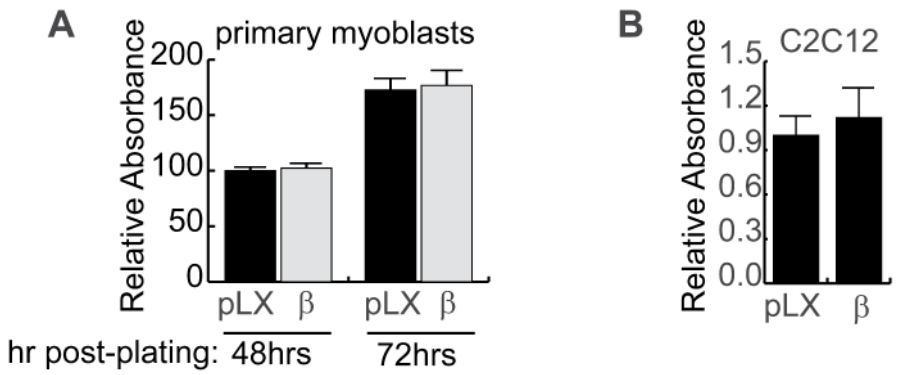

Figure 7 Overexpression of C/EBPβ in myoblasts does not affect cell growth.

(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.

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 7B).

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 8A). 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 8B). Furthermore, transient

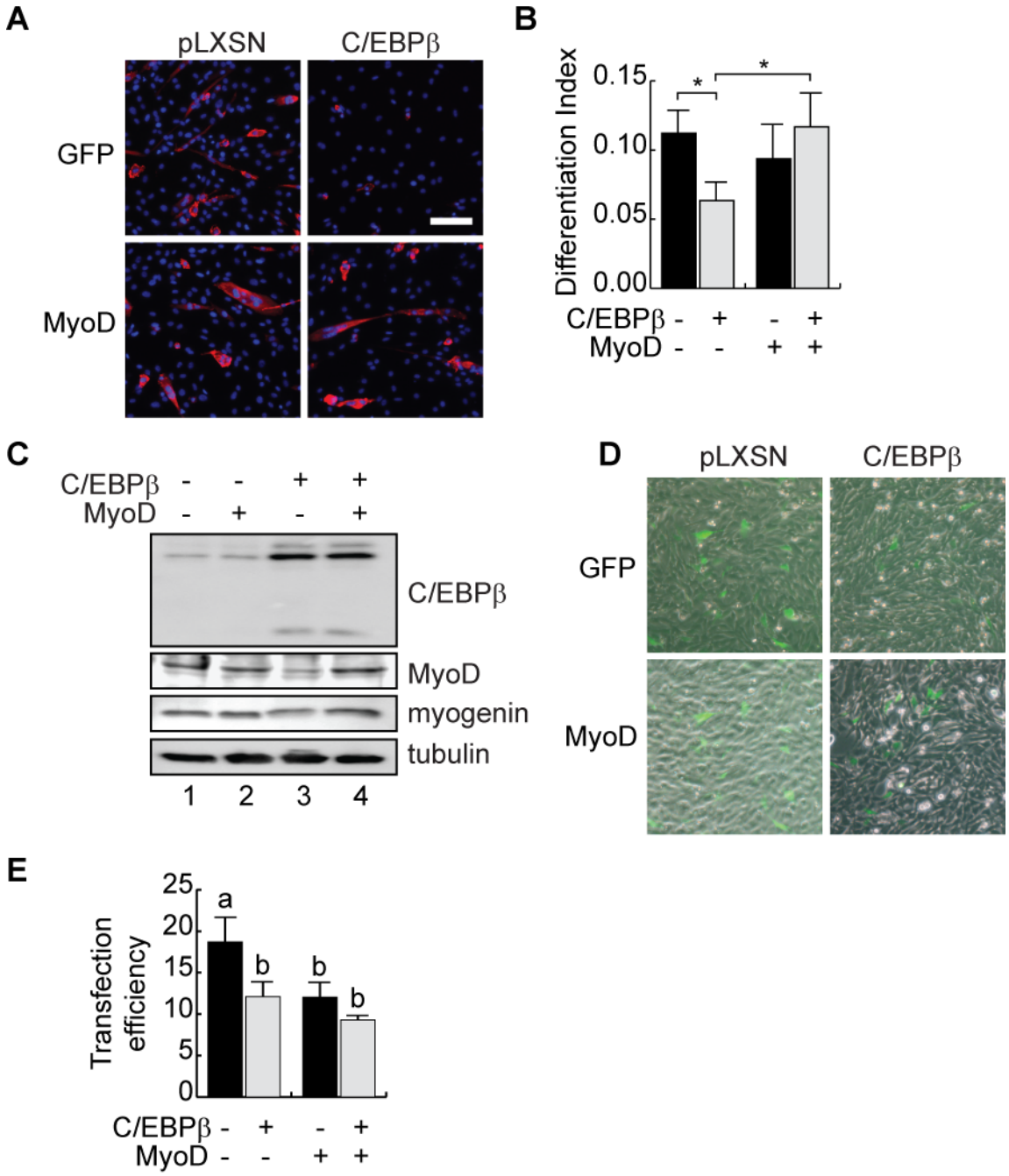

Figure 8 Restoration of MyoD protein expression rescues the differentiation defect in C/EBPβ-overexpressing C2C12 cells.

(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.

overexpression of MyoD resulted in a rescue of myogenin expression (Figure 8C), 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 8D,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 9A). 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 9B). 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 9B). Thus, we hypothesized that the increase in atrogin-1 expression

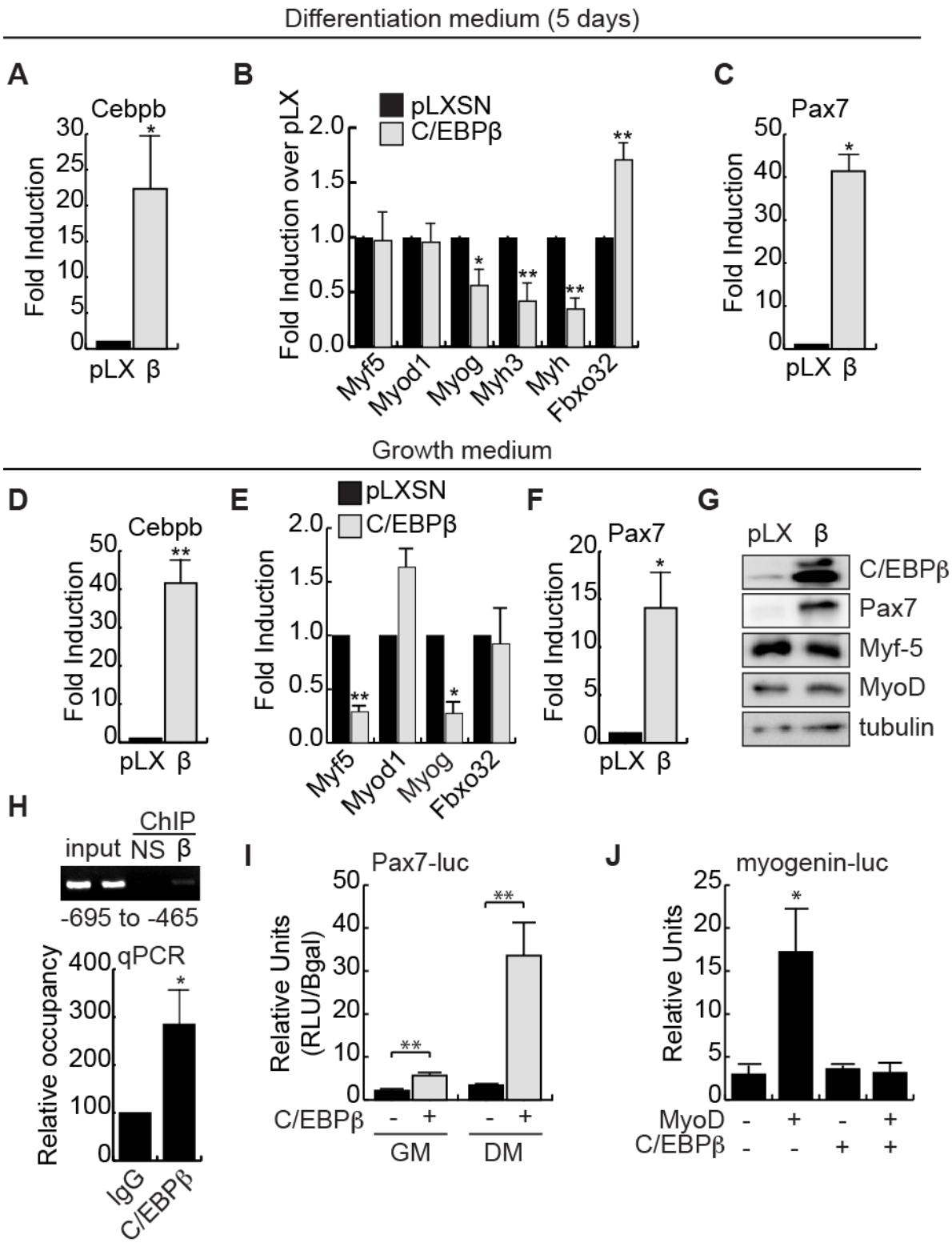

Figure 9 C/EBPβ regulates myogenic gene expression.

(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 Figure 6F). **(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 pull-down 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.

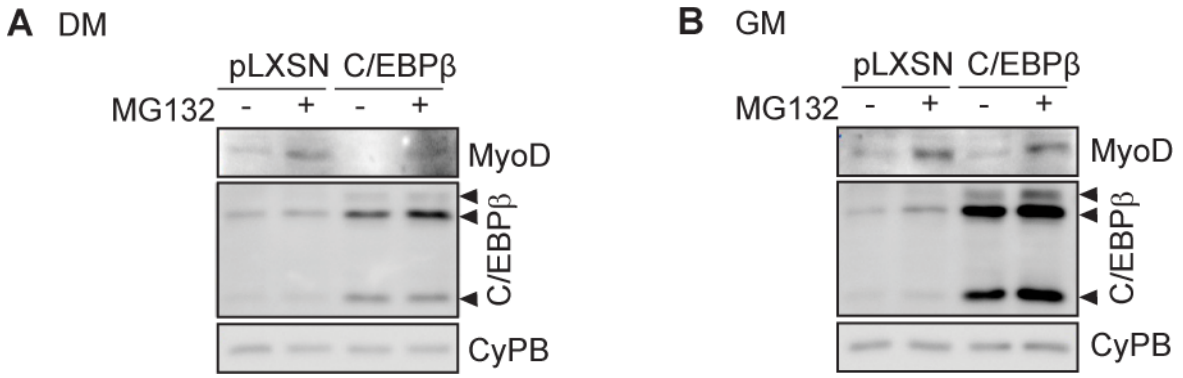

Figure 10 Loss of MyoD expression is partially rescued by the inhibition of the proteasome.

(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 pre-treated with MG132 for 2 hours prior to harvest.

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 10A). Despite the increase in *Fbxo32* expression, blocking proteasome activity with MG132 resulted in only modest and variable increases in MyoD expression 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 10).

In addition to induction of *Fbxo32* expression, *Pax7* expression was also increased 40-fold in C/EBPβ stable cell lines (Figure 9C), consistent with our western analysis (Figure 9G). 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 and 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 9D-G). In growth medium, RT-qPCR analysis revealed that *Cebpb* was expressed 40-fold over empty vector controls (Figure 9D), and both *Myf5* and *Myog* expression was significantly decreased (Figure 9E). Despite a modest increase in *Myod1* expression, this effect was not statistically significant (Figure 9E). *Fbxo32* expression was also unaffected

by C/EBPβ overexpression under growth conditions (Figure 9E), which corresponded with no change in MyoD protein expression (Figure 9G). 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 9F). 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 9G). Interestingly, Myf5 levels were only moderately decreased by C/EBPβ overexpression (Figure 9G).

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 9H). 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 9I). 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 9J). 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 knockout mouse model by breeding a floxed mouse (*Cebpb*$^{fl/fl}$) with a *Pax7*^{CreERtm} mouse (Nishijo et al., 2009) (Figure 11A). The floxed *Cebpb* mouse was bred to heterozygosity for the *Pax7*^{CreERtm} allele, and stud males were crossed with *Cebpb*$^{fl/fl}$ females (Figure 11B).

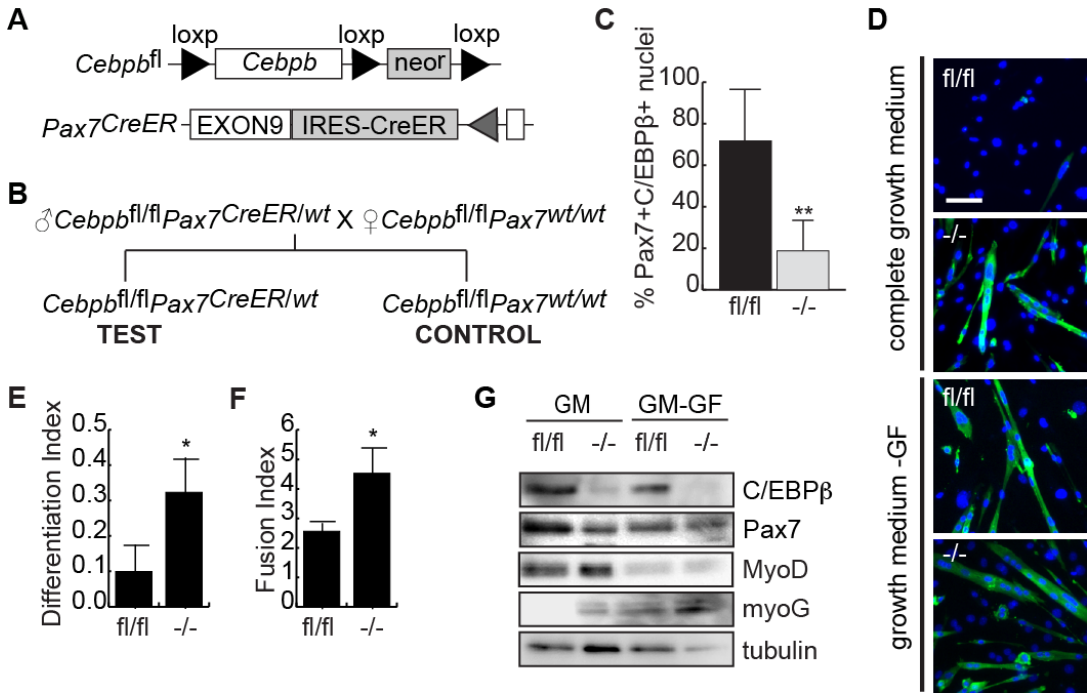

Figure 11 Loss of *Cebpb* expression in Pax7+ cells results in precocious differentiation under high serum conditions.

(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 female 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 $C/EBP\beta^{+}$ 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.

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 11C). 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 11D, 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 11D, 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 11E,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 11G). Furthermore, reduced C/EBPβ expression correlated with a decrease in Pax7 expression and a concomitant increase in myogenin expression (Figure 11G). 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 11G). MyoD levels were lowest in the most differentiated of cultures, *Cebpb*$^{-/-}$ cells withdrawn from growth factors (Figure 11G).

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 12A). 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 *Cebpb* conditional null cultures (Figure 12A). At the protein level, C/EBPβ and Pax7 protein levels were decreased following withdrawal from HGF (Figure 12B). 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.

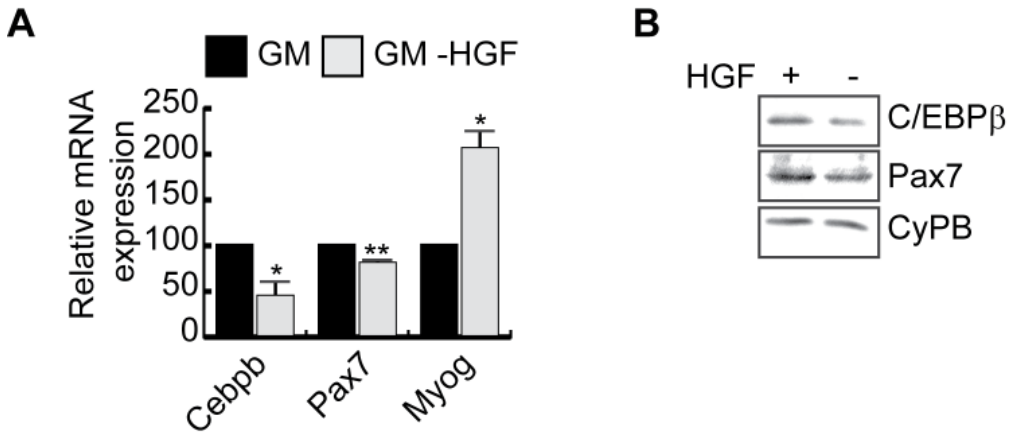

Figure 12 Withdrawal of HGF from growth medium reduces *Cebpb* expression and promotes differentiation.

(A) RT-qPCR analysis of *Cebpb*, *Pax7* and *Myog* expression in WT female 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).

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 13A,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 13B,C top). Loss of *Cebpb* expression also resulted in only a mild (17%), though not significant increase in differentiated index (Figure 13C, 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 13D). Analysis of relative mRNA expression by 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.

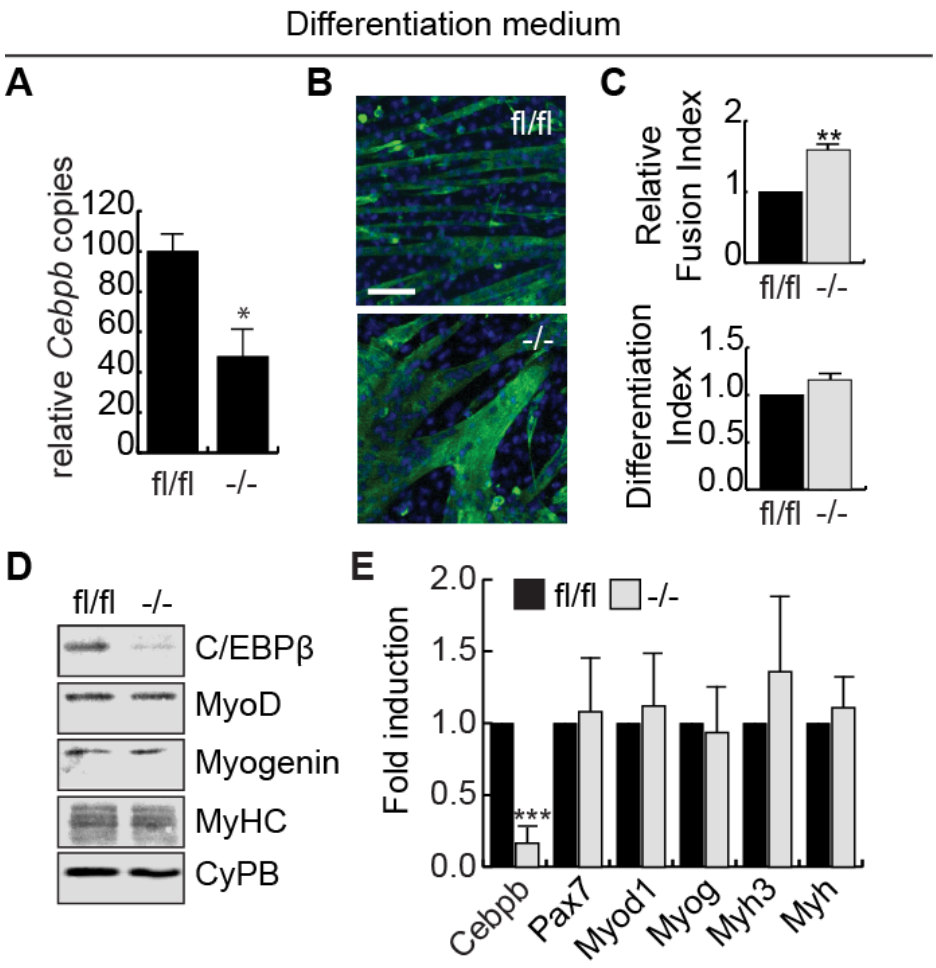

Figure 13 Loss of *Cebpb* expression in Pax7+ cells results in increased cell fusion under differentiation conditions.

Primary myoblasts from female *Cebpb*$^{fl/fl CreER-/-}$ and *Cebpb*$^{fl/fl CreER+/-}$ 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.

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 14A). The total number of Pax7+ cells in the muscle was unaffected in null mice at this time point (Figure 14B). 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 14C,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 14D). The increase in cross-sectional area was not accompanied by changes in overall fiber number (Figure 14E). 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 14F,G). Indeed, despite no difference in average cross-sectional area in female mice, there was both an

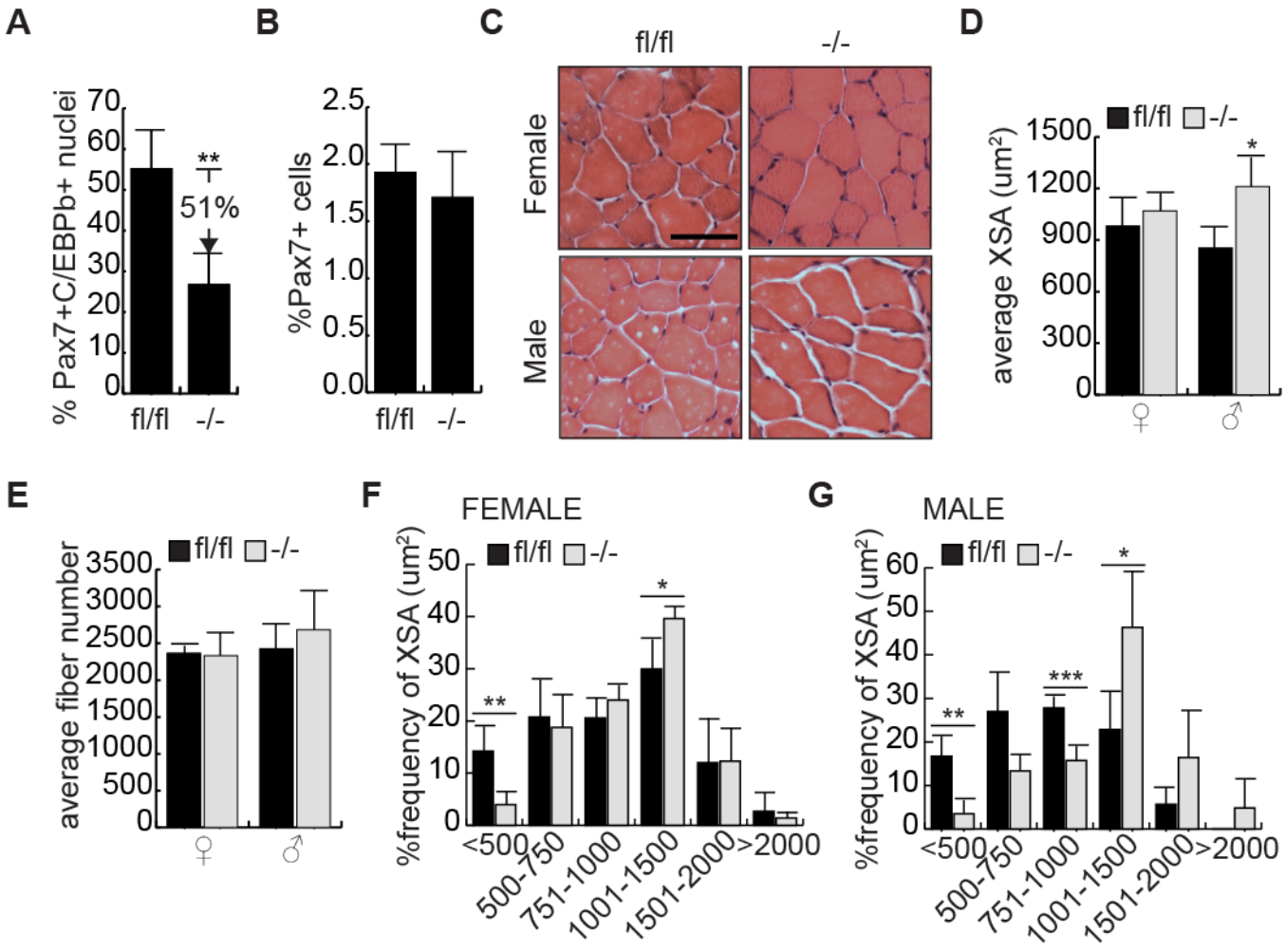

Figure 14 *Cebpb* conditional knockouts display fiber hypertrophy.

(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.

increase in larger fibers and a significant decrease in smaller fibers, suggesting that there is a modest fiber hypertrophy in the null female animals (Figure 14F). The shift in fiber size distribution was even more pronounced in male mice (Figure 14G). 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 15). 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 11C). 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 16A). 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 16A). 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

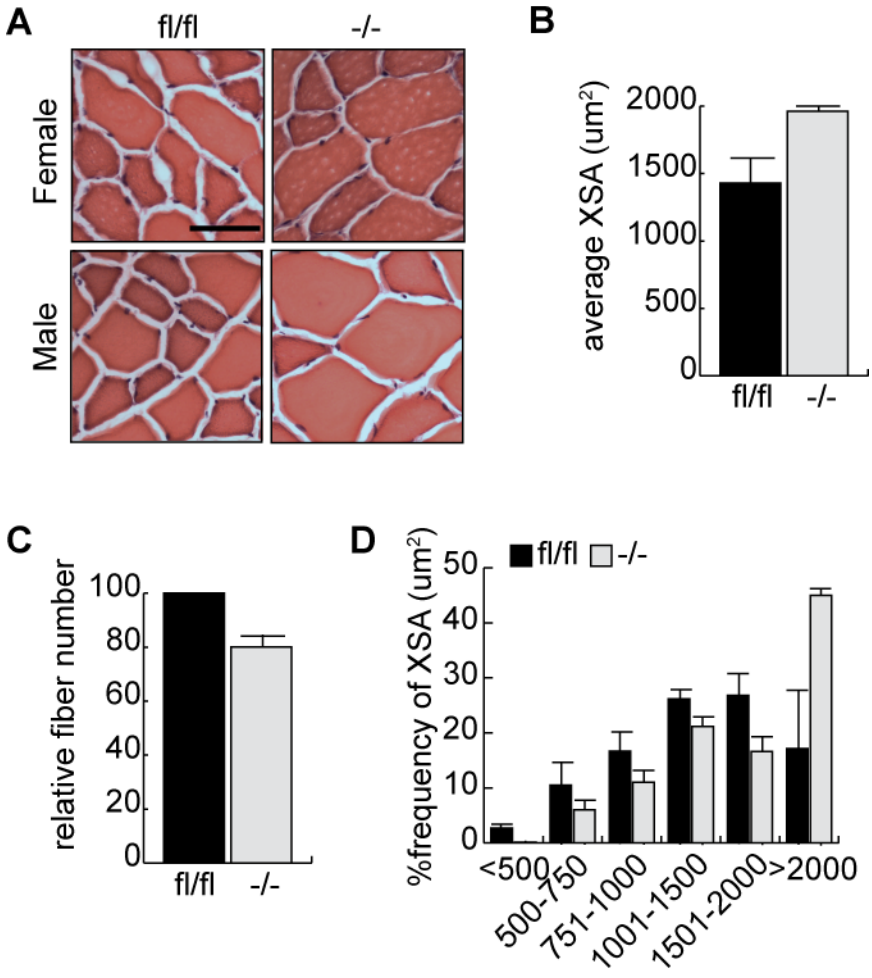

Figure 15 Fiber hypertrophy increased at day P56 in conditional knockout mice.

(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.

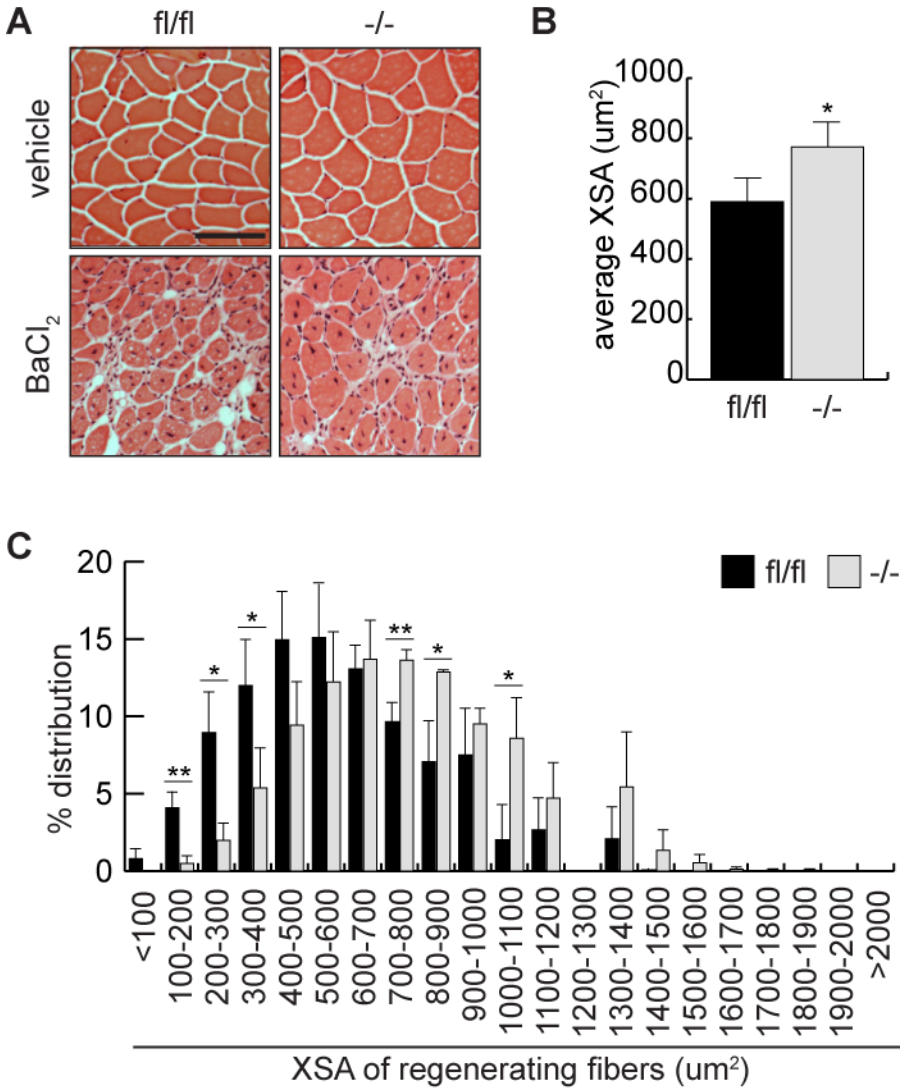

Figure 16 Muscle repair following $BaCl_2$ injury is enhanced in *Cebpb* conditional knockout animals.

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 Figure 11C). 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.

animals as compared to floxed controls (Figure 16B). 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 16C), suggesting that repair was more advanced 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 and 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-translational 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., 2002; 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 et al., 2007a; Wiper-Bergeron et al., 2007b). 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 (Argiles et al., 2006; 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 3: C/EBPB PROTECTS MUSCLE SATELLITE CELLS FROM APOPTOSIS AFTER INJURY AND IN CANCER CACHEXIA

C/EBPβ protects muscle satellite cells from apoptosis after injury and in cancer cachexia

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ABSTRACT

CCAAT/Enhancer Binding Protein beta (C/EBPβ), a transcription factor expressed in muscle satellite cells, inhibits the myogenic program and is downregulated early in differentiation. By using a satellite cell specific conditional knockout mouse model, we demonstrate that muscle regeneration following cardiotoxin injury repairs poorly with loss of C/EBPβ. In this model, we also report a decreased number of Pax7+ satellite cells (SCs) after cardiotoxin injury. This loss of muscle stem cells is attributed to an increase in SC death in mice lacking C/EBPβ. We further investigated the inflammatory response following cardiotoxin injury and we found that the levels of interleukin-1β (IL-1β), a proinflammatory cytokine, were robustly elevated following cardiotoxin injury as compared to $BaCl_2$ injury. IL-1β can induce C/EBPβ expression in myoblasts, and the stimulation of C/EBPβ expression correlated with resistance to apoptotic stimuli, whereas loss of C/EBPβ increased sensitivity to thapsigargin-induced cell death. Using cancer cachexia as a model for chronic inflammation, we found that C/EBPβ expression was increased in SCs of tumor-bearing cachectic animals. Increased expression of C/EBPβ correlated with poor myoblasts differentiation and regeneration. Further, in tumor-bearing conditional knockout animals, the SCs compartment was reduced due to increased apoptosis, indicating that C/EBPβ can promote the survival of SCs in cancer. Our findings indicate that the stimulation of C/EBPβ expression by IL-1β following muscle injury and in cancer cachexia acts to promote SCs survival, and is therefore a protective mechanism in the face of inflammation.

INTRODUCTION

Efficient regeneration of skeletal muscle is dependent on tissue-resident stem cells termed satellite cells (SCs) (Murphy et al., 2011). Quiescent and heterogeneous by nature, SCs are characterized by their histological localization between the muscle fiber sarcolemma and the basal lamina, as well as by expression of the Paired box protein Pax7 (Biressi and Rando, 2010; Kuang et al., 2007; Seale et al., 2000). Upon muscle injury, Pax7 expression declines and activated SCs progressively stimulate the myogenic basic helix-loop-helix (bHLH) regulatory factors that are required for the induction of myocyte-specific genes (Charge and Rudnicki, 2004).

CCAAT/Enhancer Binding Proteins (C/EBPs) are a family of bZIP transcription factors involved in numerous biological processes (Buck and Chojkier, 2003; Friedman, 2007; Grimm and Rosen, 2003; Rosen et al., 2000; Sebastian and Johnson, 2006). C/EBPβ null mice have defective liver regeneration (Buck et al., 1994), skin abnormalities (Zhu et al., 1999), impaired development of the mammary glands (Robinson et al., 1998), reduced adipose tissue (Tanaka et al., 1997), female sterility (Sterneck et al., 1997) and are immunodeficient (Akira et al., 1990; Screpanti et al., 1995; Tanaka et al., 1995). In healthy skeletal muscle, C/EBPβ expression is restricted to Pax7+ SCs. Highest in SCs, C/EBPβ levels decline early in differentiation and this downregulation is required for myogenesis to occur (Fu et al., 2015; Marchildon et al., 2012). Indeed, forcing C/EBPβ expression in myoblasts blocks myogenesis, and is accompanied by increased Pax7 expression and decreased MyoD, myogenin and myosin heavy chain expression. In addition, loss of

C/EBPβ in SCs results in larger myotubes in culture, muscle hypertrophy *in vivo* and enhanced muscle regeneration following a single $BaCl_2$-induced injury (Marchildon et al., 2012).

Normal skeletal muscle repair involves local inflammation that is required for efficient regeneration. M1-type macrophages are recruited early to the site of injury, produces IL-1, IL-6 and TNFα, and promote SC proliferation while inhibiting their differentiation (Bencze et al., 2012; Tidball et al., 2014). Four days after injury, M2-type macrophages become the dominant subtype in the muscle and act to decrease local inflammation by deactivating M1 macrophages (Heredia et al., 2013; Tidball and Villalta, 2010). The transient inflammatory response following acute muscle injury is accompanied by an increase in Pax7+ cells in the injured muscle that do not immediately contribute to repair (Langen et al., 2001; Merly et al., 1999; Pimorady-Esfahani et al., 1997; Toth et al., 2011). Resolution of inflammation, rather, promotes myogenesis (Arnold et al., 2007; Deng et al., 2012; Mounier et al., 2013; Ruffell et al., 2009). While acute muscle injury is accompanied by transient inflammation, chronic inflammation and dysregulated cytokine production is a feature of cachexia, characterized by both adipose tissue and skeletal muscle atrophy, and occurs in many ailments including chronic obstructive pulmonary disease, AIDS, chronic kidney failure and sepsis (Dodson et al., 2011; Kuroda et al., 2007; Saini et al., 2006; Zhou et al., 2003).

Relatively little is known about the effect of inflammation on muscle stem cell populations after acute injury, and even less under chronic conditions such as cachexia. Herein, we describe a protective mechanism where IL-1β production after

acute muscle injury or in cachexia drives increased C/EBPβ expression in muscle SCs, rendering SCs more resistant to apoptosis and preventing their differentiation. Loss of C/EBPβ expression triggers the loss of Pax7+ cells by apoptosis and impairs muscle regeneration.

RESULTS

Cardiotoxin injury increases apoptosis of C/EBPβ cKO satellite cells.

C/EBPβ conditional knockout mice (cKO), where *Cebpb* is excised in $Pax7^+$ cells, and littermate controls were injured with cardiotoxin (CTX), and repair was assessed seven days post-injury (Figure 17A,B). Unexpectedly, cKOs male mice failed to appreciably repair CTX muscle injury. In sharp contrast, cKOs repaired the injury with greater efficiency than WT controls in $BaCl_2$-induced injury as previously shown (Marchildon et al., 2012) (Figure 18). The number of fibers with centrally located nuclei, indicating regenerated fibers, was decreased by approximately 33% in cKOs after CTX injury as compared to controls with an average cross-sectional area ~24% smaller than WT controls (Figure 17C,D). Given that SCs lacking C/EBPβ can differentiate efficiently *in vitro*, we reasoned that the CTX injury might be affecting the size of the SC population in cKOs mice. While uninjured WT and cKO muscle had equivalent percentage of Pax7+ cells (~2% of total nuclei), injury increased this value approximately 2-fold in WT mice (Figure 17E). However, the

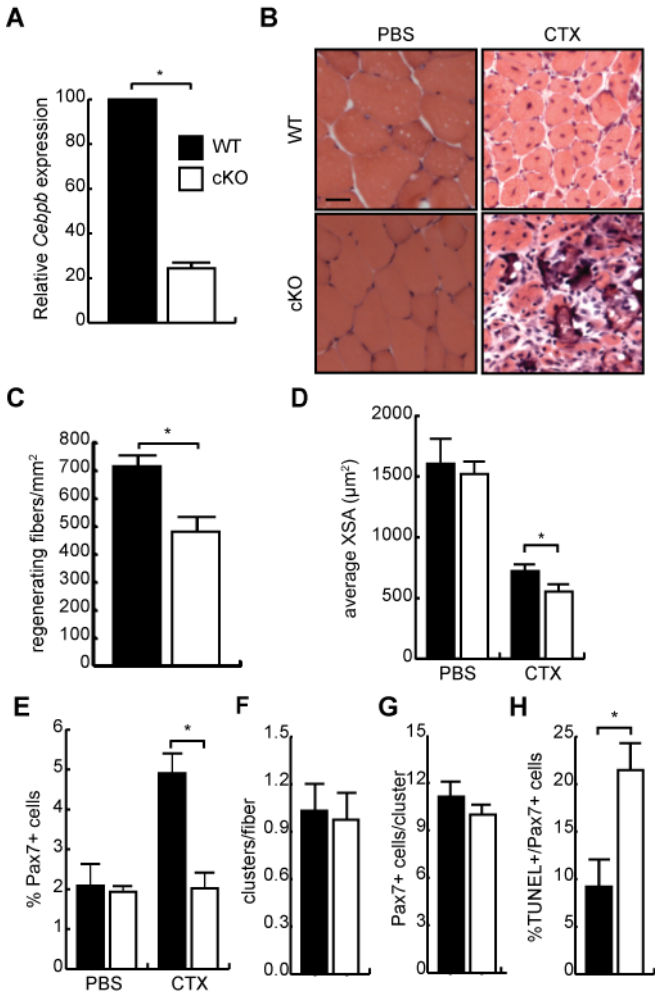

Figure 17 C/EBPβ protects muscle satellite cells from apoptosis following CTX injury.

(A) Relative *Cebpb* mRNA expression measured by qRT-PCR from myoblasts isolated from WT and cKO uninjured hind limb muscles, normalized to 18S. Both groups received IP tamoxifen treatment. *p=1x10^{-8}, n=4. **(B)** H&E-stained TA muscle cross-sections from female WT and cKO mice 7 days after injury with cardiotoxin (CTX) or sham injured with PBS. Scale bar = 20µm. **(C)** Number of regenerating muscle fibers per mm^2 in CTX-injured TA muscle from WT or cKO mice. *p=0.018, n=3. **(D)** Average fiber cross-sectional area (XSA) 7 days after

injury with CTX or sham-injured (PBS) in WT and cKO TA muscle. *p=0.047, n=4. **(E)** Percentage of Pax7+ cells (relative to total DAPI+ nuclei) found in sham-injured (PBS) and CTX-injured TA muscle from WT and cKO mice. *p=0.010, n=3. **(F)** Average number of clusters of Pax7+ cells per EDL myofiber 72 hours after isolation from WT and cKO mice. n=32 fibers. **(G)** Average number of Pax7+ cells/cluster per EDL myofibers 72 hours after isolation. n=32 fibers. **(H)** % TUNEL/Pax7+ cells (relative to total Pax7+ cells) in TA of WT and cKO mice 4 days after injury with CTX. *p=0.030, n=3.

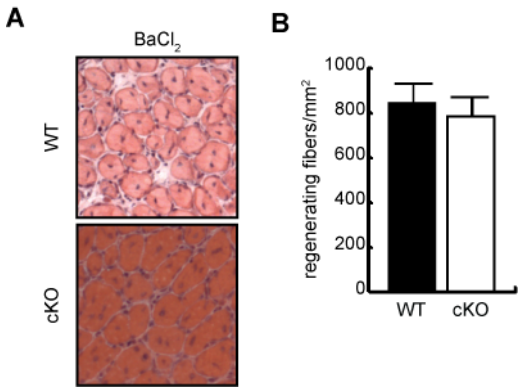

Figure 18 Loss of C/EBPβ in SCs doesn't inhibit regeneration after $BaCl_2$-injury.

(A) H&E-stained TA muscle cross-sections from female WT and cKO mice 7 days after injury with $BaCl_2$ or sham injured with PBS. n=4. **(B)** Number of regenerating muscle fibers per mm^2 in $BaCl_2$-injured TA muscle from WT or cKO mice. n=4.

increase in the Pax7+ population was not observed in the cKOs after injury, but rather remained at uninjured levels. The smaller Pax7+ population in the cKOs was not due to a failure of these cells to proliferate, as 72 hrs after myofiber isolation, equal numbers of Pax7+ SCs per cluster were observed in WT and cKO fibers, suggesting that cKO SCs activate normally (Figure 17G). Indeed, cKO myoblasts differentiate with greater efficiency than controls (Marchildon et al., 2012). We therefore investigated the apoptotic response of WT and cKO SCs following CTX injury using TUNEL staining. CTX injury provoked a ~2.5-fold increase in the number of TUNEL+/Pax7+ cells in cKOs, suggesting that after CTX injury cKOs SCs are lost by increase in apoptosis (Figure 17H).

IL-1β can stimulate C/EBPβ expression in myoblasts

Following injury to the skeletal muscle, the invasion of immune cells is prominent (McLennan, 1996; Robertson et al., 1992). Leukocytes recruited to the site of muscle injury express and secrete a constellation of immunity proteins such as cytokines that act in a paracrine fashion in the injured micro-environment (Nathan, 1987; Tidball et al., 2014). Given the polarized regenerative response to CTX and $BaCl_2$ in cKO animals, we hypothesized that a different immune response may be generated following both types of muscle injury. We first evaluated systemic inflammation post-injury by measuring plasma cytokine levels by ELISA (Figure 19). 24 hrs following injury, we did not detect any elevation in cytokine levels in the

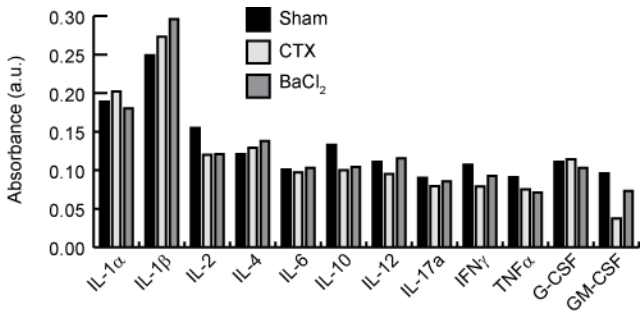

Figure 19 Undetectable elevation of cytokines in the plasma after injury.

Average absorbance values of plasma cytokines determined by ELISA. WT females C57BL/6 mice were injured with CTX, $BaCl_2$ or sham-injured. 24hrs post-injury, plasma was collected and cytokines levels were measured by ELISA following manufacturer instructions. n=4.

plasma of CTX- or $BaCl_2$-injured animals as compared to healthy animals, suggesting an absence of systemic inflammation. Therefore we investigated the local inflammation at the site of injury. We harvested CTX and $BaCl_2$ injured muscles and compared the expression levels of cytokines by qRT-PCR. Among cytokines measured, IL-1β was elevated ~6-fold in CTX-injured muscles when compared to $BaCl_2$ injury (Figure 20A). Given the high IL-1β expression in CTX-injured muscle, we asked if IL-1β could stimulate the expression of C/EBPβ in myoblasts. C2C12 and primary myoblasts were incubated with IL-1β and C/EBPβ expression was assessed by western blot (Figure 20B,C). We report a strong upregulation of C/EBPβ expression following exposure to IL-1β in both C2C12 and primary myoblasts incubated with IL-1β. Further, IL-1β treatment could increase Pax7 expression and reduce MyoD protein levels (Figure 20C).

C/EBPβ protects myoblasts from apoptosis.

C/EBPβ has previously been implicated in the survival of keratinocytes, epithelial cells of the uterus and in hepatocytes (Buck et al., 2001; Ramathal et al., 2010; Yoon et al., 2007). While quiescent SCs are known to be resistant to apoptosis, following their activation and entrance into mitosis, myoblasts become increasingly sensitive to apoptotic signals at stages where C/EBPβ level declines (Latil et al., 2012; Vahidi Ferdousi et al., 2014; Wang and Walsh, 1996). Given the increase SCs apoptosis in the CTX-injured muscle of cKO animals, we tested if

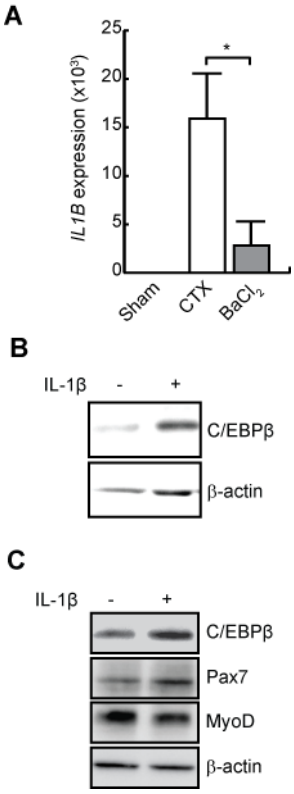

Figure 20 Il-1β upregulates C/EBPβ expression.

(A) *Il1b* expression in whole TA muscle 24 hours after injury with cardiotoxin (CTX) or $BaCl_2$. WT C57BL/6 female animals aged 8-weeks old were used. Sham injury was performed with PBS. p=0.042, n=5. **(B)** Western analysis of C/EBPβ expression in C2C12 incubated with IL-1β at 20ng/ml for 24hrs. β-actin is a loading control. **(C)** Western analysis of C/EBPβ, Pax7 and MyoD protein expression in primary myoblasts incubated with IL-1β for 24hrs. β-actin is a loading control.

C/EBPβ could promote myoblasts survival directly. C2C12 myoblasts were retrovirally transduced to express C/EBPβ or with empty virus (pLXSN) and pooled stable cell lines were treated with thapsigargin (TPG) to trigger apoptosis (Figure 21A). TPG promotes ER-stress and results in activation of caspase-12 and the intrinsic apoptotic pathway by inhibiting the sarcoplasmic reticulum calcium ATPase SERCA (Dahmer, 2005; Morishima et al., 2004; Nakagawa et al., 2000; Shiraishi et al., 2006). In the absence of treatment, approximately 10% of empty-virus controls and C/EBPβ-overexpressing myoblasts cells were dead, defined as propidium iodide-positive (PI+) and Annexin V-positive (Figure 21B). Thapsigargin treatment stimulated a two-fold increase in dead cells in empty-virus controls to approximately 20%, while ectopic expression of C/EBPβ protected against TPG-induced apoptosis, with a dead cell population of ~12% that was not statistically different from vehicle treatment. Consistent with these results, caspase 3/7 activity was decreased 66% in TPG-treated C/EBPβ-overexpressing myoblasts as compared to empty-virus controls (Figure 21C).

In primary myoblasts lacking C/EBPβ, the PI+/Annexin V+ population increased to ~27% following TPG treatment, 1.5-fold more dying cells than TPG-treated WT cultures (Figure 21D,E). Further, caspase 3/7 activity was increased by 50% in TPG-treated cKO cells as compared to WT (Figure 21F). Taken together, these data suggest that C/EBPβ regulates myoblast sensitivity to apoptosis.

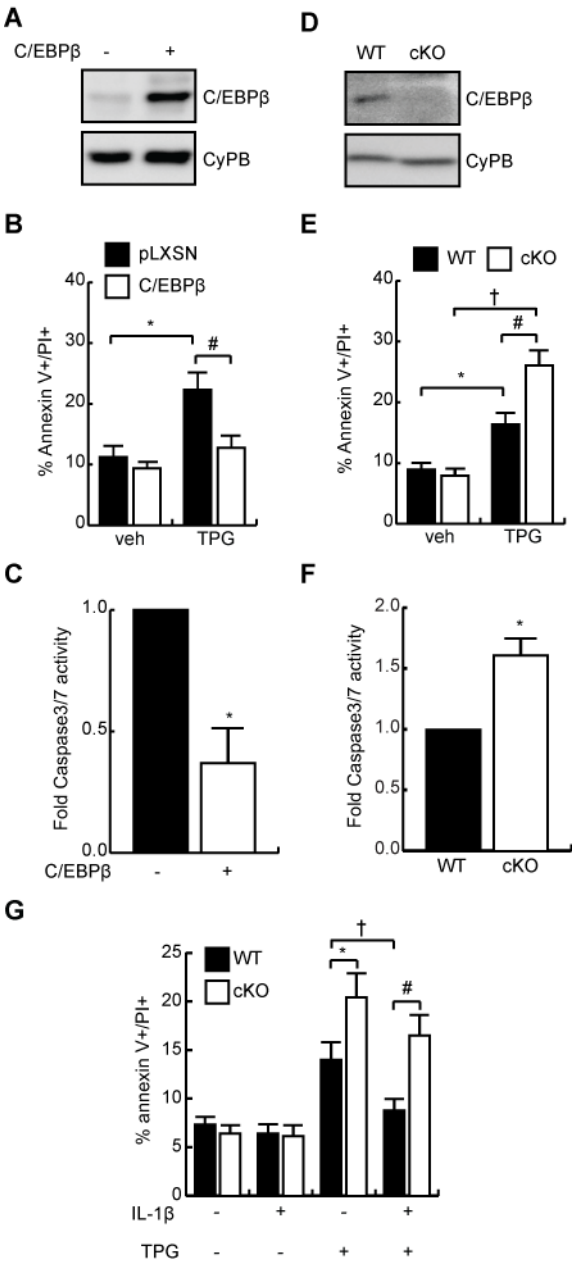

Figure 21 C/EBPβ promotes the survival of myoblasts.

(A) C/EBPβ protein expression in proliferating C2C12 cells retrovirally transduced to express C/EBPβ or with empty virus (pLXSN). Cyclophilin B (CyPB) is a loading control. **(B)** Percentage of dead cells determined by flow cytometry analysis of Annexin V and propidium iodide (PI) staining in vehicle and thapsigargin (TPG)-

treated C2C12 stable cells. *p=0.010 and #p=0.031, n=4. **(C)** Caspase3/7 activity in C2C12 overexpressing C/EBPβ treated as in (B) shown relative to C2C12 empty vector controls. *p=0.017, n=4. **(D)** Western analysis of C/EBPβ protein expression in primary myoblasts from WT and cKO mice treated with 4-OH tamoxifen for 48hrs. **(E)** Percentage of dead cells determined by flow cytometry in vehicle and TPG-treated WT and cKO myoblasts. *p=0.003, #p=0.007 and †p=$9x10^{-6}$, n=6. **(F)** Caspase3/7 activity in cKO myoblasts treated as in (E) relative to WT. *p=0.004, n=4. **(G)** Percentage of dead cells determined by flow cytometry in IL-1β-treated WT and cKO myoblasts after 24hrs in TPG, as indicated. *p=0.039, #p=0.007 and †p=0.042, n=6.

Given that IL-1β can stimulate C/EBPβ expression, we asked whether treatment with IL-1β could protect myoblasts from TPG-induced apoptosis in a C/EBPβ-dependent fashion. In WT primary myoblasts, treatment with IL-1β alone did not increase the percentage of PI+/Annexin V+ cells as compared to vehicle-treated cultures (Figure 21G). While treatment with TPG increase the percentage of WT cells dying, when IL-1β was added prior to TPG treatment to upregulate C/EBPβ, the percentage of PI+/Annexin V+ cells decreased significantly from 14% in thapsigargin-treated cells to 9% (Figure 21G). This protective effect was lost in cKO myoblasts, in which IL-1β failed to significantly reduce the population of dead cells following TPG treatment. Thus, IL-1β can protect myoblasts from apoptosis via C/EBPβ expression.

Cancer cachexia increases C/EBPβ expression in satellite cells.

Given that loss of C/EBPβ expression promotes satellite cells apoptosis in the context of acute muscle injury, we next examined the regulation of C/EBPβ expression in the context of chronic inflammation in a model of cancer cachexia. Cancer cachexia was induced in mice using the Lewis Lung Carcinoma (LLC) tumor graft model (Bennani-Baiti and Walsh, 2011; Emery, 1999). LLC cells were transplanted into WT female mice, which developed tumors (with a mean mass of 2.43±0.31g) and cachexia within four weeks of grafting. At necropsy, sham-injected animals had gained on average 1.6g, though LLC-injected animals lost 0.4g while

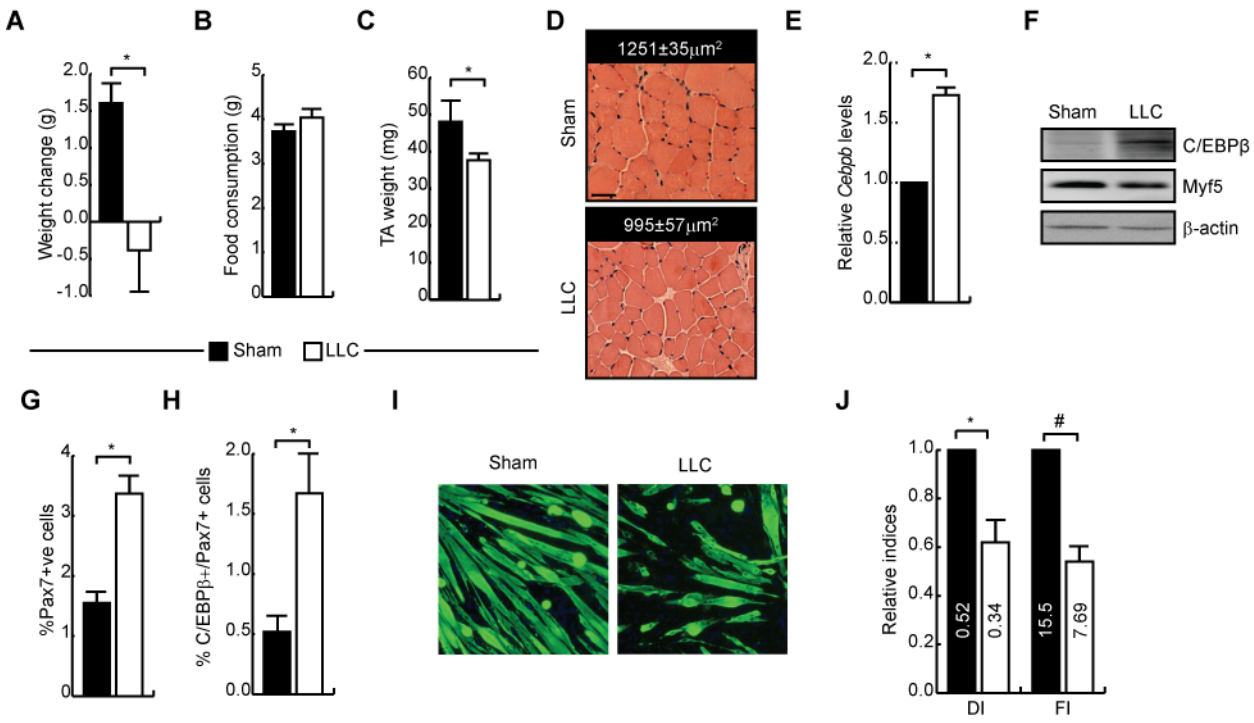

Figure 22 Cancer cachexia increases C/EBPβ expression in satellite cells and inhibits myogenesis.

(A) Average weight gain of female WT C57BL/6 sham and Lewis Lung Carcinoma (LLC)-injected mice (8 weeks old) 4 weeks after grafting. *p=0.001, n=15. **(B)** Average daily food consumption of sham and LLC tumor grafted animals. **(C)** Average TA weight from healthy and cachectic mice as in (A).*p=0.040, n=5. **(D)** H&E-stained TA cross-sections from sham and LLC-bearing mice 4 weeks after grafting. Mean fiber cross-sectional areas (XSA) are indicated ± SEM. p=0.006, n=5. Scale bar = 20μm. **(E)** *Cebpb* expression in primary myoblasts isolated from healthy and cachectic mice. *p=0.001, n=6. **(F)** C/EBPβ expression in primary myoblasts from healthy and LLC-bearing mice. **(G)** Percentage of Pax7+ cells (relative to total nuclei) in TA muscle isolated from mice as in (A). *$p=9x10^{-5}$, n=6. **(H)** Percentage of C/EBPβ/Pax7+ cells (relative to total nuclei) in TA muscle from

healthy and cachectic mice. *p=0.026, n=4. **(I)** Indirect immunostaining for myosin heavy chain expression in primary myoblasts isolated from sham and LLC tumor-bearing animals differentiated for 3 days in low serum. DAPI was used to label nuclei. n=5. **(J)** Differentiation (DI, # nuclei in MyHC+ cells/total nuclei) and fusion (FI, #nuclei/myotube) indices from cultures as in (I), shown relative to sham. Actual averages are indicated inside each bar. *p=0.003 and #p=$6x10^{-5}$, n=5.

maintaining normal appetites (Figure 22A,B). Measurement of the tibialis anterior (TA) mass in the LLC-bearing animals indicates an approximately 20% reduction (Figure 22C), and histological analysis of muscle cross-sections revealed a 20% decrease in the average fiber area in LLC injected animals as compared to sham controls, supporting the development of cachexia (Figure 22D). *Cebpb* expression in primary myoblasts isolated from healthy and tumor-bearing animals revealed a ~1.5-fold increase, as well as a significant increase at the protein level (Figure 22E,F). Further, immunohistochemical analysis of the SC population revealed that the Pax7+ population increased more than 2-fold in the TA muscles of LLC-bearing animals as compared to sham controls, indicating that SCs are present in greater numbers in cachectic mice consistent with previous observations (He et al., 2013) (Figure 22G). Further, the proportion of C/EBPβ+/Pax7+ cells was increased in the cachectic animals as compared to controls, suggesting that the cachectic milieu could also induce C/EBPβ expression in SCs (Figure 22H).

In agreement with our previous work suggesting that persistent expression of C/EBPβ in myoblasts inhibits differentiation, we tested the ability of SCs from cachectic animals to differentiate *in vitro* (Marchildon et al., 2012). To test this, we isolated primary myoblasts from cachectic and healthy mice and after in culture expansion, we induced them to differentiate in low serum conditions. Despite being removed from the cachectic milieu, the primary myoblasts isolated from cachectic mice were deficient both in differentiation and fusion, consistent with the observed stimulation of C/EBPβ expression (Figure 22I,J). Hence, in addition to muscle

atrophy and wasting, cancer cachexia can repress myogenesis that correlates with a stimulation of C/EBPβ expression.

Human cancer cachexia can promote C/EBPβ expression and block SCs apoptosis

Given that both IL-1β and LLC-induced cancer cachexia can stimulate C/EBPβ expression in myoblasts, we examined if human cancer cell lines may also stimulate C/EBPβ expression in myoblasts. To test this, the conditioned medium (CM) from human cancer cell lines was added to C2C12 myoblasts, and we assessed C/EBPβ expression by western blotting. CM from all of the tumors tested stimulated C/EBPβ expression to varying degrees, with PC-3, MCF7 and A549 lines resulting in the most robust increase in expression (Figure 23A). By contrast, the ovarian cancer cell line SKOV3 and the human colon cancer line HCT116 stimulated C/EBPβ expression the least. Incubation with CM from the PC-3 cell line also stimulated *Cebpb* mRNA expression by almost 2-fold (Figure 23B). Interestingly, while PC-3 cells expressed *Il1b,* the transcript was undetectable in SKOV3, suggesting a mechanism to explain the differential stimulation of C/EBPβ expression in myoblasts by these two cancer cell lines (Figure 23C).

Given that IL-1β protected SCs from apoptosis and that PC-3 cells express IL-1β, we asked whether the PC-3 CM could protect from TPG-induced apoptosis. WT and cKO myoblasts were incubated with PC-3 CM for six hours before

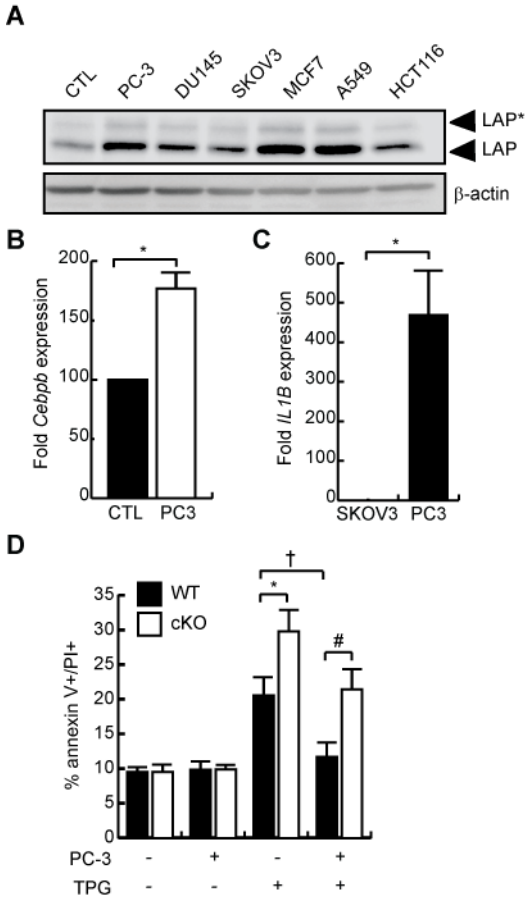

Figure 23 Human cancers stimulate C/EBPβ and promote SCs survival.

(A) Western analysis of C/EBPβ protein expression in C2C12 cells incubated with conditioned media from human cancer cell lines compared to C2C12 cells incubated with vehicle conditioned media (CTL). LAP* and LAP isoforms are indicated. β-actin is a loading control. n=3. **(B)** *Cebpb* mRNA levels in C2C12 treated 48hrs with PC-3 conditioned medium relative to non-conditioned media (CTL), normalized to 18S. *p=0.0003, n=5. **(C)** *IL1B* mRNA levels in PC-3 cells relative to SKOV3, normalized to 18S. *p=0.0088, n=5. **(D)** Percentage of dead cells assessed by flow cytometry in PC-3 conditioned media-treated WT and cKO myoblasts after 24hrs in TPG. *p=0.037, #p=0.027 and †p=0.033, n=4.

triggering of apoptosis with TPG (Figure 23D). In both WT and cKO cells, PC-3 CM did not affect the percentage of PI+/Annexin V+ cells. Similarly to what we observed following IL-1β treatment, we measured a decrease in the PI+/Annexin V+ population from 21% to 12% when WT myoblasts were pre-treated with PC-3 CM. In cKO myoblasts, PC-3 CM did not prevent apoptosis, suggesting that PC-3 CM can protect SCs from apoptosis by a C/EBPβ-dependent mechanism. Taken together, these results suggest that the induction of C/EBPβ expression by human cancer cachexia is a protective mechanism that renders myoblasts more resistant to apoptotic stimuli.

C/EBPβ is required for SCs expansion in cancer cachexia.

Our previous work has demonstrated that stimulation of C/EBPβ can block the differentiation of SCs. Whereas muscle wasting is a known symptom of cancer cachexia, the activity of SCs in cancer cachexia are poorly described. Given that cancer cachexia triggers muscle wasting and can also stimulate the expression of C/EBPβ, we hypothesized that this stimulation of C/EBPβ may be an anti-apoptosis mechanism protecting the SCs population in inflammatory disease by inhibiting SCs differentiation. To determine whether loss of C/EBPβ would sensitize satellite cells to apoptosis in the context of cancer cachexia, we grafted the LLC tumor into cKO animals (Figure 24). While there was no difference in body weight between sham-injected WT and cKO animals in the absence of tumor, both male WT and cKO

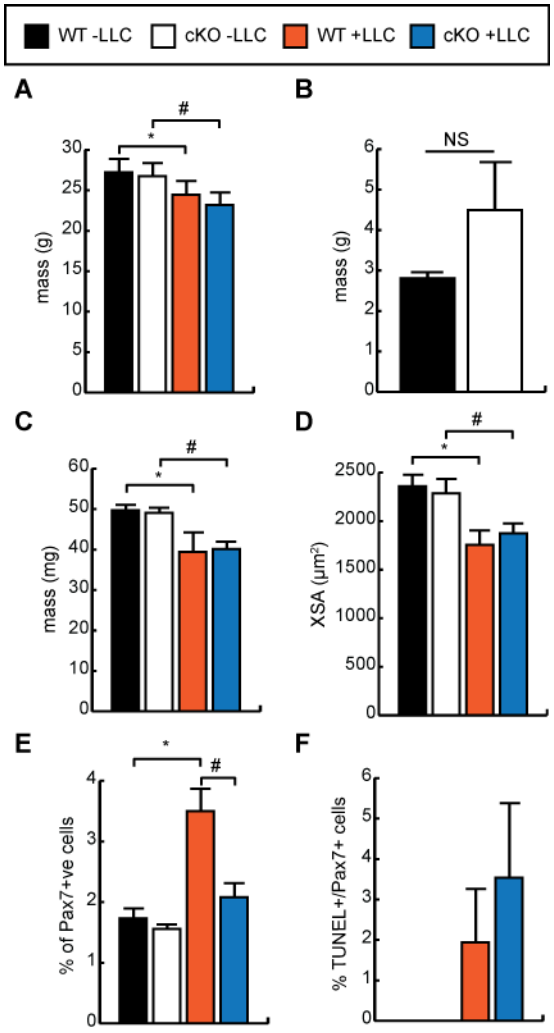

Figure 24 Loss of C/EBPβ promotes the loss of the SC compartment in cancer cachexia.

(A) Average body weight of WT and cKO mice four weeks after sham or LLC tumor graft. Eight weeks old WT and cKO male mice received IP tamoxifen injections 5 days before LLC-tumor graft. *p=0.011 and #p=0.0012, n=5. **(B)** Tumor mass at necropsy in WT and cKO animals as in (A). **(C)** TA mass in WT and cKO sham or LLC-bearing mice. *p=0.010 and #p=0.0004, n=3. **(D)** Average TA fiber cross-sectional area (XSA) from WT and cKO sham or LLC-bearing mice. *p=0.011 and

#p=0.048, n=4. **(E)** Percentage of Pax7+ cells (relative to total DAPI+ nuclei) in WT and cKO sham and LLC-bearing mice as in (A). *p=$1x10^{-4}$ and #p=0.005, n=10. **(F)** Percentage of apoptotic cells (relative to total Pax7+ cells) found in TA muscle of WT and cKO sham and LLC-bearing mice as in (A). TUNEL was used to mark apoptotic cells and SCs were labeled by IHC with a Pax7 antibody on TA cross-sections followed with DAPI staining. n=6.

animals lost approximately 10% of their body weight following tumor graft, indicative of cachexia (Figure 24A). Tumor mass was equivalent in both genotypes, though more variable in the cKOs (Figure 24B). Cachexia was accompanied by a decrease in TA weight in both WT and cKOs, though no significant differences were noted between WT and cKO tumor-bearing animals (Figure 24C). Similarly, while the average fiber cross-sectional area was decreased significantly in both genotypes with cachexia, the average muscle fiber area of cKO animals were comparable to WT cachectic animals, suggesting that loss of C/EBPβ expression in SCs did not block muscle wasting in cancer cachexia (Figure 24D). Similar findings were also documented in female cKO animals grafted with the LLC-tumor (data not shown).

Examination of the number of Pax7+ cells in uninjured WT and cKO muscle from healthy and LLC-grafted mice revealed that while the population of Pax7+ SCs in both healthy WT and cKO TA muscles were comparable, cachexia expanded the Pax7+ population from 1.7% to 3.5% in WT animals, consistent with previous reports, but not in cKO animals bearing the LLC tumor (Figure 24E) (He et al., 2013). We scored the number of TUNEL+/Pax7+ cells in TA cross-sections and found that in the absence of cancer, apoptotic SCs were not detected (Figure 24F). However, cachexia increased the percentage of TUNEL+/Pax7+ cells equivalently for both genotypes, with large variability.

C/EBPβ protects SCs from apoptosis in cancer cachexia.

Since the onset of cachexia is long, and the weight loss observed in these experiments was modest, we reasoned that apoptosis could be occurring over a long time period, making the assessment at a fixed time point difficult. To

synchronize satellite cell activation for repair, we injured both healthy and cachectic WT and cKO mice with $BaCl_2$, as this myotoxin does not on its own trigger apoptosis in the absence of cachexia. After injury, cKO tumor-bearing animals failed to repair as efficiently as WT cachectic controls (Figure 25A). Indeed, while both healthy WT and cKO animals restored TA weight similarly after injury, the TA mass of WT LLC-bearing animals was reduced to ~10% of healthy animals, whereas in cachectic cKO animals it was decreased to ~18% from healthy controls and significantly less that the tumor-bearing WT cohort (Figure 25B). This suggests that loss of C/EBPβ in SCs further impairs regeneration in cachectic animals. The average regenerating fiber cross-sectional area after injury in tumor bearing WT mice was reduced ~27% as compared to healthy controls, whereas in cKO animals, cross-sectional area after injury was reduced ~23% in cachectic animals as compared to healthy controls (Figure 25C). However, the number of regenerating fibers declined by ~37% in cKO animals indicating that the repair defect is limited to the number of regenerating fibers and not their size (Figure 25D). Taken together, these results suggest that loss of C/EBPβ can exacerbate the regeneration defect in cachectic mice.

Given that cKO animals bearing the LLC tumor had reduced regenerative capacity, we evaluated the Pax7+ SC population (Figure 25E). In tumor-bearing animals, injury expanded the Pax7+ population of WT animals when compared to healthy controls, but

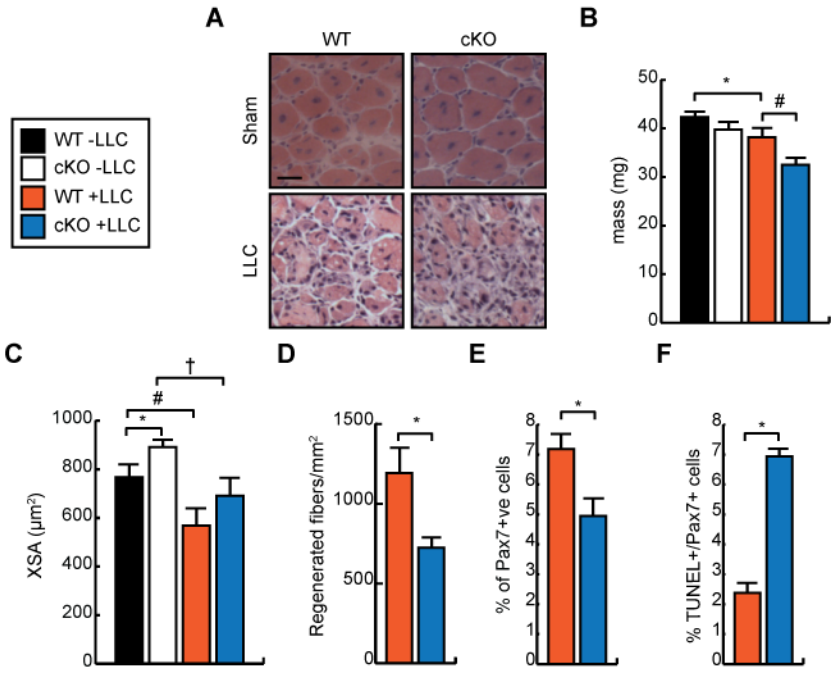

Figure 25 Increased apoptosis of SCs lacking C/EBPβ in a model of cancer cachexia.

(A) H&E stained TA cross-sections from LLC-grafted male WT or cKO mice 7 days after $BaCl_2$ injury or sham injection (PBS). Scale bar = 20μm. **(B)** Average TA mass in sham or LLC-grafted WT or cKO animals as in (A). *p=0.046 and #p=0.018, n=5. **(C)** Average TA fiber XSA in sham or LLC-grafted $BaCl_2$-injured WT or cKO mice as in (A). *p=0.048, #p=0.036 and †p=0.022, n=5. **(D)** Number of regenerating muscle fibers per mm^2 7 days after $BaCl_2$ injury to the TA in LLC-grafted WT and cKO mice. *p=0.025, n=5. **(E)** Percentage of Pax7+ cells (relative to total DAPI+ nuclei) 7 days following $BaCl_2$ injury in TA of WT and cKO LLC-bearing mice. *p=0.014, n=5. **(F)** Percentage of apoptotic cells (relative to total Pax7+ cells) found in $BaCl_2$-injured TA of WT and cKO LLC-bearing mice. *$p=9x10^{-6}$, n=4.

this was not matched in the injured TA of cachectic cKO animals. Indeed, the Pax7+ population in the cKO tumor-bearing animals was ~37% smaller than in the WT cachectic animals. The apoptosis levels were low in healthy animals after injury regardless of genotype, but in cKO animals with cachexia we observed a ~3-fold increase in the percentage of TUNEL+/Pax7+ cells compared to WT tumor-bearing animals, suggesting that C/EBPβ acts to protect satellite cells from apoptosis in the context of cachexia (Figure 25F).

DISCUSSION

Satellite cells and myotubes are known to be relatively resistant to apoptotic stimuli (Burgess et al., 1999; Latil et al., 2012). Injury and activation of satellite cells increase the vulnerability of activated SCs to apoptosis. However, the mechanism by which satellite cells withstand apoptosis is poorly defined. Pax7 expression is considered protective from apoptosis, 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 (Oustanina et al., 2004; Relaix et al., 2006). Recently, Brg1, a component of the Swi/Snf chromatin remodeling complex was shown to be required for maintaining viability in myoblasts, and this through regulation of Pax7 expression (Padilla-Benavides et al., 2015). Interestingly, Pax7 is also a target of C/EBPβ in proliferating myoblasts and in differentiating cultures (Marchildon et al., 2012). Induction of C/EBPβ by IL-1β stimulates Pax7 expression and thus, may act through this factor to protect muscle SCs from apoptosis. In

addition, Myod1$^{-/-}$ myoblasts are resistant to apoptosis both *in vitro* and *in vivo* and C/EBPβ is a potent inhibitor of MyoD protein expression, making it straightforward to speculate that C/EBPβ could also modulate sensitivity to apoptosis through the control of MyoD expression (Asakura et al., 2007). C/EBPβ has also been shown to directly inhibit caspase activity, and to regulate p53 activity and expression, both of which could also regulate sensitivity to apoptotic signals (Buck et al., 2001; Yoon et al., 2007). Interestingly, the activity of caspase-3 has been shown to be required for myogenesis (Larsen et al., 2010). Hence, whereas myoblasts overexpressing C/EBPβ are resistant to apoptosis and inhibit caspase-3 activity, we cannot exclude the possibility that myogenesis inhibition of this cell culture may be the cause of this apoptosis resistance. In acute injury, when the inflammation is short-lived, regeneration is restrained and resolution of the inflammation would be expected to reduce C/EBPβ expression allowing for the initiation of myogenesis (Arnold et al., 2007; Deng et al., 2012; Mounier et al., 2013; Ruffell et al., 2009). This is indeed consistent with the known time frame of muscle repair after injury (Arnold et al., 2007). In chronic inflammation, the levels of proinflammatory cytokines remain high, leading to an expansion of the Pax7+ population, but a defect in muscle repair. Loss of C/EBPβ expression in the context of cancer cachexia does not exacerbate muscle wasting, but does decrease the satellite cells compartment through apoptosis, and further impairs muscle regeneration (Figure 26). It remains unknown whether the SC numbers and activity recover when the inflammatory milieu

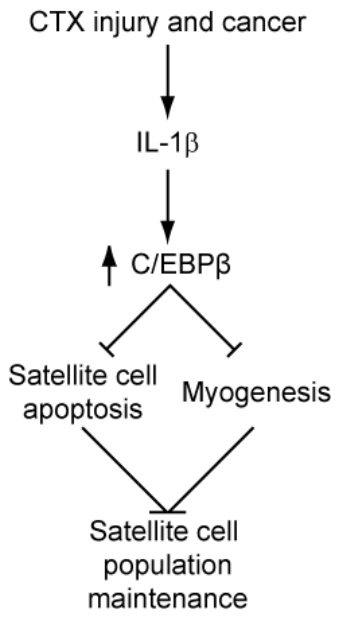

Figure 26 Model of SCs survival by inflammation and C/EBPβ.

Acute muscle injury or cancer cachexia can stimulate the production of IL-1β. In myoblasts, IL-1β signaling stimulates C/EBPβ expression resulting in two consequences: promotion of satellite cells survival and inhibition of muscle regeneration. The inhibition of satellite cells apoptosis and of satellite cells differentiation together promotes the maintenance this muscle stem cell population.

resolves, but given that tumor resection can improve cachexia, it is likely that the persistence of C/EBPβ in muscle satellite cells in the cachectic animal is transient (He et al., 2013). As such, the induction of C/EBPβ in satellite cells acts as a sensor for inflammation providing both survival signals and concomitantly a blockade of regeneration.

While a pro-survival role for C/EBPβ has been described in the development of cancer (hepatocellular carcinoma and melanomas), our results identify a function for C/EBPβ in an adult stem cell population. In *gallus gallus* C/EBPβ (NF-M) promotes survival in hematopoietic progenitor cells, suggesting that our findings could extend to a broad range of stem cell populations as well as a diverse range of organisms (Muller et al., 1995).

One of the limitations of murine cachexia models is the relatively short term in which muscle wasting is studied. Unlike human cachexia, the wasting in mice persists for only a few weeks before the cachexia is too profound for humane methods. As such, it is impossible to know from our experiments, in which the animals were sacrificed at approximately 10% weight loss with relatively mild cachexia, whether the induction of C/EBPβ expression by the cachectic environment persists as cachexia worsens or whether the cells eventually become desensitized to the effects of proinflammatory cytokines. In the case of cytokine resistance, we would expect loss of C/EBPβ expression in cachexia to trigger apoptosis of activated muscle precursors resulting in loss of the regenerative response and a more rapid loss of muscle mass. Indeed, an increase in satellite cell apoptosis in a cachexia model with more severe weight loss has been observed (He

et al., 2014). Moreover, muscle biopsies from patients suffering of gastrointestinal cancers had increased DNA fragmentation, typical of apoptosis, suggesting that loss of muscle cells contributes to wasting in humans (Busquets et al., 2007). These findings would suggest that anti-inflammatory therapy could release the C/EBPβ-imposed blockade of muscle repair and therefore counteract the loss of muscle protein observed in cachexia (Reid et al., 2013).

MATERIALS AND METHODS

Cell culture and differentiation.

C2C12 cells (ATCC) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Primary WT and cKO myoblasts cultures, obtained by enzymatic digestion and pre-plating, were maintained in DMEM supplemented with 20% FBS, 10% horse serum (HS), 10ng/ml basic fibroblast growth factor and 2ng/ml hepatocyte growth factor (Peprotech). *In vitro* activation of the Cre DNA recombinase was done by addition of 2μM 4-OH tamoxifen (Sigma). Primary myoblasts were stimulated to differentiate in DMEM supplemented with 10% HS and 2% FBS. Replication-incompetent retroviruses were produced by calcium phosphate transfection of the Phoenix packaging cell line. Infection of C2C12 myoblasts was perform as described (Springer and Blau, 1997). pLXSN-C/EBPβ plasmid has been described previously (Wiper-Bergeron et al., 2003).

Lewis lung carcinoma (LLC) and MCF7 cells were maintained in DMEM supplemented with 10% FBS. PC-3 and DU145 were maintained in RPMI 1640

supplemented with 10% FBS and 2mM L-glutamine. A549 cells were maintained in F-12K (Sigma) media containing 10% FBS. HCT116 and SKOV3 cells were maintained in McCoy (Wisent) media containing 10% FBS. All cell lines were kept in a humidified atmosphere at 37°C with 10% CO_2. To prepare conditioned media, we collected medium from confluent cancer cells cultures after 48 hours and after 0.45µm filtration, mixed this 1:1 with fresh media, which was added to C2C12 or primary myoblast cultures.

Ex vivo myofibers culture and staining.

6 to 8-week old WT and cKO mice were sacrificed and EDL muscles were digested 1h in 0.2% collagenase (Sigma) at 37°C with agitation. Loosen fibers were dissociated with decreasing bore-size Pasteur pipettes and cultured for three days in DMEM supplemented with 15% FBS (Wisent), 2% chick embryo extract (Accurate chemical) and penicillin and streptomycin. For staining, suspension fibers were washed in warm DMEM, fixed in 4% paraformaldehyde for 10minutes and quenched in 1% glycine for 5miniutes. Fibers were permeabilized in 0.3% triton X-100 and blocked 1hr in 5% goat serum and 2% BSA. Pax7 primary antibody (DSHB, 1/100) was incubated over-night at 4°C. The secondary antibody used were biotin-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, 1/500) with Cy3-strepavidin (Jackson Immunoresearch, 1/1000) 1h each. 1µg/ml DAPI (Sigma) was added for 5minutes.

Animal models.

All animal handling procedures conformed to the guidelines established by the University of Ottawa Animal Care Service and the Canadian Council on Animal Care. Mice carrying a C/EBPβ-floxed allele (Sterneck et al., 2006) and the mouse carrying the Pax7-CreER^{tm} allele (Nishijo et al., 2009) were maintained in a mixed genetic background, to generate control (WT, Cebpb$^{fl/fl}$Pax7$^{+/+}$) and conditional null (cKO, Cebpb$^{fl/fl}$Pax$^{CreER/+}$) mice. To allow growth of LLC cells in transgenic animals, C/EBPβ$^{fl/fl}$ and Pax7$^{CreER/+}$ mice were backcrossed to C57BL/6 (Jackson) mice for nine generations. For all LLC experiments, six to eight week old male and female mice were used. Young WT and cKO littermates received daily intraperitoneal (IP) injections of tamoxifen [1mg/20g] for five days. For the induction of cancer cachexia, 5x10^5 sub-confluent LLC cells washed in PBS were injected subcutaneously or PBS for sham animals.

Tibialis anterior muscle injury.

For injuries, 30µl of cardiotoxin (Latoxan, France) at 10^{-5} M or 50µl of 1.2% $BaCl_2$ (Sigma) both dissolved in PBS were injected into the left TA muscle using a 28 1/2 gauge syringe. PBS was injected in the right TA muscle for control. At necropsy, TA muscle was dissected and fixed in 10% formalin and paraffin embedded or flash frozen in melting isopentane.

Immunocytochemistry, Immunohistochemistry and TUNEL.

In situ TUNEL assays were performed according to manufacturer instructions (Roche) and counter-stained with DAPI [0.5µg/ml] for 5min. For immunohistochemistry, muscle sections were air-dried 30min at 65°C and fixed 10min in 4% paraformaldehyde. After washes, antigen-retrieval was done for 20min at 92°C with citrate buffer (10mM citric acid, 0.05% Tween-20, pH 6.0) and sections were cooled to 20°C. Sections were permeabilized 10min in 0.5% Triton X-100, washed and blocked 1 hour in 5% normal donkey serum (Jackson Immunoresearch). Antibodies used for detection were: mouse anti-Pax7 (DSHB, 1/100), rabbit anti-Laminin (Abcam 11575, 1/100), and rabbit anti-C/EBPβ (Santa Cruz Biotechnology SC-150, 1/100) biotin-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, 1/500) with Cy3-strepavidin (Jackson Immunoresearch, 1/1000), and Dylight488-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, 1/500). All primary antibodies were added on sections and incubated at 4°C over-night. Secondary antibody labeling was done for 1hr.

Reverse-transcriptase quantitative PCR.

Total RNA was purified at indicated times with the RNeasy kit (Qiagen). 1µg of purified RNA was DNase digested for one hour at 37°C (Ambion) and cDNA was synthesized using the iScript kit (Bio-Rad). cDNA was PCR amplified on a Stratagene MX3005p real-time thermocycler using a iTaq universal SYBR Green kit (Bio-Rad). Relative transcript expression was computed using the ΔΔCt method.

Caspase3/7, Annexin V and propidium iodine staining.

C2C12 or primary myoblasts were incubated with IL-1β (Sigma) at 20ng/ml for 24hrs. To trigger apoptosis, myoblasts were treated with 100nM thapsigargin for 24hrs (Sigma). Recombinant IL-1β or PC-3 CM was added 6hrs before thapsigargin. Caspase3/7 activity was assessed following manufacturer instructions (Promega). For assessment of apoptosis by flow cytometry, myoblasts were collected by trypsin, washed in ice-cold PBS and resuspended in Annexin V buffer (10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$, pH 7.4) and labeled with Annexin V and propidium iodide according to manufacturer instructions (Life technologies). Cells were analyzed by flow cytometry on a Beckman CyAn ADP instrument and dot plots were made and analyzed with Kaluza software.

Microscope acquisition and imaging.

For histological images, muscle sections were pictured with brightfield light microscope CX42 (Olympus) using a Qcapture 3 camera. Representative images are presented and were equally processed by Photoshop (Adobe) by adjusting levels uniformly. For fluorescent images, pictures were taken on a DMI3000B (Leica) epifluorescence microscope using infinity 3 (Lumenera) camera. For processing, individual pictures were level adjusted uniformly and pasted on a single color channel in Photoshop. Representative merged pictures are shown.

Antibodies and Western blot.

Whole cell lysate were prepared in IPH buffer (50mM Tris pH 7.5, 150mM NaCl, 0.5% NP-40, 5mM EDTA, 1mM DTT and 1X protease inhibitor cocktail) and briefly sonicated on ice. For detection the following antibodies were used: mouse anti-Myod1 (SC-32758, 1/500), rabbit anti-C/EBPβ (SC-150, 1/500) rabbit anti-Myf5 (SC-302, 1/500) from Santa Cruz Biotechnology, mouse anti-Pax7 (pax7, 1/200) both from DSHB, rabbit anti-Cyclophilin B (Abcam, 1/10,000) and mouse anti-β-actin (Sigma, 1/10,000).

Statistical analysis, sample size, randomization and blinding.

For bar graphs, the bar is the mean and the error bars are the standard error of the mean (SEM). For statistical analysis, two samples were subjected to a two-tailed student t-test assuming equal variance and normal distribution. Different populations were identified as having a p-value less than 0.05. All *in vitro* experiments were performed on a minimum of three independent trials. All *in vivo* experiments were performed on a minimum of five animals per group.

Inflammatory cytokines ELISA.

WT C57BL/6 female mice aged eight weeks (Charles River) received a CTX, $BaCl_2$ or were sham-injury to the TA as indicated. 24hrs later, mice were sacrificed and blood was collected by cardiac-puncture in heparin-treated syringes. Plasma was

separated by centrifugation and inflammatory cytokines levels were assess by ELISA following manufacturer instruction (Qiagen MEM-004A).

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CONFLICT OF INTEREST DISCLOSURE

The authors do not have any competing commercial interests or other conflict of interest to declare.

CHAPTER 4: GENERAL DISCUSSION

C/EBPβ: a new regulator of skeletal muscle SCs

Indirect IHC examination of C/EBPβ localization revealed that C/EBPβ is not equally distributed in muscle (Figure 5). Rather, in healthy muscle C/EBPβ expression is highest in Pax7+ SCs. Our time course analysis of C/EBPβ expression *in vitro* suggests that C/EBPβ expression is high in isolated SCs and its expression decreases as myoblasts differentiate in myotubes. In addition, we found that *Cebpb* transcript and protein are lower in differentiated myotubes when compared to SCs. This pattern of expression correlates with microarray analysis comparing quiescent SCs, myoblasts and myotubes (Fukada et al., 2007).

While the majority of Pax7+ cells express C/EBPβ *in vivo*, a small fraction of SCs in TA muscle cross-sections did not express C/EBPβ. Given that SCs are a heterogeneous stem cell population by nature, it would be informative to describe the expression of C/EBPβ in the different SCs populations (Cornelison and Wold, 1997; Sacco et al., 2008). One way to address the pattern of expression of C/EBPβ in different population of SCs is by analyzing the transcriptome of a large number of single SCs. Single SCs can be isolated by FACS from a skeletal muscle of a Pax7-nGFP transgenic mouse, and the expression of known SCs markers of each single SC may be measured by classic qRT-PCR (Sacco et al., 2008; Sambasivan et al., 2011). One population of SCs has never expressed *Myf5* and are considered to be more stem-like cells. It would be interesting to determine if this population expresses C/EBPβ and whether C/EBPβ can influence their cell fate decisions.

C/EBPβ isoform expression are dynamically regulated in SCs myogenesis

The pattern of C/EBPβ expression copies the pattern of Pax7 expression as both are stimulated in SCs and myoblasts, and downregulated in myotubes (Figure 5). Interestingly, both genes act to maintain SC populations and are important for cell survival (Asakura et al., 2007; Buck et al., 2001; Relaix et al., 2006), suggesting that C/EBPβ and Pax7 act in the same pathway. Indeed, C/EBPβ is sufficient to stimulate Pax7 expression, though it is not required for this function (Figure 9), and our results suggest that this may be a more important role in the postnatal animal.

In vitro, western analysis of C/EBPβ isoform expression during SCs differentiation indicates that LAP isoform is the most highly expressed (Figure 5). LAP*, the longest isoform of C/EBPβ is also expressed in myoblasts, but the shortest inhibitory isoform LIP is almost undetectable, suggesting that LAP is the most functionally important C/EBPβ isoform during myogenesis. LAP is the strongest transcriptional activator and given its high expression in myoblasts, this suggests that LAP may promote the expression of important targets for the maintenance of the SCs phenotype, including Pax7 (Figure 9). In contrast to LAP, LAP* has 21 extra amino acids at the N-terminus that allows it to recruit an ATP-dependent SWI/SNF chromatin remodeling complex (Kowenz-Leutz and Leutz, 1999; Kowenz-Leutz et al., 2010). Brg1, a component of this complex, is known to promote SCs proliferation and survival by stimulating the expression of Pax7 in proliferating myoblasts (Padilla-Benavides et al., 2015). Interestingly, Padilla-Benavides *et al.* hypothesized that LAP* may recruit Brg1 to the *Pax7* locus to stimulate its expression. This hypothesis is consistent with our present findings that

C/EBPβ stimulates Pax7 and also promotes SCs survival (Figure 9). To determine if C/EBPβ and Brg1 form a transcriptional complex, chromatin immunoprecipitation could be used to determine if they bind to the same region of DNA at the *Pax7* promoter. CRISPR/Cas9 mutation of the start codon preventing LAP* to be translated would be one model to establish a physiological role for LAP* in skeletal muscle.

Alternatively, it could be of interest to determine if the LIP isoform of C/EBPβ, which is not expressed in satellite cells from healthy muscle, is expressed in diseased muscle. If so, it would be expected that this isoform would act as a dominant negative for LAP*/LAP, and based on the results presented herein, would drive a more robust regenerative response at the expense of maintenance of the stem cell pool and increase sensitivity to apoptosis with inflammation.

C/EBPβ is an inhibitor of myogenic differentiation

To better define the functions of C/EBPβ in SCs, we used classic gain and loss of function models. When myoblasts ectopically expressing C/EBPβ were cultured to confluency and then differentiated, the formation of myotubes was inhibited when compared to empty vector controls (Figure 6). This data supports the hypothesis that increased levels of C/EBPβ can block myogenic differentiation. The inhibition of myogenic differentiation by C/EBPβ was rescued upon introducing a mutation in the DNA-binding domain of C/EBPβ, indicating that DNA-binding was required for the inhibition (Li, 2011).

C/EBPβ-overexpressing cultures had decreased expression of MyoD, myogenin and MyHC, while Myf5 expression remained unchanged in both control and C/EBPβ overexpressing cultures (Figure 6). Pax7 expression was also strongly upregulated in cells ectopically expressing C/EBPβ. Given that the expression of MyoD is upstream of both myogenin and MyHC, both repression of MyoD expression and stimulation of Pax7 are two plausible targets for C/EBPβ-dependent inhibition of myogenic differentiation. Transient transfection of MyoD in C/EBPβ overexpressing cells could partly rescue the differentiation defect measured in C/EBPβ overexpressing cells, restoring Myog and MyHC in C/EBPβ overexpressing cells (Figure 8). This demonstrates that part of the differentiation defect seen in C/EBPβ-overexpressing cells is caused by decreased MyoD expression and is consistent with previous reports documenting the phenotype of $MyoD^{-/-}$ mouse (Megeney et al., 1996; Sabourin et al., 1999). In addition, forced expression of Pax7 has been shown to repress myogenic differentiation in some models (Olguin and Olwin, 2004; Zammit et al., 2006b). Given that $MyoD^{-/-}$ SCs represent an intermediate between SCs and myoblasts, this may suggests that myoblasts overexpressing C/EBPβ have a phenotype that is less myoblasts and more muscle SC-like (Sabourin and Rudnicki, 2000). Indeed, we hypothesize that SCs overexpressing C/EBPβ have a phenotype resembling the MyoD null cells.

The evidence presented above all involves *in vitro* models, but a formal *in vivo* demonstration would be ideal to re-enforce the inhibition of myogenic differentiation by C/EBPβ. One way to address the differentiation potential of ectopic C/EBPβ myoblasts is to perform a transplant assay of the aforementioned C/EBPβ-

overexpressing stable cell line into a regenerating muscle of a WT recipient mouse. Another way to address this would be to create a transgenic mouse using the Pax7 promoter (Tg:Pax7-C/EBPβ) such that any cell expressing Pax7 will express high levels of C/EBPβ. In the context of this thesis, a positive regulatory loop between C/EBPβ and Pax7 would be created in this transgenic mouse model. Following injury, we hypothesize that the Tg:Pax7-C/EBPβ mouse will not display regenerated muscle fibers but instead would display an accumulation of activated SCs.

Creation of a SCs-specific C/EBPβ conditional knockout mouse model

The C/EBPβ$^{-/-}$ mouse has many defects in both immunity and metabolism (Table 4) all of which could negatively impact skeletal muscle development and regeneration in a muscle-extrinsic manner (Heredia et al., 2013; Rabinovsky et al., 2003; Serrano et al., 2008; Staiger et al., 2009). The first report assessing the phenotype of the C/EBPβ$^{-/-}$ mouse indicated a normal skeletal muscle histology, though a detailed analysis was not provided, but nonetheless confirming that C/EBPβ is not required for development of skeletal muscle (Screpanti et al., 1995; Tanaka et al., 1995; Tanaka et al., 1997). However, upon close examination of the hindlimb muscles of C/EBPβ$^{-/-}$ mice, we noted that the average fiber cross-sectional area was smaller when compared to WT littermates (Li, 2011), in contrast to our observations in a conditional knockout animal (Figure 14). The decrease in muscle size could be caused by impaired insulin signaling to the skeletal muscle and a decrease in both blood growth hormone levels, growth factor levels and glucose concentration seen in the C/EBPβ$^{-/-}$ mouse (Croniger et al., 2001; Liu et al., 1999; Staiger et al., 2009; Wang et al., 2000). All these various phenotypes made the

investigation of the roles of C/EBPβ in skeletal muscle complicated to interpret in the total absence of C/EBPβ.

One of the limitations of our tamoxifen-dependent conditional knockout model is the incomplete excision of C/EBPβ, resulting in variable phenotypes. While we measure efficient recombination of C/EBPβ in normal Pax7+ SCs (Figure 11), we did not specifically measure recombination levels post-injury. In the context of uninjured muscle, SCs turnover is low and therefore recombination escapers are unlikely to "spontaneously" increase in numbers and mask and repopulate the niche (Mourikis et al., 2012). Using a similar Pax7^{CreERtm} model as described in this thesis, three groups found that IP injections of tamoxifen lead to a partial phenotype following muscle injury, and the excision could be increased by supplementing the chow with tamoxifen (Gunther et al., 2013; von Maltzahn et al., 2014). The incomplete excision following IP injection of tamoxifen prior to injury permitted the repopulation of the SCs niche by recombination escaper cells, resulting in a mosaic and partial phenotype (Lepper et al., 2009; von Maltzahn et al., 2014). Recombination escaper cells are often present when using a transgenic animal that requires tamoxifen to promote the excision of the target gene (Mourikis et al., 2012). However, in models of regeneration, SCs increase in numbers rapidly and recombination-escaper cells can repopulate the niche efficiently.

In our model, single injury to the C/EBPβ cKO SCs resulted in an decrease number of SCs due to impaired self-renewal and precocious differentiation (Figure 29), while recombination escapers may increase in abundance in the same model, allowing escapers to replenish the SC niche (von Maltzahn et al., 2014). Upon

performing a second injury, we measure a significant decrease in the regenerative capacity of C/EBPβ cKO SCs and this may be an under penetration of the C/EBPβ cKO phenotype given the replenishment of the niche by WT recombination-escapers. Repeating this exact protocol by addition of tamoxifen to the chow in the course of serial injury will likely increase the severity of the C/EBPβ cKO phenotype (von Maltzahn et al., 2014).

HGF stimulates C/EBPβ in SCs and block myogenic differentiation

HGF, one of the growth factor added to myoblast growth medium, was previously shown to promote C/EBPβ expression in hepatocytes following partial hepatectomy (Cho and Kim, 2003; Greenbaum et al., 1998; Shen et al., 1997). In our experiments, removal of HGF from the cell media triggered a decrease in C/EBPβ and Pax7 levels and promoted precocious differentiation (Figure 12), suggesting that HGF may inhibit myogenesis through stimulation of C/EBPβ expression (Miller et al., 2000). *In vitro*, addition of HGF has been shown to promote mitosis and to block myoblast differentiation and fusion, consistent with the phenotype of myoblasts ectopically expressing C/EBPβ (Walker et al., 2015).

HGF binds to the cell-surface receptor c-met and following binding, c-met activates the intracellular PI3-kinase pathway (Cho and Kim, 2003). Knockout models of c-met or HGF are embryonically lethal, characterized by a failure to develop the placenta, liver and skeletal muscle (Bladt et al., 1995; Schmidt et al., 1995; Uehara et al., 1995). Interestingly, C/EBPβ is highly expressed and has a role in these three tissues (Table 4). HGF signaling is required in the embryo for the migration of stem cells from the dermomyotome to the budding limbs. In the adult,

muscle regeneration is defective in a mouse model with a SCs-specific knockout of c-met. The decreased muscle regeneration seen in c-met cKO mice originate from decreased motility of mutant cells, though SCs lacking c-met activate, proliferate and differentiate normally when compared to WT cultures. Although HGF has been shown to promote activation, proliferation and to block differentiation of SCs, it is therefore unlikely that HGF signaling is required to support these functions (Allen et al., 1995; Kastner et al., 2000; Tatsumi et al., 1998; Webster and Fan, 2013). Given the pleiotropic functions of HGF, one interesting question to address is whether C/EBPβ mediates the effects of HGF in SCs. Given that c-met cKO SCs have a decrease motility, it may be interesting to address whether C/EBPβ cKO SCs have a similar phenotype.

Although the evidence presented here suggests that HGF is upstream of C/EBPβ expression in cultured myoblasts, in hepatocytes, C/EBPβ is a transcriptional activator of HGF expression, therefore creating a positive feedback loop (Jiang and Zarnegar, 1997; Tomida and Saito, 2004). C/EBPβ can bind to the proximal promoter of HGF and can stimulate its expression, which is further potentiated by inflammatory cytokine treatment. Moreover, previous work has established that myoblast cell lines express both c-met and HGF, and that both genes are repressed upon differentiation, correlating with the pattern of C/EBPβ expression during myoblast differentiation (Anastasi et al., 1997). It would be interesting to investigate whether C/EBPβ cKO SCs have an impaired expression of HGF and c-met, placing C/EBPβ upstream of this pathway.

Increased myotube size with loss of C/EBPβ

One difference that was notable when comparing WT and C/EBPβ$^{-/-}$ myotubes was the increase in myotube size with loss of C/EBPβ (Li, 2011), a phenotype also observed using our cKO mouse model (Figure 13). Thus, C/EBPβ expression is dispensable for differentiation of myoblasts, but may be involved in limiting myoblast fusion, as its loss results in bigger myotubes in culture and fibers with increased area *in vivo*.

The ERK signaling pathway via Klf4 was shown to promote muscle cell fusion (Sunadome et al., 2011). Kruppel-like zinc finger (Klf) transcription factors are a family of zinc finger transcription factors important for cell proliferation and differentiation. In the absence of Klf4, myoblasts differentiation occurred normally but myotubes produced were significantly smaller, while overexpression of Klf4 increased myotube size, suggesting that Klf4 might promote myotube fusion. Interestingly, in adipogenesis, C/EBPβ represses Klf4 and in the absence of C/EBPβ, Klf4 expression was increased (Birsoy et al., 2008). By analogy with this adipogenic cross-regulation, loss of C/EBPβ in myoblasts may result in increased levels of Klf4 and this may be a hypothetical mechanism by which myotube size increases C/EBPβ cKO myotubes. In support of this hypothesis, *Klf4* message is increased 4-fold in C/EBPβ cKO myoblasts (unpublished findings).

Work from other members of the lab identified the transcription factor Smad2 as a target of C/EBPβ (Lamarche et al., 2015). In C/EBPβ cKO myoblasts, decreased Smad2 expression could inhibit downstream signaling by myostatin (myostatin-resistance), allowing for enhanced muscle growth. Furthermore, work by

other groups has mapped and characterized binding sites for C/EBPβ on the myostatin promoter, demonstrating that C/EBPβ is sufficient to stimulate the 1.2Kb myostatin promoter (Allen et al., 2010). Together, multiple lines of evidence converge on the hypothesis that C/EBPβ is an upstream regulator of the myostatin pathway in skeletal muscle.

C/EBPβ promotes SCs self-renewal following injury

Given that the fate of activated SCs is balanced between differentiation and self-renewal, loss of C/EBPβ shifts the self-renewal/differentiation balance toward differentiation, producing an increase in muscle fiber area and decreased Pax7+ SCs after injury (Figure 29).

Notch signaling is active in quiescent SCs and its activity declines as SCs becomes activated and differentiate (Mourikis et al., 2012). Loss of the Notch effector Rbpj in SCs decreases the expression of Pax7 and increases the expression of both MyoD and Myog. In addition, $Rbpj^{-/-}$ SCs were progressively lost with time, and was not attributed to apoptosis or cell-fate changes. This suggests that Notch signaling is required for the maintenance of SCs *in vivo* (Mourikis et al., 2012). Taken together, this phenotype resembles the phenotype of C/EBPβ cKO animals. Indeed, Notch signaling has been shown to promote C/EBPβ expression in skin epidermis (Wang et al., 2008), potentially placing C/EBPβ as a downstream effector of Notch in SCs.

Sprouty1 is an inhibitor of RTK cell-surface receptors, of which c-met is a member. Sprouty1 is highly expressed in quiescent SCs, decreases significantly

following activation post-injury, and restimulated in self-renewing SCs (Shea et al., 2010). This suggests that Sprouty1 may be an important gene for the maintenance of SCs quiescence. Mice with a conditional loss of Sprouty1 in SCs repair injured muscle normally, but following injury, SCs population declines by 40% as compared to controls. In addition, while no difference in the number of Myog+ differentiating cells were noted in a regenerating muscle with loss of Sprouty1, a two-fold increase in myoblast apoptosis was measured in Sprouty1 null muscle following injury. This suggests that Sprouty1 is required for the replenishment of the SCs population and it also suggest that Sprouty1 inhibits SC apoptosis in regeneration. Interestingly, we also report normal regeneration in the C/EBPβ cKO model (Figure 16), a 45% decline in self-renewing SCs following injury (Figure 29), and increased sensitivity to apoptosis in regenerating C/EBPβ cKO muscle (Figure 17). With important overlap in the biological functions of C/EBPβ and Sprouty1, it would be interesting to assess if Sprouty1 and C/EBPβ are in the same molecular pathway. Indeed, conditional knockout of Sprouty1 has been shown to decrease C/EBPβ expression in bone marrow stem cells (Urs et al., 2010).

Increase apoptotic sensitivity of SCs loss of C/EBPβ

SCs are resistant to many apoptotic insults. However, upon cell-cycle re-entry in response to injury, activated SCs become sensitive to apoptotic signals. In addition, following the triggering of myoblast differentiation, a significant proportion of myoblasts do not differentiate but instead undergo apoptosis (Wang and Walsh, 1996).

The mechanisms by which C/EBPβ promotes survival of myoblasts remains to be elucidated. One candidate is Bcl-2, highly expressed in SCs, and whose expression is regulated by C/EBPβ in cancer (Heckman et al., 2003; Vahidi Ferdousi et al., 2014; Yoon et al., 2007). Further, C/EBPβ regulates MyoD and Pax7 expression, which have both independently been shown to regulate myoblasts sensitivity to apoptosis.

The promotion of SC survival is of great importance for stem cell therapy approaches. Stem cells transplantation is a conceptual treatment for muscle-wasting conditions such as Duchenne muscular dystrophy (Gussoni et al., 1999). The underlying concept of stem cell transplantation involve the correction of a genetic muscle wasting disorder by intra-muscular injection of healthy donor SCs. Unfortunately, transplantation experiments in animals and in clinical trials were largely unsuccessful in part because of the significant apoptosis of transplanted cells (Fan et al., 1996; Gussoni et al., 1992; Mendell et al., 1995). Here, we suggest that manipulation of C/EBPβ expression is one way we could block apoptosis of transplanted myoblasts. Treatments that promote transient C/EBPβ expression in myoblasts may improve the survival of transplanted muscle stem cells without negatively impacting their differentiation. In agreement with the findings of this thesis, co-injection of muscle stem cells with macrophages in the *mdx* mouse improved engraftment (Lesault et al., 2012).

Cardiotoxin and barium chloride injuries reveal differences in skeletal muscle regeneration responses

The inhibition of muscle regeneration in the C/EBPβ cKO mouse following CTX injury (Figure 17) contrasts with the $BaCl_2$ injury (Figure 16), where regeneration was enhanced. As both injuries were performed on identical transgenic mouse lines in the same genetic background, with equivalent excision of C/EBPβ, the difference in regeneration post-injury must be due to different effects of the toxins on satellite cells, either directly or indirectly. In the literature, most reports investigating the biology of muscle regeneration limit their study to one toxin in a specific model with few studies directly comparing CTX and $BaCl_2$ injuries. Work by Boldrin et al. investigated the activation of SCs using different injury methods. They found that the number of activated SCs following $BaCl_2$ injury was approximately 30% higher than after CTX injury (Boldrin et al., 2011). This may signify an increased activation of SCs with $BaCl_2$ injury, or decreased proliferation of SCs with CTX injury, or an increase SCs loss with CTX injury, thus reflecting SC intrinsic and extrinsic effects. Interestingly, donor SCs grafted into the *mdx* muscle previously injured with $BaCl_2$ injury engrafted with higher efficiency compared to after CTX injury (Boldrin et al., 2011).

We tested the plasma levels of pro-inflammatory cytokines following CTX and $BaCl_2$ injury but we failed at measuring any elevation over basal levels. However, upon measurement of cytokines level locally in the injured TA, a number of cytokines were abundant following both types of injuries. One of the cytokines that was highly stimulated in CTX injury when compared to $BaCl_2$ is IL-1β, suggesting

that the immune response to the injury itself is different in these two models (Figure 20). Hours following injury to the skeletal muscle, macrophages and other immune cells are infiltrating the muscle and blood-infiltrating macrophages are required for regeneration (Lescaudron et al., 1999; Pimorady-Esfahani et al., 1997). The first wave of macrophages in muscle regeneration is M1 macrophages, which have a pro-inflammatory phenotype and produce high levels of TNFα, IL-1β, and IFNγ cytokines. These macrophages may be the source of pro-inflammatory cytokines that act to protect SCs from apoptosis in early activation and proliferation. Indeed, macrophages can promote myoblast proliferation and inhibit their differentiation (Bencze et al., 2012; Merly et al., 1999).

Post-injury inflammation in the absence of C/EBPβ

One open question at the moment is whether leukocytes are correctly recruited at the site of injury in C/EBPβ cKO SCs. This is a critical question given that others have shown that macrophages can block apoptosis of myoblasts by a cell-cell contact mechanism (Chazaud et al., 2003; Sonnet et al., 2006). Conditioned media from macrophages can also provide mitogens to myoblasts, including HGF, FGF, TGFβ and IGF-I and IGF-II which can, alone and synergistically, promote the proliferation of myoblasts (Chazaud et al., 2003; Robertson et al., 1993). Surprisingly, macrophages have an even greater influence on myoblast proliferation when cell-cell contact is permitted (Chazaud et al., 2003). The protective effects of macrophages on myoblasts are thought to be mediated by receptors VLA-4, LFA-1, PECAM-1 and CXCR1 (Sonnet et al., 2006).

Leukocytes can directly communicate in a paracrine manner with muscle SCs, via inflammatory cytokines produced following damage (Robertson et al., 1993). Given that C/EBPβ is a mediator of inflammation, that the C/EBPβ null mouse is immunocompromised and that C/EBPβ SCs apoptose in the context of CTX injury, C/EBPβ is an obvious candidate gene that can modulate gene expression in response to inflammatory response (Table 4). Myoblasts are also a source of a number of pro-inflammatory cytokines that are involved in the recruitment of macrophages to the site of injury (Chazaud et al., 2003; De Rossi et al., 2000). For example, IL-6 and IL-1α expression are constitutive in myoblasts (De Rossi et al., 2000). Although myoblasts do not produce IL-1β and TNFα at the basal level, they can stimulate the expression of these two cytokines upon exposure to inflammatory cytokines (De Rossi et al., 2000). In human myoblasts, IL-1β was previously shown to be produced without the need of an inflammatory trigger (Authier et al., 1999). This is consistent with the hypothesis that myoblasts cells can promote the local recruitment of blood monocytes via components of immunity. In light of these findings, it is hypothesized that myoblasts can behave in part as immune cells (De Rossi et al., 2000). C/EBPβ can promote directly the expression of many of the cytokines mentioned here, and thus may be a central regulator of myokine production (Huber et al., 2012; Poli, 1998). As such, it would be informative to measure the expression of IL-1β in C/EBPβ cKO SCs, and to address whether IL-1β acts in an autocrine manner to promote SCs survival (Figure 21). Further, a more in depth analysis of myokine production in the context of muscle growth and repair would be informative, to better understand the signals produced.

In contrast to pro-inflammatory cytokines, anti-inflammatory cytokines IL-4 and IL-13, produced by M2 macrophages, promote the differentiation of myoblasts (Heredia et al., 2013). IL-10 can deactivate the M1-macrophages, thereby reducing inflammation, to promote myoblast differentiation. However, when IL-10 is injected into the injured muscle and M1 macrophages are deactivated early, SCs differentiate precociously, decreasing the regenerative response (Perdiguero et al., 2011). It would be interesting to evaluate the impact of these anti-inflammatory cytokines on myoblasts C/EBPβ expression, as a means of explaining the precocious differentiation observed *in vivo*.

SCs and inflammation in context: SCs dysfunction and muscle wasting in cancer cachexia

Using the Lewis Lung Carcinoma model, we measure an expansion of Pax7+ SCs in the cachexic muscle (Figure 22). This was also reported by other groups and in patients, suggesting that the expansion of SCs in cancer cachexia is a reproducible observation (He et al., 2013; Penna et al., 2010). We also report that animal models of cancer cachexia such as the LLC tumor have inhibited myogenesis *in vitro* and *in vivo* (Figure 25). Indeed, regeneration following both CTX and $BaCl_2$ injury was inhibited in LLC-tumor bearing recipient mouse. This suggests that circulating factors produced by the neoplasm or by the host in response to the neoplasm can negatively impact muscle regeneration (He et al., 2013). In addition, cancer cachexia can also negatively impact chronic regeneration as seen in the *mdx* mouse grafted with the LLC tumor (He et al., 2013).

Given the increased number of Pax7+ SCs in the hindlimb muscle of both cachexic animals and patients, He et al investigated whether Pax7 expression in SCs was causal to muscle wasting in cancer cachexia (He et al., 2013). Following tumor grafting into $Pax7^{+/-}$ mice, both muscle weight and fiber area were increase when compared to $Pax7^{+/+}$ grafted with the same tumor. This is a proof of principle that decreased Pax7 expression is sufficient to improve muscle wasting in models of cancer cachexia (He et al., 2013). Interestingly, we have demonstrated that inflammation can stimulate C/EBPβ expression and also that C/EBPβ is a transcriptional activator of Pax7 (Figure 20). Therefore, we predict that increase expression and number of Pax7 SCs in models of cachexia may be promoted by C/EBPβ stimulation.

Future directions

Loss of C/EBPβ in myoblasts results in increased myofiber size but the underlying mechanism for this phenotype remains unknown. Multiple lines of evidence suggest that C/EBPβ may act upstream of the myostatin pathway by promoting the expression of both Smad2 and myostatin. Hence, in the absence of C/EBPβ, the myostatin pathway is downregulated and an in increased muscle size is measured. The relevance C/EBPβ for the myostatin pathway could be addressed by creating a muscle-fiber conditional knockout model. Indeed, creating a novel conditional mouse model using the *Cebpb*^{fl} allele with a muscle creatin kinase (*MCK*^{Cre}) driver would be appropriate. Increasing the understanding of C/EBPβ

negative role in the promotion of muscle fiber size may offer new therapeutic targets for the treatment of muscle atrophy conditions.

We have demonstrated that C/EBPβ can promote SCs survival but the mechanism and downstream target for this effect remains to be determined. In hepatocytes, phosphorylation of Thr217 of C/EBPβ was shown to be both sufficient and required to promote hepatocytes survival. This is a candidate post-translational modification of C/EBPβ that may promote SCs survival and mutation of Thr217 to a phosphorylation-dead residue would substantiate this mechanism. In conditions of chronic inflammation and muscle wasting, treatment of patients with anti-inflammatory drugs may decrease muscle wasting but they may also increase the sensitivity of SCs for apoptosis. However, co-treatment with a drug that can promote pThr217 of C/EBPβ might allow SCs survival.

Loss of C/EBPβ decreases the number of Pax7+ SCs following injury, and it also exacerbates serial muscle regeneration. Given that Cre-recombination is tamoxifen-dependent and that complete excision rates are difficult to obtain, it may be valuable try this protocol with constant tamoxifen administration in the animal's chow, which has been shown to increase the excision rate, and also the penetrance of the phenotype. Alternatively, creating a new mouse model using the $Cebpb^{fl}$ and a $Myf5^{Cre}$ driver would allow uniform excision rates and presumably demonstrates a more contrasting phenotype. By using any of these two models, we predict a decrease SCs self-renewal following regeneration and this would translate into a loss of muscle repair after a second injury.

Conclusion

This thesis establishes novel functions of the bZIP transcription factor CCAAT/Enhancer Binding Protein beta in skeletal muscle SCs. In adults, C/EBPβ expression is highest in quiescent SCs and progressively declines with differentiation. Upon genetic loss of C/EBPβ expression, differentiation of muscle stem cells occurs normally. However, the resulting C/EBPβ cKO generated muscle fibers have an increase size both *in vitro* and *in vivo*. This suggests that C/EBPβ may restrict muscle fusion or hypertrophy.

In the presence of C/EBPβ, myogenesis is inhibited and SC resistance to apoptosis improved. In chronic inflammation models, such as cancer cachexia, C/EBPβ expression is stimulated at least in part by IL-1β. Cachexic mice lacking C/EBPβ expression in SCs are not protected from muscle wasting, and have an increased sensitivity to apoptosis.

Following activation, proliferation and differentiation of SCs, a small fraction of SCs escape the differentiation path and return to the SCs phenotype, thus creating a pool of reserve SCs. We demonstrate that C/EBPβ is sufficient to promote the expression of the universal muscle stem cell gene Pax7. In C/EBPβ cKO SCs, the propensity of SCs to self-renew is decreased, such that serial injuries to the mutant muscle results in decreased regeneration capacity and a decrease in satellite cell number.

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APPENDIX 1: SATELLITE CELL MAINTENANCE IS DEPENDENT ON C/EBPB EXPRESSION

INTRODUCTION

The mammalian skeletal muscle has a phenomenal capacity to be regenerated. This remarkable feature of the skeletal muscle is attributed to a small population of muscle-resident stem cells that are anatomically distinct from muscle fiber cells. Indeed, muscle stem cells are found peripherally to the muscle fiber sarcolemma lying under the epimysium. Following their original anatomical identification in *Xenopus leavis*, muscle stem cells were named satellite cells (Mauro, 1961). Whereas the role of satellite cells (SCs) is to contribute to make skeletal muscle in the neonatal and juvenile age, SCs are also strictly required to repair muscle injuries in adults (Sambasivan et al., 2011; Schultz, 1996). Upon injury, the myofiber nuclei cannot proliferate to replace dying areas of the multinucleate syncytium because muscle cells have irreversibly exited mitosis (Moss and Leblond, 1971). SCs can also contribute to muscle fiber remodeling in adults as shown by their involvement for muscle fiber elongation, hypertrophy or fiber-type switch following different exercise regimens (Charifi et al., 2003; Darr and Schultz, 1987; Rosenblatt et al., 1994). Although SCs are mitotically quiescent and have a low turnover rate during daily occupations, SCs become activated and perform exponential mitoses following trauma to the muscle fiber (Bischoff, 1975, 1986; Schultz et al., 1978; Snow, 1977). In fact, SCs harvested from isolated muscle fibers can expend as myoblasts and make new multinucleated myotubes *in vitro* (Rosenblatt et al., 1994). Moreover, transplantation of a single healthy myofiber into a SC-depleted host recipient mouse has provided compelling evidence that

SCs can generate new myofibers, and also many new functional SCs (Collins et al., 2005).

Pax7 is a paired-box transcription factor present at high expression level in every SC (Harel et al., 2009; Seale et al., 2000). Pax7 expression declines mildly in activated SCs while Pax7 is not found in myotubes, suggesting that Pax7 is important for SCs function. Examination of a mouse model with a targeted null mutation in Pax7 indicated that this gene is required for the maintenance of muscles SCs (Seale et al., 2000). At birth, the $Pax7^{-/-}$ mouse appears normal but display peri-natal muscle growth retardation and early death, highlighting the requirement of SCs for neonatal muscle growth (Kuang et al., 2006; Seale et al., 2000). Whereas at birth SCs are found in the hindlimb muscle of the $Pax7^{-/-}$ mouse, they are progressively lost by apoptosis (Relaix et al., 2006). In addition, inhibited proliferation of muscle stem cells isolated from $Pax7^{-/-}$ newborns indicated SC-intrinsic functions of Pax7 for myoblasts mitosis (Kuang et al., 2006). Pax7 expression is also required for the stimulation of Myf5 and MyoD expression in myoblasts and in adult SCs following injury (McKinnell et al., 2008; von Haehling and Anker, 2014). Likewise, overexpression of a dominant negative Pax7 mutant in myoblasts repressed the expression of the myogenic transcription factors Myf5 and MyoD, as well as decreased the proliferation rates of mutant myoblasts (Collins et al., 2009; Relaix et al., 2006). On the other hand, ectopic expression of Pax7 in myoblasts increased proliferation, stimulated Myf5 expression, and inhibited precocious differentiation of myoblasts as determined by the inhibition of myogenin expression (Olguin et al., 2007; Soleimani et al., 2012; Zammit et al., 2006a).

Furthermore, ectopic expression of myogenin represses Pax7 expression, suggesting that Pax7 and myogenin (MyoG) reciprocally regulate one another (Olguin et al., 2007). Pax7 has been shown to significantly stimulate more than 43 genes in C2C12, and Pax7 also stimulated these genes in fibroblast cells, suggesting a direct Pax7 stimulation (McKinnell et al., 2008). In an adult context, Pax7 is also required for the maintenance of the SC population as mice with a conditional deletion of Pax7 progressively loose every SCs (Gunther et al., 2013). This loss of SCs in adults is further illustrated by the failure to regenerate a skeletal muscle injury in this Pax7 conditional knockout model (Lepper et al., 2011; von Maltzahn et al., 2014). Inhibition of muscle regeneration in adult mice was also reported upon ablation of the SCs compartment via a transgenic mouse model expressing the diphtheria toxin receptor by virtue of the Pax7 promoter (Lepper et al., 2011; Sambasivan et al., 2011). Together, these contemporary mouse models indicate that Pax7 expression, as well as Pax7+ SCs are required for muscle regeneration. However, following examination of the previous mouse models, the muscle homeostasis remained unchanged by either conditional knockout of Pax7, or by ablation of Pax7+ SCs in mature mouse, indicating the dispensability of SCs for muscle function in the absence of injury (Gunther et al., 2013; Sambasivan et al., 2011). Together, SCs are unquestionably required and sufficient for skeletal muscle regeneration.

C/EBP is a family of six basic leucine-zipper (bZIP) transcription factors. C/EBPβ, the second member of this family, has been shown to regulate the biology of many types of adult stem cells such as mesenchymal stem cells, haematopoietic

stem cells, mammary stem cells and keratinocytes stem cells (LaMarca et al., 2010; Smink and Leutz, 2012; Tanaka et al., 1997; Wang and Friedman, 2002; Zhu et al., 1999). However, the role of C/EBPβ in skeletal muscle satellite cells remains unknown. Previously, we established that C/EBPβ was a negative regulator of adult myogenesis (Marchildon et al., 2012). Ectopic expression of C/EBPβ blocked the myogenic program *in vitro*, and conditional knockout of C/EBPβ in adult SCs promoted muscle regeneration following injury. We also demonstrated that the universal SCs molecular marker Pax7 was a direct target of C/EBPβ. In order to regenerate a skeletal muscle injury a multiple of times, SCs have to restock their compartment following each bout of repair (Luz et al., 2002; Zammit et al., 2004). Thus, the maintenance of Pax7 expression in a small population of SCs is required. Given the importance of SCs as well as of Pax7 expression for muscle regeneration, one element of crucial importance is the definition of the transcriptional factors controlling the expression of Pax7. We hypothesize that C/EBPβ is required to maintain Pax7 expression to sustain SCs numbers in the adult after injury. Herein, by using a C/EBPβ conditional knockout mouse model, we provide evidence that C/EBPβ is required for the maintenance of SCs population after injury. Knockdown of C/EBPβ decreases the expression of Pax7 and promotes an early differentiation of myoblasts. *In vivo*, loss of C/EBPβ in SCs after injury decreases the SCs population compartment and abrogates the repair of multiple muscle injuries.

MATERIALS AND METHODS

Primary myoblast culture.

Young (4-8 week old) mice, were sacrificed by CO_2 asphyxiation, and all hindlimb muscles were dissected, minced with a razor blade, and digested for 2hrs in 0.2% collagenase/dispase (Roche) at 37°C with constant shaking. Primary myoblasts were isolated by selective plating as previously described (Marchildon et al., 2012). Primary cultures were maintained in DMEM supplemented with 20% FBS (Gibco), 10% horse serum (Sigma), penicillin and streptomycin (Wisent), 10ng/ml bFGF and 2ng/ml HGF (Peprotech). To trigger the excision of Cebpb, proliferating cells were cultured with 2μM 4-OH tamoxifen (Sigma) dissolved in ethanol for 48hrs. To differentiate primary myoblasts, cultures at 80% confluency were changed to DMEM with 2% FBS, 10% HS, penicillin and streptomycin for 48 hours.

Mouse models, tamoxifen treatments and muscle injury.

$Cebpb^{fl/fl}$;$Pax7^{CreERtm/+}$ (and control: $Cebpb^{fl/fl}$;$Pax7^{+/+}$) were generated as previously described (Marchildon et al., 2012; Nishijo et al., 2009; Sterneck et al., 2006). To excise *Cebpb*, animals were subjected to five daily intraperitoneal injections of tamoxifen (Sigma) dissolved in corn oil at a dosage of 2mg/20g. For injury, mice (6-8 weeks old) were anesthetized by isoflurane inhalation, and 50μl of 1.2% $BaCl_2$ was injected in the left TA muscle. Animals were given chow and water ad libitum. All animal handling procedures conformed to the guidelines established by the University of Ottawa Animal Care Service and the Canadian Council on Animal Care.

Quantitative PCR analysis.

To collect RNA, total RNA was purified using RNeasy kit (Qiagen) following manufacturer instructions. RNA was digested with DNase (Ambion) for 30minutes at 37°C and cDNA was synthesized with iScript kit (Biorad) following manufacturer instructions. qPCR was performed using iTaq SYBR universal kit (Biorad) following manufacturer instructions on a Stratagene MX3005p thermocycler. The following primers oligonucleotides were used: *Cebpa*-f: 5'-TTCGGGTCGCTGGATCTCTA-3', *Cebpa*-r: 5'-CCCGAGAGGAAGCAGGAATC-3', *Cebpb*-f: 5'-TCGAACCCGCGGACTGCAAG-3', *Cebpb*-r: 5'-CGACGACGACGTGGACAGGC-3', *Cebpd*-f: 5'-AGAACCCGCGGCCTTCTAC-3', *Cepbd*-r: 5'-ATGTAGGCGCTGAAGTCGAT-*3',* *Cebpz*-f: 5'-CACCACCACACCTGAAAGCAGAACC-3', *Cebpz*-r: 5'-TGCGTGTGACCTCTGTTGGCC-3', *Pax7*-f: 5'-GACGACGAGGAAGGAGACAA-3', *Pax7*-r: 5'-CGGGTTCTGATTCCACATCT-3', *Myf5*-f: 5'-CTGAGGGAACAGGTGGAGA-3', *Myf5*-r: 5'-CTGCTGTTCTTTCGGGACCAG-3', *Myod*-f: 5'-TGGCATGATGGATTACAGCG-3', *Myod*-r: 5'-CCACTATGCTGGACAGGCAGT-3', *18S*-f: 5'-CGCCGCTAGAGGTGAAATC-3', *18S*-r: 5'-CCAGTCGGCATCGTTTATGG-3'. All primers were designed using the Primer3 algorithm. All amplifications were normalized to 18S levels. Relative transcript expression was computed by using the ΔΔCt method.

Antibodies and western analysis.

Whole cell lysates were produced in IPH (50mM Tris pH 7.5, 150mM NaCl, 0.5% NP-40, 5mM EDTA) supplemented with 1mM DTT (Bioshop) and 1x protease

inhibitor cocktail (Roche). Lysates proteins content were quantified using a modified Lowry kit (Biorad) and proteins were separated on a 12% SDS-PAGE gel. Primary antibodies used were: rabbit anti-C/EBPβ (SC-150; 1/500), rabbit anti-MyoD (SC-304; 1/500), rabbit anti-Myf5 (SC-302; 1/500) all from Santa Cruz biotechnology, mouse anti-Pax7 (Pax7-c; 1/100, DSHB) and rabbit anti-cyclophilin B (ab16045; 1/10,000, Abcam) was used as loading control. Secondary antibodies used were: HRP-conjugated anti-rabbit IgG (NA934; 1/4,000) and HRP-conjugated anti-mouse IgG (NA931; 1/4,000) from GE healthcare.

Histology, Immunohistochemistry and immunocytochemistry.

Cell cultures were washed in PBS and fixed for 10minutes in 4% paraformaldehyde. Following washes, cells were permeabilized in 0.3% Triton X-100 for 15 minutes and then blocked 1hr in 5% normal donkey serum (Jackson Immunoresearch). Primary antibodies used were: mouse anti-Pax7 (Pax7 supernatant; 1/10, DSHB) and rabbit anti-MyHC (sc-20641; 1/100, Santa Cruz biotechnology), all incubated overnight at 4°C. Secondary antibody used were: Biotin-conjugated $F(ab')_2$ donkey anti-mouse IgG (715-066-150; 1/500), Alexa-488 $F(ab')_2$ donkey anti-rabbit IgG (711-546-152; 1/500), Cy3-strepavidin (016-160-084; 1/1,000) all from Jackson Immunoresearch. For TA-muscle histology, 8 micron-thick frozen sections were adhered to a microscope slide, and sections were stained with haematoxylin & eosin following standard procedures. For IHC, frozen sections were dehydrated 30 minutes at 65°C, fixed 10 minutes in 4% paraformaldehyde and antigen retrieval was performed for 20 minutes at 92°C in (10mM citric acid with 0.05% tween-20). Following washes, sections were permeabilized for 10 minutes in 0.5% Triton X-100

(1% Triton X-100 for F1.652) and blocking and staining was performed as above. Additional primary antibodies used were: rabbit anti-Ki67 (ab15580; 1/200), rabbit anti-laminin (ab11575; 1/200) from Abcam and mouse anti-eMyHC (F1.652-C; 1/100) from DSHB. TUNEL staining was performed prior to IHC staining following manufacturer instructions (Roche). Nuclei were label with DAPI (Sigma, 1µg/ml) for 5 minutes.

Microscopy imaging and acquisition.

Histological muscle sections were taken on a bright-field light microscope CX42 (Olympus) using a Qcapture 3 camera. Florescent microscopy pictures were taken on a DMI3000B (Leica) epifluorescence microscope using infinity 3 (Lumenera) camera. Representative images are presented and were equally processed by Photoshop (Adobe) by adjusting levels uniformly.

Statistical analysis, sample size, randomization and blinding.

On each graph, the bar represents the mean and the error bar represents the standard error of the mean. Two samples were subjected to a two-tailed Student t-test assuming equal variance and normal distribution for statistical analysis, and having a p-value <0.05 identified different populations. For all *in vitro* experiments, a minimum of 3 biological repeats was analyzed. For all *in vivo* experiments, a minimum of 5 animals per genotype was analyzed. $BaCl_2$ muscle injury was performed blinded.

RESULTS

C/EBPβ promotes Pax7 expression and reserve cells formation in vitro

Our previous work has established a negative role for C/EBPβ in adult myogenesis. We previously demonstrated that C/EBPβ could stimulate the expression of Pax7, the universal SC marker, but whether loss of C/EBPβ decreased the expression of Pax7 remained undetermined (Marchildon et al., 2012). To address whether loss of C/EBPβ decreased the expression of Pax7, we cultured both WT and cKO myoblasts in the presence of 4-OH tamoxifen for two days, resulting in excision of *Cebpb* as assessed by qRT-PCR and western analysis (Figure 27A-C). Loss of C/EBPβ expression in myoblasts resulted in a ~50% decrease expression Pax7 mRNA and protein (Figure 27D-F), without affecting the expression of the myoblasts markers Myf5 and MyoD (Figure 27G-I). Hence, loss of C/EBPβ in proliferating primary myoblasts specifically decreases the expression of satellite cell marker Pax7.

While the expression of Pax7 was specifically decreased in cKO myoblasts, loss of C/EBPβ expression did not promote obvious growth and differentiation defects and cKO cells were comparable to WT. Since cKO cells have a decrease expression of Pax7, we used an *in vitro* reserve cell approach to test the ability of cKO cells to produce undifferentiated self-renewing SCs. Following the differentiation of myoblasts, mononucleated Pax7+ cells that remains

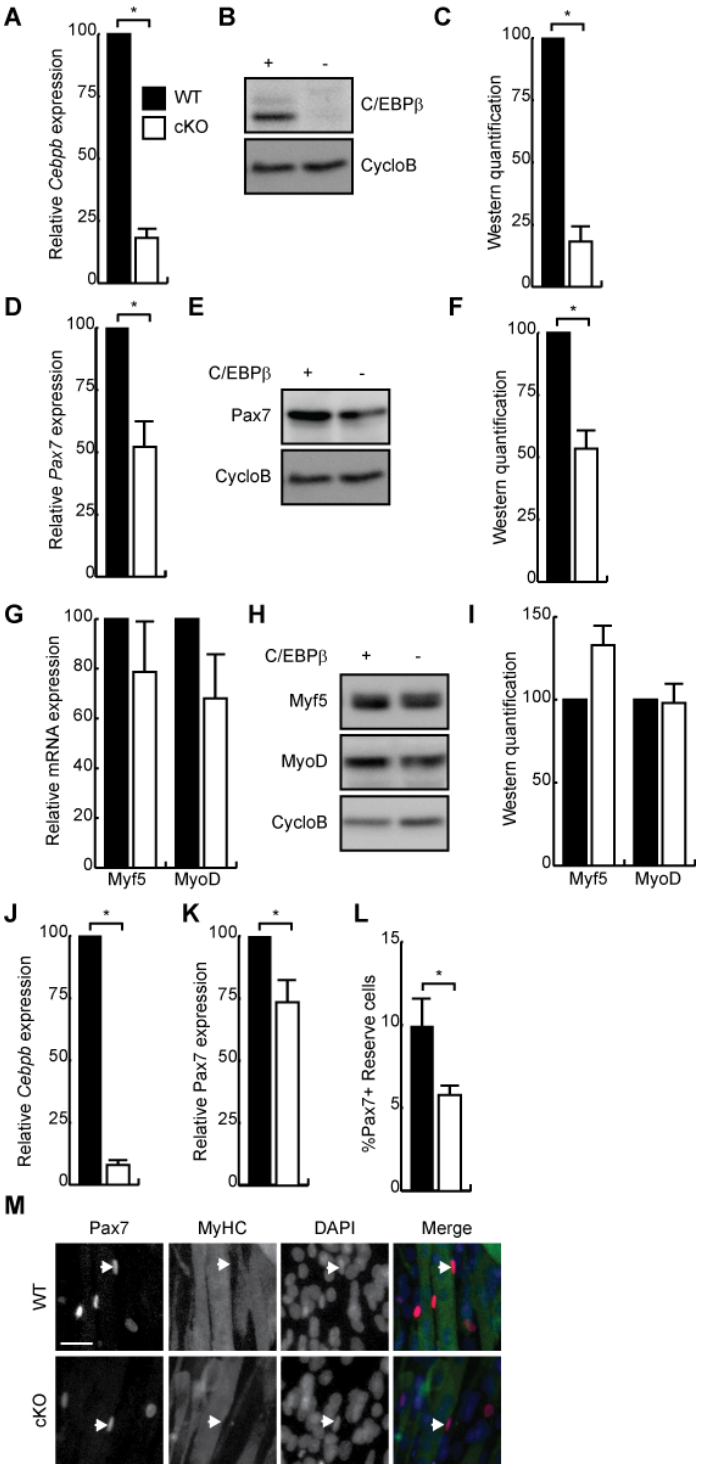

Figure 27 Loss of C/EBPβ in Pax7+ SCs decreases the propensity to make reserve cells *in vitro*.

(A) Relative *Cebpb* expression in primary myoblasts measured by qPCR. Primary myoblasts from WT and cKO animals were collected from hindlimb muscles and following purification, cultures were treated for 48hrs with 4-OH tamoxifen [2μM]. Transcript was normalized to 18S. *$p=2x10^{-13}$, n=6. **(B)** Western analysis of C/EBPβ expression in WT and cKO cells treated as in (A). Cyclophilin (CycloB) serves as loading control. n=6. **(C)** Western quantification of C/EBPβ expression in WT and cKO myoblasts relative to WT expression and treated as in (A). *$p=8x10^{-6}$, n=6. **(D)** Relative *Pax7* expression in WT and cKO myoblasts treated as in (A) and measured by qPCR. Transcript was normalized to 18S. *$p=5x10^{-4}$, n=6. **(E)** Western analysis of Pax7 expression in WT and cKO cells treated as in (A). CycloB serves as loading control. n=4. **(F)** Western quantification of Pax7 expression in WT and cKO myoblasts relative to WT expression and treated as in (A). *p=0.003, n=4. **(G)** Relative expression of *Myf5* and *MyoD* in WT and cKO myoblasts treated as in (A) and measured by qPCR. Transcripts were normalized to 18S. n=6. **(H)** Western analysis of Myf5 and MyoD expression in WT and cKO cells treated as in (A). CycloB serves as loading control. n=4. **(I)** Western quantification of Myf5 and MyoD expression in WT and cKO myoblasts relative to WT expression and treated as in (A). n=4. **(J)** Relative *Cebpb* expression in WT and cKO myotubes differentiated for three days and measured by qPCR. WT and cKO were treated with 4-OH tamoxifen in proliferation for two days before differentiation. Transcript was normalized to 18S. *$p=9x10^{-15}$, n=6. **(K)** Relative *Pax7* expression in WT and cKO myoblasts treated as in (J) and measured by qPCR. Transcript was normalized to 18S. *p=0.018, n=4. **(L)** Average percentage of Pax7+ cells in WT and cKO myotubes cultures

differentiated as in (K). *p=0.044, n=4. **(M)** Indirect immunofluorescence of WT and cKO differentiated cultures as in (J). Cultures were labeled with Pax7 and myosin heavy chain (MyHC), and nuclei were label with DAPI. Arrowhead points to a Pax7+ reserve cell. Scale bar = 20μm.

undifferentiated are called reserved cells and are a model of satellite cell self-renewal (Halevy et al., 2004; Yoshida et al., 1998; Zammit et al., 2004). In differentiated cKO cultures, both *Cebpb* and *Pax7* expression remain decreased when compared to WT cells (Figure 27J,K). To address whether this decrease in Pax7 expression also inhibits the number of reserve cells created, we used indirect immunocytochemistry to label Pax7-expressing cells (Figure 27L,M). Consistent with the measured lower *Pax7* expression, conditional loss of C/EBPβ inhibited the production of Pax7+ reserved cells by approximately 50% when compared to WT control cultures, suggesting that C/EBPβ is important for the creation of reserve cells *in vitro*.

Work by others has suggested a role for other C/EBP members in skeletal muscle and therefore loss of C/EBPβ in myoblasts may result in expression compensation by other C/EBP members (Fukada et al., 2007; Loinard et al., 2012; Penner et al., 2002; Yang et al., 2005). First, we assessed the expression of C/EBP member α, β, δ and ζ in WT myoblasts (Figure 28A). qRT-PCR analysis of myoblasts indicates that *Cebpb* is the member whose expression is the highest and *Cebpb* expression is at least 2.5-fold higher than another C/EBP. In addition, mRNA analysis of other C/EBP members in WT and cKO myoblasts indicates that loss of C/EBPβ did not result in compensation by another C/EBP gene (Figure 28B).

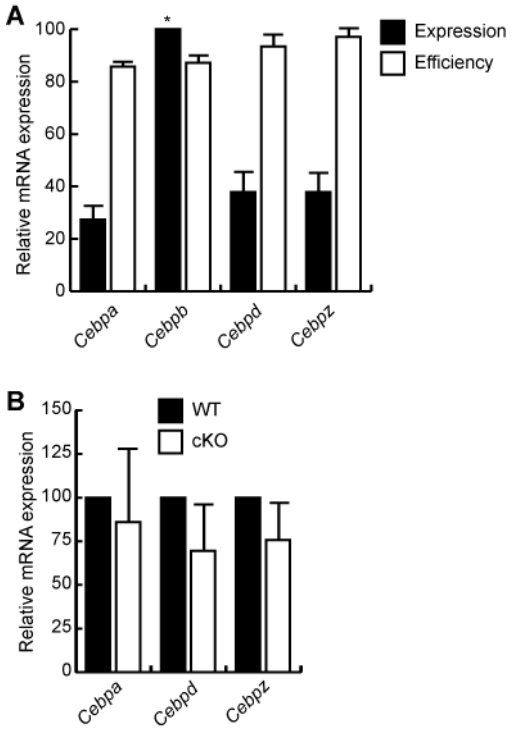

Figure 28 Loss of *Cebpb* is not compensated by other C/EBP members.

(A) Relative expression of *Cebpa*, *Cebpb*, *Cepbd* and *Cebpz* in WT myoblasts measured by qPCR. Primary myoblasts from WT animals were collected from hindlimb muscles and four days following purification, mRNA was collected and analyzed. Transcript was normalized to 18S. Average qPCR reaction amplification efficiency is indicated. *$p=2x10^{-5}$ compared to *Cebpd*, n=4. **(B)** Relative expression of *Cebpa*, *Cebpd* and *Cebpz* in WT and cKO myoblasts treated as in (A) and measured by qPCR. Cultures were treated for 48hrs with 4-OH tamoxifen [2μm] before analysis. n=4.

Together, C/EBPβ is the most abundant C/EBP member in myoblasts and its loss doesn't generate a compensatory response, suggesting that C/EBPβ may have the most prominent role in SCs among other C/EBPs.

C/EBPβ promotes the maintenance of SCs in vivo

Given that C/EBPβ can stimulate the expression of Pax7 *in vitro*, we asked if loss of C/EBPβ might decrease the number of SCs *in vivo*. To address whether C/EBPβ promote SCs numbers *in vivo*, we injected both WT and cKO animals with tamoxifen for five days, and we scored the number of Pax7+ SCs by IHC on the TA muscle cross-sections four days (day 0) later (Figure 29A). No significant differences in the number of Pax7+ SCs were noted, suggesting that Pax7+ SCs are not loss spontaneously with loss of C/EBPβ (Mourikis et al., 2012). Then, we hypothesized that the number of cKO SCs might be decrease following muscle injury given the exponential growth and proliferation of SCs after injury. To test this, we injured WT and cKO TA muscle with $BaCl_2$ and we monitored the number of Pax7+ SCs after injury (Figure 29A,B). As expected, Pax7+ cells numbers were lower in cKO animals at all time points examined post-injury (Figure 29A,B). Although the number of Pax7+ cell was lower after injury in cKO animals, this did not result in a decrease regenerative capacity of cKO animals because cKO animals have an enhanced or equal regenerative capacity when compared to WT littermates (Marchildon et al., 2012).

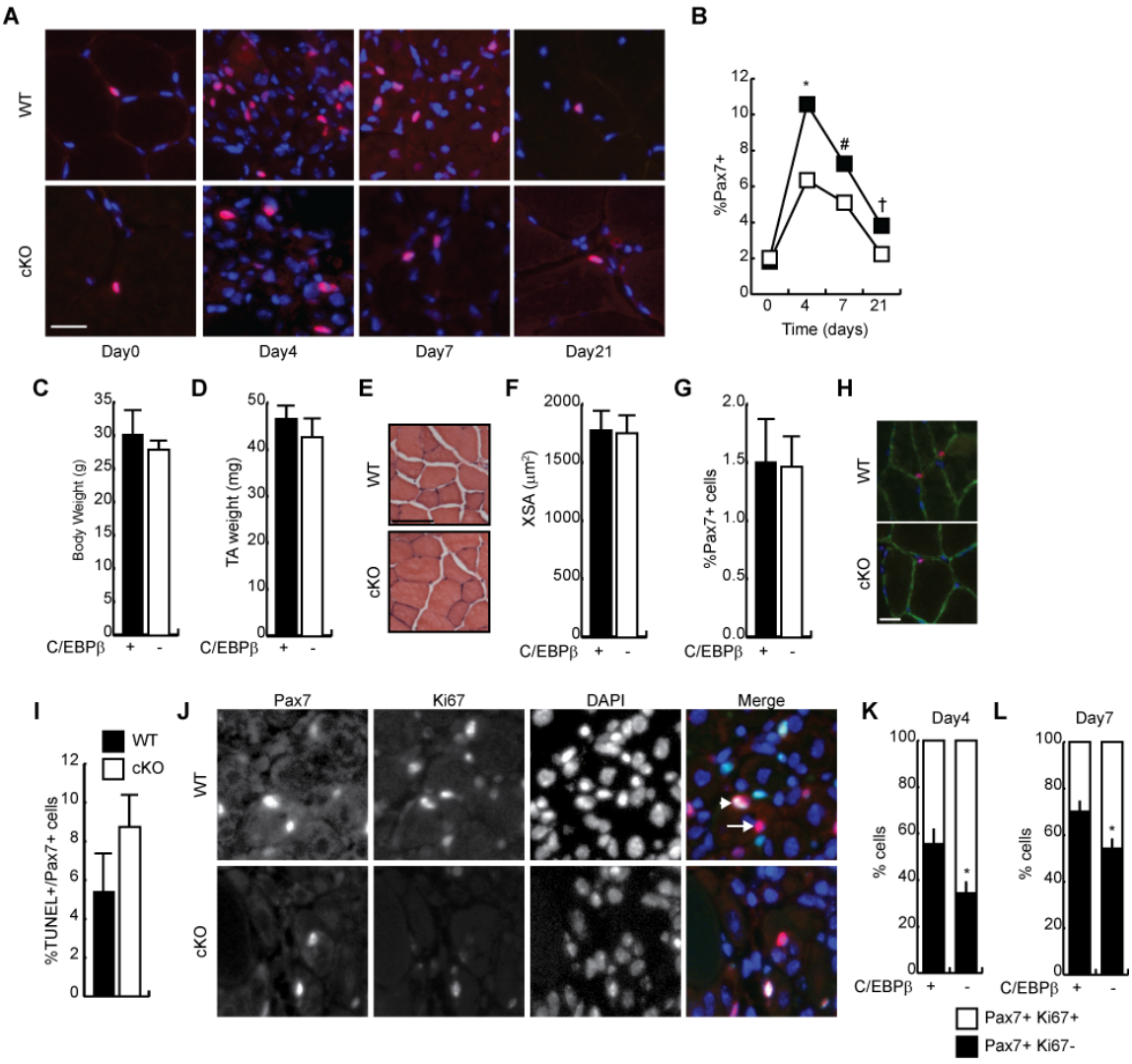

Figure 29 Decreased percentage of Pax7+ cells in cKO animals following $BaCl_2$ muscle injury.

(A) Indirect immunofluorescence images for Pax7 in WT and cKO TA-muscle cross-sections. WT and cKO female animals were injected daily with tamoxifen (2mg/20g) for five days and three days later, animals were injected intramuscularly (TA) with 50μl of 1.2% $BaCl_2$. Animals were analyzed at 4, 7 and 21 days post-injury. At sacrifice, TA muscle was dissected, flash-frozen in melting isopentane and cross-sections were label with Pax7. Nuclei were label with DAPI. Representative field of

view are presented. n=5. Scale bar = 20μm. **(B)** Average percentage of Pax7+ cells (relative to total DAPI+ nuclei) in WT and cKO animals treated as in (A). *p=0.0001, #p=0.005, †p=0.005, n=5. **(C)** Average body-weight of WT and cKO male animals four months following tamoxifen injections. WT and cKO animals were injected daily for five days with tamoxifen at a dose of 2mg/20g and were sacrificed four months later. n=3. **(D)** Average TA muscle weight of WT and cKO animals treated as in (C). n=3. **(E)** H&E-stained TA muscle cross-sections from WT and cKO animals as in (C). n=3. Scale bar = 50μm. **(F)** Average fiber cross-section area of WT and cKO animals treated as in (C). **(G)** Average percentage of Pax7+ SCs (relative to total DAPI+ nuclei) in WT and cKO TA muscle cross-sections. Animals were treated as in (C). n=3. **(H)** Indirect immunofluorescence for Pax7 in TA muscle cross-sections of WT and cKO animals treated as in (C). Basal lamina is identified with laminin (green) and nuclei were labeled with DAPI. n=3. Scale bar = 20μm. **(I)** Average percentage of TUNEL+ & Pax7+ (relative to total Pax7+ cells) in WT and cKO animals treated as in (A) and analyzed 4 days post-injury. n=4. **(J)** Indirect immunofluorescence images for Pax7 and Ki67 in WT and cKO animals treated as in (A) and analyzed 4 days post-injury. Representative field of view are presented. Arrow represents a Pax7+Ki67- cell. Arrowhead represents a Pax7+Ki67+ cell. n=4. **(K)** Average percentage of Pax7+ Ki67+ cells (relative to total Pax7+ cells) in WT and cKO animals as in (A) and 4 days post-injury. *p=0.027, n=4. **(L)** Average percentage of Pax7+ Ki67+ cells in WT and cKO animals as in (A) and 7 days post-injury. *p=0.017, n=5.

Given that C/EBPβ is required for the maintenance of SCs following $BaCl_2$ injury, we asked if C/EBPβ might be required to maintain SCs numbers after an extended period and without muscle injury. Indeed, the time frame of the tamoxifen treatment and $BaCl_2$ injury performed above were short and therefore the possibility that C/EBPβ might be required to maintain SCs numbers after long periods could not be addressed. To test whether C/EBPβ is required to maintain Pax7+ SCs numbers after an extended period, we tamoxifen treated WT and cKO animals to excise *Cebpb* and analyzed the TA-muscle four months later (Figure 29C-H). Despite loss of C/EBPβ, there were no significant changes noted in muscle biology, histology or SCs number when compared to WT animals. Thus, we conclude that C/EBPβ is not required to maintain SCs numbers in the absence of muscle injury.

The lower number of Pax7+ SCs in cKO animals following injury could be attributed to an increase SC death. To test this, Pax7+ SCs were labeled with TUNEL to mark apoptotic SCs (Figure 29I). Four days post-injury, no statistically relevant differences in the number of apoptotic SCs were noted between WT and cKO animals, suggesting that the decrease number of Pax7+ SCs in cKO mice is not a result of increase apoptosis. Therefore, we tested whether cKO SCs proliferated normally as compared to WT SCs following injury. To test this, we labeled SCs with Ki67 which marks all stages of the cell-cycle (Scholzen and Gerdes, 2000). Four and seven days following injury, the percentage of Pax7+Ki67+ cells was statistically increased in cKO animals as compared to WT, suggesting that cKO SCs are more proliferative and that the loss of SCs following injury might be

due to an unusual cell-cycle kinetic and a failure of SCs self-renewal in the absence of C/EBPβ (Figure 29K,L).

Decreased muscle regeneration in cKO animals following serial injury

Satellite cells are absolutely required for skeletal muscle regeneration (Murphy et al., 2011). Given the decrease number of SCs with loss of C/EBPβ after $BaCl_2$ injury, we hypothesized that the cKO skeletal muscle might not regenerate well following a second injury given its lower number of SCs. To examine the requirement of C/EBPβ for SC self-renewal *in vivo*, two serial injuries were performed, separated by 21 days (Figure 30). At sacrifice, histological analysis of WT and cKO TA muscles indicated that the average cross-sectional area of regenerating fibers was decreased by ~44% in cKO animals seven days after the second injury (Figure 30A,B). Newly regenerated fibers were identified with IHC to the embryonic isoform of myosin heavy chain (Whalen et al., 1990). We also assessed the long-term recovery of WT and cKO animals 21 days after the second TA-muscle injury. The average TA weight of cKO animals was ~18% less than controls, while the average TA-muscle fiber area was decreased by ~21% in cKO animals, indicating that cKO muscle is less able to repair serial injuries (Figure 30C-E).

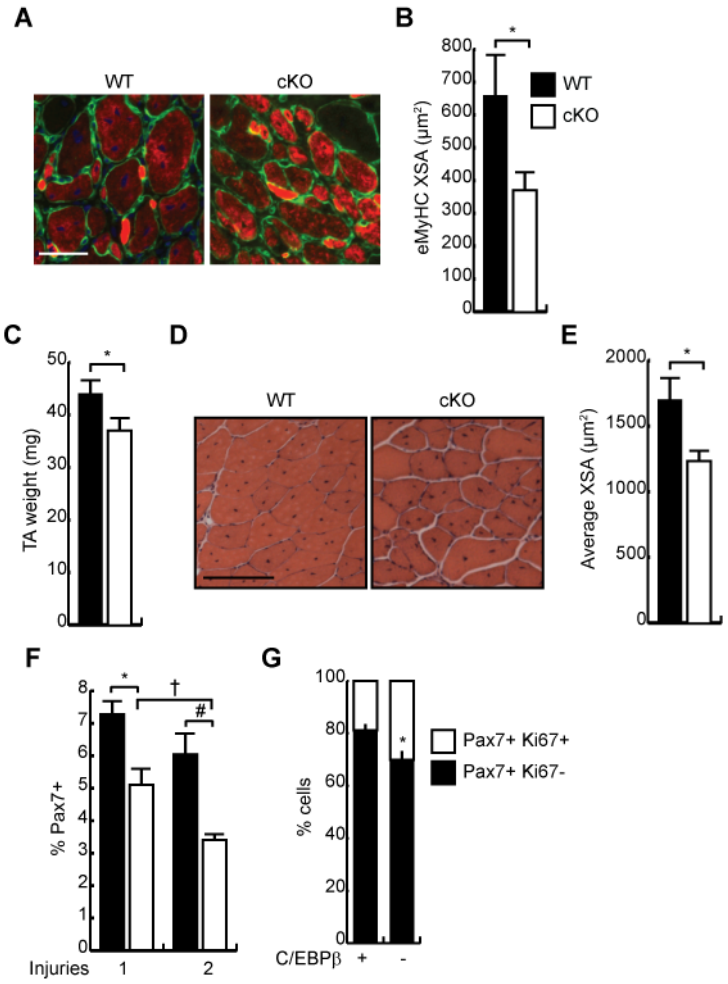

Figure 30 Decreased regenerative capacity of cKO SCs following serial injuries.

(A) Indirect immunofluorescence images for embryonic myosin heavy chain (red) and laminin (green) of WT and cKO animals. WT and cKO male animals were injected daily for five days with tamoxifen at a dosage of 2mg/20g, and three days later, one TA was injured with 50µl of 1.2% $BaCl_2$. 21 days following this first muscle injury, an identical muscle injury was performed on WT and cKO TA muscle and animals were analyzed seven days later. TA muscle was dissected, flash-frozen in melting isopentane and cross-sections were label with eMyHC and laminin. Nuclei

were label with DAPI. n=5. Scale bar = 50μm. **(B)** Average eMyHC+ TA muscle fiber area of WT and cKO animals treated as in (A) seven days after the second muscle injury. *p=0.038, n=5. **(C)** Average TA muscle weight of WT and cKO male animals treated as in (A) and 21 days following the second injury. *p=0.029, n=5. **(D)** H&E-stained TA muscle cross-sections from WT and cKO animals treated as in (A) and 21 days following a second muscle injury. Representative field of view are presented. n=5. Scale bar = 50μm. **(E)** Average TA muscle fiber cross-sectional area of WT and cKO animals treated as in (A) 21 days after the second muscle injury. *p=0.028, n=6. **(F)** Average percentage of Pax7+ SCs in WT and cKO animals seven days after the first and seven days after the second $BaCl_2$-injury. WT and cKO animals were injected treated as in (A) and were analyzed after the first and after the second injury. *p=0.005, #p=0.001, †p=0.004, n=6. **(G)** Average percentage of Pax7+ Ki67+ cells (relative to total Pax7+ cells) in WT and cKO animals as in (A) and seven days after the second injury. *p=0.008, n=6.

Further, seven days after the second injury, cKO animals had ~45% fewer Pax7+ cells than WT, a further loss of the Pax7+ compartment when compared to a single injury (Figure 30F). Similar to a single injury, a ~37% increase in the percentage of Pax7+Ki67+ cells was observed in cKO muscle as compared to WT (Figure 30G). Taken together, this data suggests that C/EBPβ is required for the maintenance of the SC compartment following injury.

DISCUSSION

The regulation of Pax7 expression for SCs function is critical. Therefore, the identification of transcriptional activators of Pax7 provides useful mechanistic understanding of SCs function. Herein, using a C/EBPβ conditional knockout mouse model, we show that C/EBPβ expression is required to maintain the expression of Pax7. Both in culture and in animals, the loss of C/EBPβ expression decreases the expression of Pax7, and also the number of Pax7+ SCs. In serial muscle injury models, we demonstrate that the functional loss of SCs in the C/EBPβ cKO mouse model decreases muscle fiber area, indicative of decrease regeneration. The findings of this study are in agreement with previous reports that investigated the function of Pax7 in SC using genetic models. In both the $Pax7^{-/-}$ and the C/EBPβ cKO model presented here, mutant SCs have a proliferative defect such as their numbers progressively declines *in vitro* and after injury *in vivo* (Oustanina et al., 2004; Relaix et al., 2006; Seale et al., 2000). Furthermore, the efficient differentiation measured in C/EBPβ cKO SCs is consistent with the reported

phenotype of Pax7-deficient SCs and suggests that lost of neither Pax7 nor C/EBPβ impairs myogenesis (Gunther et al., 2013; Zammit et al., 2006b). Interestingly, we did not measure a loss of SCs in the C/EBPβ cKO under sedentary homeostasis, suggesting a dispensability of C/EBPβ in the absence of injury. On the other hand, SCs were progressively lost in Pax7-deficient SCs sedentary mouse models, suggesting that Pax7 is required to maintain the SCs compartment in day-to-day life (Gunther et al., 2013). This requirement for Pax7 has been demonstrated using a $Myf5^{Cre}$; $Pax7^{loxp/loxp}$ genetic mouse model in which Cre DNA recombinase is chronically activated in SCs. In our model described herein, we have injected cKO mice with tamoxifen for only five days (Figure 11). Thus, while we would have expected a similar lost of SCs in our cKO mouse model, one reasonable explanation for this absence of phenotype is the inability of our tamoxifen regimen to efficiently excise C/EBPβ on a longitudinal study.

Despite the mildness of the injured-muscle phenotype of this mouse model, this is consistent with other reports that have used a similar $Pax7^{CreERtm}$ allele to conditionally excise a gene of interest (Gunther et al., 2013; Lepper et al., 2009; von Maltzahn et al., 2014). Although we were expecting a more severe muscle regeneration deficit in the C/EBPβ cKO mouse model, inefficient Cre DNA-recombinase might explain this mild phenotype. In fact, a partial muscle phenotype after injury has been demonstrated to originate from an incomplete activation of Cre DNA-recombinase following tamoxifen treatment (Brack, 2014). Whereas Cre is activated in most of Pax7+ SCs following five daily tamoxifen injections, few SC remain Cre-inactive with this tamoxifen regimen and are functionally like WT

(Gunther et al., 2013; von Maltzahn et al., 2014). Given the incredible regenerative and self-renewal capacity of a single SC, a few Cre-inactive SCs are sufficient to mask the phenotype of Cre-active knockout SCs. In order to increase the penetrance of the aforementioned C/EBPβ cKO model, concomitant tamoxifen injections with muscle injury has been shown to increase the global activation of Cre, just as providing rodent chow supplemented with tamoxifen.

In many myopathies, the abundance of SCs declines sharply. Indeed, repeated rounds of muscle degeneration and regeneration trigger a chronic circle of SCs activation, differentiation and self-renewal, which eventually exhaust the population of SCs. This is evident in models of Duchenne muscular dystrophy where SCs exhaustions precede muscle fiber exacerbation. In fact, SCs function has been demonstrated to be a determinant in myopathies. This was well illustrated in mice by crossing an *mdx* mouse with a DNA telomerase null mouse (Sacco et al., 2010). Loss of telomerase function decrease SCs self-renewal and amplify the pathology of the *mdx* mouse. Similar findings were reported previously by crossing the *mdx* mouse with the MyoD null (Megeney et al., 1996). In agreement with the decline of the SC population in C/EBPβ cKO muscle presented herein, we speculate that loss of C/EBPβ would exacerbate the pathology of Duchenne muscular dystrophy by accelerating the depletion of the SC population.

APPENDIX 2: PHENOTYPES OF THE C/EBPB NULL MOUSE

Table 4 Review of the most prominent phenotypes of the C/EBPβ null mouse.

Tissue	Stage	Phenotype	Mechanism	Reference
Liver/hepatocytes	Fetal/newborn	Failure to metabolize glycogen. Hypoglycemia	Failure to stimulate production of PEPCK	(Croniger et al., 1997)
	Post-natal	Decrease hepatocytes proliferation, hypoplasia	Decrease expression of cyclin B and E, decrease MKP-1, increase p21	(Greenbaum et al., 1998)
	Adult	Decrease acute-phase protein expression. Hypoglycemia. Decrease liver glucose production, decrease cAMP production. Attenuation of diet-induced non-alcoholic steatohepatitis	Decrease PEPCK and PKA expression, increase expression of PDE3 Decrease expression of TNFα, CRP, PPARγ and FAS	(Cappelletti et al., 1996; Croniger et al., 2001; Liu et al., 1999; Rahman et al., 2007)
Liver regeneration	Adult	Decrease proliferation of hepatocytes following partial hepatectomy. Apoptosis of hepatocytes	C/EBPβ promotes the expression of E2F target genes by recruiting CBP/P300. RSK-mediated phosphorylation of C/EBPβ Thr-217 inhibits caspases activity	(Buck et al., 2001; Wang et al., 2007)
Skin/keratinocytes	Newborn	Impaired keratinocyte differentiation, hyperplasia.	Decrease keratin 10 expression	(Lopez et al., 2009)
	Adult	Decrease neoplasm formation and increase keratinocytes apoptosis Refractory to skin cancer	Increase p53 activity. Increase expression of p53 and $p19^{ARF}$, but independently of $p19^{ARF}$. Increase apoptosis	(Ewing et al., 2008; Sterneck et al., 2006; Yoon et al., 2007; Zhu et al., 1999; Zhu et al., 2002)
Bone/osteoblasts	Embryo	Delayed bone formation, inhibition of chrondrocytes maturation.	Decreased expression of Runx2 target genes, synergy between C/EBPβ and Runx2	(Staiger et al., 2009)
	Adult	Osteopenia. Increase bone resorption, hyperactive osteoclasts	Decrease expression of MafB	(Smink et al., 2009)
Ovary	Adult	Inhibition of granulosa cells differentiation	Elevated expression of inhibin α. Increase expression of Ptgs2	(Burkart et al., 2005; Fan et al., 2009; Sterneck et al., 1997)

			and P450 aromatase. LH activates C/EBPβ via ERK1/2	
Mammary gland/epithelial cells	Adult	Defective ductal morphogenesis, reduced branching. Decrease oncogenic transformation. Decrease sensitivity to hormonal-dependent mitosis	Decrease cyclin D1 activity. Increase expression of TGFβ and Smad2, decrease expression of cyclin E and cdk2 activity	(Grimm et al., 2005; Lamb et al., 2003; Robinson et al., 1998)
Uterus/epithelial cells	Adult	Decrease proliferation of uterine epithelial cells in pregnancy, inhibition of decidualization. Uterine epithelium growth-arrest and increase apoptosis	Decrease sensitivity to pregnancy hormones. Decrease expression of E2F3, increase expression of p27, p53, ATM, ATR, p63	(Mantena et al., 2006; Ramathal et al., 2010)
Brain	Adult	Decrease volume of dead brain tissue following infarcts, decrease apoptosis	Decrease inflammation	(Kapadia et al., 2006)
Adipose tissue/obesity	Fetal/newborn	Decrease adipogenesis. Decrease epididymal fat	Decrease expression of PPARγ, PEPCK, aP2	(Tanaka et al., 1997)
	Adult	Refractory to high fat diet induced obesity and to fatty liver. Increase respiration rates and decrease blood levels of triglycerides, free fatty acids and cholesterol. Decrease pro-inflammatory cytokines expression in obesity	Decrease expression of acetyl-CoA carboxylase and fatty acid synthase. Increase expression of UCP-1 and UCP-3 Increase expression of LXRα, SCD1, DGAT2	(Millward et al., 2007; Rahman et al., 2012)
Brown adipose tissue	Newborn	Decrease lipid accumulation	Decrease UCP-1, Elovl3, PGC-1α, PPARγ expression	(Kajimura et al., 2009; Tanaka et al., 1997)
	Adult	Failure to promote thermogenesis upon cold temperature exposure	Decrease expression of LPL	(Carmona et al., 2005)
Pancreas/Islet cell	Adult	Ameliorates hyperglycemia in diabetic mouse	Decrease activity of Atf6α and GRP78	(Matsuda et al., 2010)
Lungs/epithelium	Adult	Decrease pulmonary fibrosis	Decrease expression of pro-inflammatory cytokines, decrease expression of a-smooth muscle actin and collagen	(Hu et al., 2007)
Immune system/myeloid cells	Adult	Apoptosis of Myc/Raf infected myeloid cells. Compromised immunity following	Decrease expression of IGF-1. MEK1/2 activates C/EBPβ. Inhibition of G-CSF signaling	(Tanaka et al., 1995; Wessells et al., 2004)

		intravenous pathogen infection. Deficient macrophages activity		
Immune system/lymphocytes	Adult	Hyperplasia of B-lymphocytes in thymus and lymph nodes. Impaired B-lymphocytes differentiation	Absence of IL-12. Decrease expression of CXCL12	(Screpanti et al., 1995; Yoshioka et al., 2014)
Spleen/lymphatic system	Adult	Spleenomegaly, lymphoadenopathy, peripheral lymph node enlargement, myeloid cells in lymph nodes	Increase IL-6 production	(Screpanti et al., 1995)
Bone marrow	Adult	Hyperplasia of haematopoietic stem cells	Increase levels of IL-6	(Screpanti et al., 1995)
Haematopoietic stem cells/granulopoiesis	Adult	Decrease cytokine-induced granulopoiesis. Inhibition of neutrophils and granulocytes differentiation	Decrease IL-3 expression. G-CSF stimulates C/EBPβ. Apoptosis of neutrophils, failure to respond to G-CSF	(Akagi et al., 2008; Hirai et al., 2006)
Skeletal muscle/myofibers	Adult	Resistant to tumor-induced muscle atrophy	Decrease expression of Atrogin-1	(Zhang et al., 2011)

APPENDIX 3: PUBLIC MICRO-ARRAY DATABASES OF C/EBPB TARGETS

Table 5 Public micro-array databases of C/EBPβ target genes.

Tissue	Age	Model	Reference
Mesenchymal stem cells	Adult	α2-collagen^{CreER} x Cebpb$^{f/f}$	(Hu et al., 2012)
Keratinocytes	Newborn	Keratin14^{Cre} x Cebpb$^{f/f}$	(Lopez et al., 2009)
Uterine epithelium	Adult	Cebpb$^{-/-}$ 18hrs estrogen treatment	(Ramathal et al., 2010)
C2C12 myoblasts		ShRNA Cebpb	(Kajimura et al., 2009)
Hep3B hepatoma		C/EBPβ-LAP and C/EBPβ-LIP inducible expression	(Saint-Auret et al., 2011)
3T3-L1 preadipocytes		SiRNA Cebpb	(Guo et al., 2012)
Macrophages	Adult	Cebpb$^{-/-}$ macrophages J2-virus transformed	(Wessells et al., 2004)