The regulation of satellite cell function and myogenesis by isoforms of C/EBPβ

Hwabin Lee

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the M.Sc. program in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

ABSTRACT

Adult skeletal muscles have remarkable regenerative capacity. Muscle regeneration occurs when muscle tissue experiences injury, causing a population of normally quiescent muscle-resident stem cells, called satellite cells, to become activated. The CCAAT/enhancer binding proteins known as C/EBPs are transcription factors belonging to the bZIP family. Previous work from our lab has identified C/EBPβ as an important negative regulator of myogenesis. C/EBPβ expression is localized to muscle satellite cells and is downregulated upon induction to differentiate, mirroring the loss of Pax7 expression in early myogenesis. C/EBPβ expression also negatively regulates MyoD protein expression. Leaky ribosomal scanning of the *Cebpb* mRNA produces three C/EBPβ isoforms: LAP*, LAP and LIP, though the individual role of each of these isoforms has not been investigated in myoblasts. This thesis focuses on determining the role of each of the C/EBPβ isoforms during skeletal muscle differentiation. Forced expression of the C/EBPβ-LIP isoform in myoblasts led to a decrease in Myf5, MyoD, and myogenin expression under differentiation conditions when compared to empty vector controls. Further, the fusion of cells was greatly reduced following differentiation. C/EBPβ-LIP expressing cells also demonstrated a growth defect, with pronounced G1 arrest and features of senescence. In contrast, myoblasts expressing the C/EBPβ-LAP isoform has impaired differentiation, though this was not as pronounced as in C/EBPβ-LIP expressing cells and proliferated normally. While LIP is not normally expressed in primary myoblasts from healthy muscle, the ratio of LIP:LAP was increased in primary myoblasts isolated from mdx mice, an animal model for Duchenne muscular dystrophy. These findings suggest that the regulation of C/EBPβ isoform expression could regulate stem cell stamina and may contribute to defects in muscle regeneration in disease.

ACKNOWLDEGEMENTS

I would like to express my sincere appreciation to my supervisor, Dr. Nadine Wiper-Bergeron, for patiently and respectfully guiding and encouraging me throughout my degree.

I would like to acknowledge my thesis advisory committee members Dr. Qiao Li and Dr. Ilona Skerjanc for their support and direction.

I would like to acknowledge a wonderful group of people in the lab for making my two years of graduate studies a very enjoyable experience. Neena Lala-Tabbert, Dechen Fu, Catherine St-Louis, Francois Marchildon and Emilie Lamarche.

I would like to acknowledge the following people and groups of people for helping me to get through this: Jinsoo Shin, Emil Al-Kadi, Hamood Al-Sudais, Li Lab, Trinkle-Mulcahy Lab, SGI members and CMM council members 2014-2015.

I would like to acknowledge NSERC and the OGS program for their generous scholarships.

Special thanks to my mom and my dad for providing the fortunate opportunity to pursue higher education in Canada.

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1 Muscle Regeneration and Satellite Cells

Adult skeletal muscles have remarkable regenerative capacity. Muscle regeneration occurs when muscle tissue experiences injury, causing a population of normally quiescent muscle-resident stem cells (called satellite cells due to their satellite position on the muscle fiber) to become activated (Snow, 1978). Satellite cells are committed progenitors, making up only a small fraction of all myonuclei - less than 5% in adult skeletal muscle (Bischoff and Heintz, 1994). Intense research has laid the foundation for our current view on satellite cell function: the maintenance and regeneration of skeletal muscle mass (Morgan and Patridge, 2003; Charge and Rudnicki, 2004). Several molecular markers of satellite cells have been identified which include cell surface markers (eg. c-Met, α7-integrin, Syndecan-3 and 4), adhesion molecules (eg. m-cadherin), CD34 (established marker of the hematopoietic stem cell lineage) and the myogenic transcription factor Pax7 (Irintchev *et al*., 1994; Heslop *et al*., 2000; Cornelison and Wold 1997; Seale *et al*., 2000; Tedesco *et al*., 2010).

1.2 The myogenic regulatory factors (MRFs) and Pax7

Early studies in the field of skeletal myogenesis led to the discovery of the myogenic regulatory factors (MRFs): MyoD, Myf5, myogenin, and MRF4 (Myf6) (Konigsberg, 1963; Yaffe, 1968; Braun *et al*., 1989; Davis *et al*., 1987; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Wright *et al*., 1989). Since their discovery, the mechanisms by which the MRFs regulate commitment to the myogenic lineage, skeletal muscle development and adult skeletal myogenesis have been intensely studied.

The myogenic regulatory factors are a family of basic helix loop helix (bHLH) transcriptional factors that share a conserved bHLH domain responsible for DNA binding and

dimerization (Hu *et al*., 1998; Murre *et al*., 1989). Myf5 is required for the regulation of proliferation, while MyoD is required for entry into the muscle differentiation program (Megeney *et al* 1996). Myogenin and MRF4 are required for myogenic differentiation and fusion for the formation of myotubes (Rudnicki and Jaenisch, 1995). Because of functional redundancy, either Myf5 or MyoD is required for the normal development of skeletal muscle; however, the loss of both Myf5 and MyoD results in a total loss of skeletal muscle progenitors and the complete loss of skeletal muscle (Rudnicki *et al*., 1993). MyoD null mice are normal at birth and the skeletal muscle phenotype is normal; however, they exhibit impaired muscle regeneration after injury in the postnatal animal and satellite cells isolated from MyoD-/- mice are defective in differentiation suggesting that MyoD has an important role in postnatal muscle regeneration (Megeney *et al*., 1996; Cornelison *et al*., 2000). Normal numbers of myoblasts are present in myogenin knockout mice but these mice exhibit a severe deficiency in mature skeletal muscle (Hasty *et al*., 1993). MRF4 knockout mice exhibit normal skeletal muscle with a higher level of myogenin suggesting increases in myogenin levels are able to compensate for loss of MRF4, but MRF4 cannot compensate for loss of myogenin (Zhang *et al*., 1995). Therefore, Myf5 and MyoD are expressed first in progenitor cells of the myogenic lineage and act as determination factors, while myogenin and MRF4 are secondary MRFs required for terminal differentiation and fusion.

Pax7 belongs to the family of paired box transcription factors and has been identified as the primary molecular marker for satellite cells since it is expressed uniformly in both quiescent and activated satellite stem cell populations (Charge and Rudnicki, 2004; Seale *et al*., 2000; Kuang *et al* 2007). Although the Pax7-/- mouse undergoes normal embryonic development, embryonic loss of Pax7 results in severe muscle atrophy due to a complete absence of satellite cells necessary for postnatal growth and regeneration (Seale *et al*., 2000; Oustanina *et al*., 2004). Pax

7 has an impact on the function and the number of satellite cells in the developing animal and during postnatal growth (prior to postnatal day 21), as the conditional inactivation of Pax7 between P7-11 impaired the regeneration severely (Lepper *et al*., 2009).

1.3 CCAAT/Enhancer Binding proteins.

The CCAAT/enhancer binding proteins, known as C/EBPs, are transcription factors belonging to the bZIP family and are known to play an important role in cell proliferation, apoptosis, and differentiation in a number of cell types including adipocytes, hepatocytes, keratinocytes, and hematopoietic cells (Birkenmeier *et al*. 1989; Cao *et al*. 1991; Flodby *et al*. 1996; Ramji and Foka 2002; Nerlov *et al*. 2007). There are six C/EBP family members: C/EBPα, β, δ, ε, γ and ζ, (Descombes *et al*., 1990; Williams *et al*., 1997). C/EBP transcription factors are characterized by the presence of a highly conserved C-terminal basic region followed by a leucine zipper motif which allow DNA binding and dimerization among family members, respectively (Ramji and Foka 2002). Dimerization is a prerequisite for DNA binding to the CCAAT box motif found in many gene promoters (Ramji and Foka 2002). By contrast, the N-terminal domain of C/EBP factors, which houses the activation domain, is far less conserved with lower than 20 percent sequence similarity (Ramji and Foka 2002).

1.4 CCAAT/Enhancer Binding protein beta.

C/EBPβ is ubiquitously expressed and has been shown to play a role in cell differentiation, apoptosis and inflammation. C/EBPβ was first identified as a DNA-binding protein responsible for the actions of IL-6 and as a consequence was named NF-IL-6 (Akira *et al*., 1990). *Cebpb* is an intronless gene, producing a single mRNA (Cao *et al*., 1991; Descombes and Schibler, 1991) though leaky ribosomal scanning of the *Cebpb* mRNA produces three C/EBPβ protein isoforms: Liver-enriched Activator protein * (LAP*), LAP, and LIP (Liver-enriched inhibitory protein)

(Cao *et al*., 1991; Descombes and Schibler, 1991; O'Rourke *et al*., 1997; Gigliotti and DeWille, 1998). The three isoforms are produced using one of three in frame AUG codons that can serve as start sites (Fig. 1). The first AUG codon, found at the 5'end of the mRNA, is used to produce the full length LAP* product. The second AUG produces LAP, a protein product lacking the first 21 amino acids of LAP*, but retaining all of the activation function. The third AUG produces LIP, an inhibitory form of C/EBPβ that can bind DNA and dimerize with C/EBP factors but lacks activation domains that would normally allow for the activation of transcriptional responses by C/EBPβ (Descomebes and Schibler 1991; Raught *et al*., 1995; Calkhoven *et al*., 2007).

While the isoforms are largely produced by ribosome scanning of the mRNA, the ratio of LAP*, LAP and LIP appears to be regulated by eukaryotic translation initiation factors (eIFs) and the kinases PKR and mTOR (Hershey, 1991; Morris, 1995; Calkhoven *et al*. 2007). PKR and mTOR signaling modulate the activity of eIF-2 and eIF-4E, such that increased eIF function promotes the expression of LIP, while decreased eIF function shifts the expression to favour full-length isoforms in the pre-adipocyte 3T3-L1 cell line.

The differential expression of LAP*, LAP and LIP has been studied in the regenerating liver (Luedde *et al*., 2004). Mice injected with an adenovirus to express LAP had delayed S phase entry with only 7% BrdU-positive hepatocytes compared to 20% in the control group (Luedde *et al*., 2004) indicating a negative effect of LAP on hepatocyte proliferation. In contrast, LIP overexpression increased the level of cell cycle markers including cyclin A and cyclin E, emphasizing the distinct functions of LAP and LIP in hepatocyte proliferation (Luedde *et al*., 2004).

In mesenchymal stem cells, the LIP isoform was shown to be essential for osteoblast differentiation (Hata *et al.*, 2005). Overexpression of LIP in C3H10T1/2 mesenchymal stem cells inhibited adipocyte differentiation while drastically enhancing osteoblast differentiation in the presence of BMP2 (Bone Morphogenetic Protein 2). Moreover, the overexpression of LIP in C2C12 cells increased BMP2-induced alkaline phosphatase activity and stimulated Runx2-dependent alkaline phosphatase activity. Importantly, an oligonucleotide precipitation assay and chromatin immunoprecipitation experiment showed that LIP can directly bind to the osteocalcin gene promoter. *In vivo*, the correlation between LIP expression and osteoblastogenesis was not retained as transgenic mice expressing only the LIP isoform demonstrated attenuated bone formation and osteopenia (Harrison *et al.*, 2005). This discrepancy is likely due to the lack of activating forms of C/EBPβ in the animal model, suggesting that it is the relative amounts of each factor that is important in the regulation of differentiation processes such as osteoblastogenesis.

1.5 The cell cycle

Proper progression through the cell cycle is essential for cell differentiation. There are four distinct phases in the cell cycle that are tightly controlled: G_1 (gap 1), S (synthesis), G_2 (gap 2) and M (mitosis). Cells can commit to enter the cell cycle in the G_1 phase or undergo a reversible cell cycle arrest known as the quiescent state (G_0) in the absence of nutrients and growth factors. S phase allows the synthesis of DNA and it is bounded by two gaps, G_1 and G_2, which take place prior to DNA replication and mitosis (M), respectively. Each of these phases is controlled by cell cycle checkpoints, which ensure the proper completion of each phase by temporary cell cycle arrest (Hartwell and Weinert, 1989).

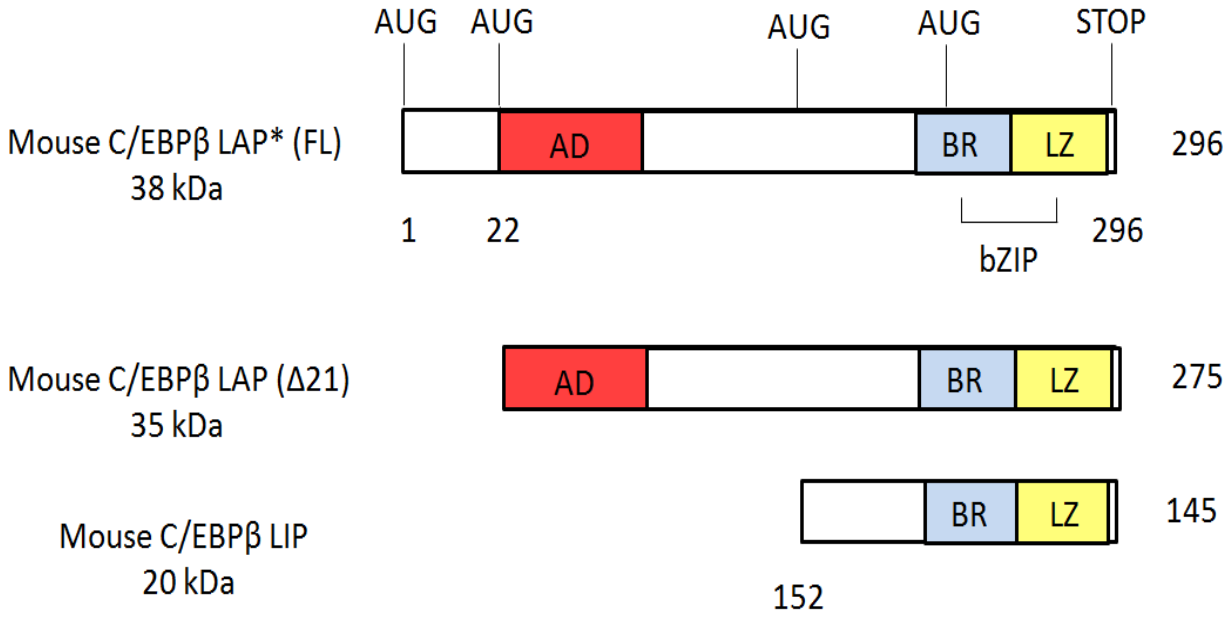


FIGURE 1. A SCHEMATIC REPRESENTATION OF C/EBPβ ISOFORMS LAP*, LAP AND LIP. The C/EBPβ gene contains three possible AUG codons that can serve as start sites. CCAAT/enhancer-binding protein beta (C/EBPβ), Activation domain (AD), Leucine zipper (LZ), DNA basic binding region (BR), Basic leucine zipper domain (bZIP).

A family of serine/threonine protein kinases known as cyclin-dependent kinases (CDK) are the key regulatory proteins activated during the cell cycle, are responsible for cell cycle progression, and are themselves regulated by subunits called cyclins.

Unlike CDK levels which are constant throughout the cell cycle, the expression of cyclins rises and falls during a specific phase of the cell cycle due to ubiquitin-mediated proteolysis (Pines 1991; Glotzer *et al.* 1991; Slingerland and Pagano, 1998). Different types of cyclins are responsible for binding to CDK proteins at specific phase of the cell cycle. The activation and expression of particular CDK-cyclin complexes during specific cell cycle stages allows a stepwise entry into each phase throughout the cell cycle. The G_1 to S phase transition of the cell cycle is critical for the control of cell proliferation and the mechanism of transcriptional activation is conserved from yeast to humans (Bertoli *et al*., 2013). CDK-cyclin D complexes are required for G_1 while CDK-cyclin E complexes regulate the entry into S phase (Ohtsubo *et al* 1995). The level of D-type cyclins peaks in the early stages of G_1, which then interact with CDK4 and CDK6 (Ohtsubo *et al.,* 1995). In late G_1, E-type cyclins interact with CDK2 promoting cell cycle progression into S phase (Ohtsubo *et al.,* 1995). A CDK-cyclin A complex is required during S phase for the completion of DNA replication (Walker& Maller, 1991). Negative cell cycle regulators known as cyclin kinase inhibitors (cki, INK4 family and the KIP family) regulate CDK-cyclin complexes which in turn regulate members of the pocket protein family (Rb, p107, and p130). Inhibition of cyclin-CDK complexes by these two families results in G_1 cell cycle arrest by preventing the G_1 to S cell cycle transition.

1.6 Cell Cycle and myogenesis

In order for myoblasts to undergo terminal differentiation into mature myotubes, cells must exit the cell cycle in low serum concentrations. Unlike quiescent cells, the post-mitotic

multinucleated myotubes cannot re-enter the cell cycle and proliferate in the presence of growth factor (Tiainen *et al.*, 1996). This permanent withdrawal from the cell cycle allows myoblasts to commit to the differentiation program (Andres and Walsh, 1996). The expression of cyclin A and cyclin D1 are downregulated during differentiation (Wang and Nadal-Ginard, 1995) while the overexpression of CDK2/cyclin A and cyclin D1 in C2C12 myoblasts inhibited the transcriptional activation of the muscle-specific genes such as MyoD and myogenin (Guo and Walsh, 1997). Furthermore, many studies have also investigated interactions between MyoD and the cell cycle regulators that lead to cell cycle arrest of myoblasts (Gu *et al*., 1993; Martelli *et al*., 1994). Rb is a critical regulator of G_1 during cell cycle progression that can also regulate skeletal muscle differentiation. Both the Rb mRNA and protein are upregulated during terminal differentiation and an accumulation of hypo-phosphorylated Rb occurs in early differentiation (Endo and Goto, 1992; Gu *et al*., 1993). The direct interaction between MyoD and hypo-phosphorylated Rb has also been shown using HeLa cells, emphasizing Rb as an important regulatory mechanism from the cell cycle exit to muscle differentiation (Gu *et al*., 1993).

1.7 C/EBPβ in proliferation

Many studies have identified C/EBPβ as a key regulator controlling the differentiation and proliferation of multiple cell types. While the role of C/EBPα is strictly antiproliferative, the effects of C/EBPβ are more complex, having both a negative and positive role in cell proliferation. Similar to C/EBPα, C/EBPβ has growth inhibiting activities in hepatocellular carcinoma, lymphoid cells, epidermal keratinocytes, and primary fibroblasts (Buck *et al*., 1994; Screpanti *et al*., 1995; Zhu *et al*., 1999; Sebastian *et al*, 2005). Conversely, growth promoting activities of C/EBPβ have been shown in mammary epithelial cells in mice and human, macrophage tumor cells and normal hepatocytes (Robinson *et al*., 1998; Seagroves *et al*., 1998;

Bundy and Sealy, 2003; Wessells *et al*., 2004; Luedde *et al*., 2004). The effect of C/EBPβ on proliferation is therefore context specific.

Interestingly, C/EBPβ is regulated by Rb. Earlier studies have shown a physical interaction between all three isoforms of C/EBPβ and the hypophosphorylated form Rb (Chen *et al*., 1996). Using transiently transfected cells, Rb has been shown to increase the transcriptional activity of C/EBPβ, indicating the positive regulation by Rb (Chen *et al*., 1996).

The role of each isoform on proliferation has also been investigated in several tissues. The proliferative activity of LIP has been demonstrated in the adult rodent mammary gland during pregnancy (Ranghi *et al*., 1995). Using transient transfections, all three forms of C/EBPβ have been shown to regulate the cyclin D1 promoter. While LAP activates the cyclin D1 promoter the activity of LIP and LAP* at the cyclin D1 promoter has the opposite effect (Bundy *et al*., 2003; Eaton *et al*., 2001). C/EBPβ$^{-/-}$ mouse embryo fibroblasts (MEFs) cannot undergo mitotic clonal expansion (MCE) and fail to accumulate triglyceride during adipogenesis (Tang *et al*., 2003). The LAP isoform can rescue both MCE and differentiation but not LIP isoform suggesting the distinct role of C/EBPβ isoforms (Tang *et al*., 2003). A distinct role of each isoform on cell proliferation has also been shown in hepatoma cells. Overexpression of C/EBPβ LAP arrests hepatocytes in G1/S transition but expression of LIP overcomes the effect of LAP on the cell cycle emphasizing the importance of the LIP/LAP ratio in cell cycle progression (Buck *et al*., 1994). One of the central mechanisms driving cell proliferation by LIP proposed is repression of the E2F complex. C/EBPβ has been shown to repress the E2F complex which is dependent on the members of Rb protein family (Sebastian *et al*., 2005). LIP lacking activation domains, which can work as a dominant negative, has been shown to disrupt the E2F1-Rb complexes

(Orellana *et al.*, 2010). This important finding on the mechanism of LIP in cell proliferation advances our knowledge on the anti-proliferative effects of C/EBPβ.

1.8 Cell-cycle arrest and senescence

In the absence of nutrients and growth factors, cells undergo a reversible cell cycle arrest known as quiescence. However, unlike quiescent cells that can resume proliferation in the presence of nutrients, post-mitotic cells cannot re-enter the cell cycle after differentiation. Cell cycle checkpoints induce temporary cell cycle arrest to ensure the detection of any errors during the cell cycle. Continual stress and excessive damage can also cause a permanent withdrawal from cell cycle known as cellular senescence.

Senescence markers

Senescent cells can be detected by distinct morphological changes and markers, which are not completely specific on their own. Therefore, multiple markers are used to confirm the senescent cells. Flat and enlarged cell morphology is often associated with senescent cells, while the expression of cell cycle inhibitors, tumour suppressors, DNA damage markers, heterochromatin markers such as histone 3 Lys9 trimethylation (H3K9me3) and the absence of proliferation markers such as Ki67 staining and the 5-bromodeoxyuridine incorporation (BrdU) support the senescent phenotype. One of the main features that senescent cell display is the decline in DNA replication during S phase, which can be detected by several proliferation markers including the BrdU and Ki67. BrdU labeling can be used to determine cell proliferation by detecting the presence of newly synthesized DNA, as BrdU is a nucleotide analog that is incorporated during the S phase. Nuclear antigen Ki67 can be used as a proliferative marker because it is specific to proliferating cells. The Ki67 protein is absent in cells in G_0 but is present

in all active phases of the cell cycle including G_1, S, G_2 and M phases, which allows to determination of the percentage of proliferating cells in a culture (Gerdes *et al*., 1984). One specific marker that has been identified to stain senescent cells is senescence-associated β-galactosidase activity at pH 6 which was discovered in 1995 (Dimri *et al*., 1995). Senescent cells can be stained for β-galactosidase activity by using the β-galactosidase substrate X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). The formation of an insoluble blue substrate can be detected in senescent cells but not in quiescent or in terminally differentiated cells (Dimri *et al*., 1995).

1.9 C/EBPβ is a regulator of skeletal muscle regeneration

Loss of *Cebpb* in a mouse model results in severely reduced white adipose tissue, immunodeficiency and increased insulin sensitivity in skeletal muscle (Tanaka *et al*., 1997; Wang *et al*., 2000). Mice lacking C/EBPβ have decreased plasma levels of insulin, glucose and free fatty acid indicating decreased gluconeogenesis during fasting. More importantly, the skeletal muscle from the mice lacking C/EBPβ had greater insulin-stimulated glucose transport activity and increased phosphatidylinositol 3-kinase (PI3K) and Akt serine phosphorylation activity that are important cellular mediators of cell growth and protein synthesis (Wang *et al*., 2000). These results indicate increased glucose metabolism in skeletal muscle of C/EBPβ$^{-/-}$mice.

C/EBPβ is expressed in muscle and the upregulation of C/EBPβ expression has been shown in many of muscle wasting models (Penner, Gang *et al*. 2002; Giresi, Stevenson *et al*. 2005; Allen, Bandstra *et al*. 2009). In order to understand the *in vivo* role of C/EBPβ in skeletal muscle, our lab has investigated the expression pattern of C/EBPβ. Indirect immunofluorescence revealed that C/EBPβ expression was localized to Pax7+ cells indicating C/EBPβ, predominantly the LAP isoform, is expressed in satellite cells of healthy muscle but not in myonuclei (Marchildon *et al*.,

2012). In addition, C/EBPβ expression was down-regulated upon induction to differentiate, mirroring the loss of Pax7 expression. Persistent C/EBPβ expression negatively regulated the expression of MyoD and myogenic differentiation (Marchildon *et al*., 2012, Fu *et al*., 2015). Loss of C/EBPβ expression, mostly LAP protein, in satellite cells increased cell fusion and promoted muscle differentiation. Thus, our data suggest that the downregulation of C/EBPβ expression is critical for the expression of MyoD and for differentiation to occur. Moreover, *Cebpb* conditional knockout mice showed 30% increase in the average cross-sectional area of regenerating fibers compared to the control group following an injury to muscles (Marchildon *et al*., 2012).

2. RATIONALE

Our laboratory has identified that C/EBPβ is an important negative regulator of myogenesis (Marchildon *et al*., 2012). Previous studies in our laboratory have investigated the role of C/EBPβ in myogenic differentiation using ectopic expression of C/EBPβ in C2C12 myoblasts and primary myoblasts and a conditional null mouse model (Marchildon *et al*., 2012). However, little is known about the role of the LAP* LAP and LIP isoforms individually in skeletal muscle differentiation. Studies investigating the role of individual C/EBPβ isoforms in myogenesis are lacking

3. OBJECTIVES and HYPOTHESIS

3.1 Hypothesis

Given that overexpression of C/EBPβ (LAP*, LAP and LIP) inhibits myogenesis, I hypothesize that over-expression of the LAP isoform alone in C2C12 myoblasts will inhibit

differentiation and fusion of myocytes. I further predict that over-expression of the LIP isoform alone will act as a dominant negative and enhance cell differentiation and cell fusion.

3.2 Objectives

1. To characterize the impact of the LAP and LIP isoform on myogenesis (differentiation and fusion) using overexpression studies.
2. To examine the impact of the LAP and LIP isoform on proliferation and cell cycle progression in C2C12 myoblasts.

4. MATERIALS AND METHODS

4.1 Constructs

A retroviral vector encoding LAP was generated by site-directed mutagenesis QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Mississauga, Ontario, Canada) of pLXSN-C/EBPβΔ21using the following primers: forward Primer (5'-GACGACGCGCCCGCCCTGGCGGCCGGTTTCC 3'); reverse primer (5' GGAAACCGGCCGCCAGGGCCGGGCGCGTCGTC 3') and according to manufacturer's instructions. The C/EBPβΔ21 construct which lacks the first 21 amino acids in the N-terminal was originally generated by C. St-Louis. DNA sequencing was performed to verify the mutation from M to L at second AUG of C/EBPβΔ21 (DNA Sequencing Facility, Ontario Genomics Innovation Centre). The pLXSN-C/EBPβ LIP (*M. musculus)* construct was kindly provided by Dr. Abdou. The pLXSN-C/EBPβ LIP dbd- construct was generated by D. Fu and has two point mutations, A235P and K238P, in the C-terminal bzip domain preventing DNA binding.

4.2 Cell culture

C2C12 myoblasts (American Type Culture Collection, Manassas, VA, USA) were cultured and maintained in growth medium containing Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose, 110 mg/L sodium pyruvate, and 584 mg/L L-glutamine) (Wisent, Saint-Bruno, QC, Canada), 10% heat-inactivated fetal bovine serum (HI-FBS) (Invitrogen, Carlsbad, CA, USA) and Non-Essential Amino Acids (NEAA). Cultures were maintained at subconfluent densities to maintain differentiation potential and were passaged every two days. To induce differentiation of C2C12 myoblasts, DMEM supplemented with 2% heat-inactivated horse serum (HI-HS) was added to near-confluent C2C12 cells (80~90% density).

4.3 Retrovial Transfection and Infection

Calcium phosphate transfection was used to create replication incompetent pLXSN-based retroviruses (Clontech Laboratories, Inc., Mountain View, CA, USA) from Phoenix™ Ampho packaging cells. Phoenix cells were grown in a 60mm dish (70-80% confluent) and 3mL of growth medium containing 25 µM chloroquine diphosphate (Sigma-Aldrich, Oakville, ON, Canada) was added to each dish. 10ug of Plasmid DNA was diluted in a final volume of 500uL which contained 428 µl of ddH_2O and 62 µl of $CaCl_2$. 500 µl of 2X HBS (10mM KCl, 50mM HEPES pH 7.05, 12mM dextrose, 1.5mM Na_2HPO_4 and 280mM NaCl) and 10uL of plasmid DNA suspended in 500uL were quickly added dropwise to the Phoenix cells. The Phoenix cells were incubated for 10 hours at 37C° after which media was refreshed. The medium containing virus was collected 48hours post-transfection and filtered using a 0.45 µm syringe filter (Millipore, Billerica, MA, USA) to remove cellular debris. 10cm dishes of 50-60% confluent C2C12 cells were infected with 1mL of viral supernatant in 5mL of DMEM containing 6ug/mL polybrene (Sigma-Aldrich, Oakville, ON, Canada) and incubated for 15 minutes at 37C°. The dishes were wrapped with parafilm and centrifuged for 30 minutes at 1100g. Selection with G418 (Sigma-Aldrich, Oakville, ON, Canada) at a final concentration of 400ug/mL was started 48 hours after infection media for 10 days.

4.4 Western Analysis and Immunodetection

Cells were washed twice with 1X PBS and collected in IPH buffer (50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, and 0.5% NP-40, protease inhibitors (1X), 1mM DTT). Each sample was sonicated twice for 10 seconds at 10% power and incubated on ice for 30 minutes, and centrifuged at 13 000 rpm for 10 minutes (4°C) to remove debris. Supernatant was transferred to a new tube. The Thermo Scientific Pierce BCA protein Assay Kit was used to determine the

protein concentrations. 25µg of protein sample were loaded onto 10% or 12% SDS-PAGE gel and run at 200V for approximately 45 minutes, and transferred onto PVDF transfer membrane at 100V for 1.5 hours at room temperature. The PVDF membrane was then incubated with blocking solution (2% milk) for 30 minutes. After blocking, the membrane was incubated overnight with primary antibody diluted in either 1X PBS-T (1X phosphate buffered saline with 0.05% Tween-20) or blocking solution (2% milk) at 4°C. Blots were washed three times with 1X PBS-T for 5 minutes each. Detection was achieved by incubation with anti-rabbit or anti-mouse secondary antibody at a concentration of 1:5000 (GE Healthcare, Buckinghamshire, UK) for one hour at room temperature. The blots were washed three times with 1X PBS-T for 5 minutes each and antigen-antibody complexes were detected using the Fujifilm LAS-4000 chemiluminescent detection system was used to capture images.

4.5 Immnnocytochemistry

In order to assess differentiation and fusion of C2C12 myoblasts retrovially transduced to express C/EBPβ-LIP or LAP, immunohistochemistry was performed using anti-myosin heavy chain antibody (MF-20, DSHB). Cells were washed twice with 1X PBS and fixed in ice cold methanol for 10 minutes at room temperature. Cell were washed again twice with 1X PBS and permeabilized for 15 minutes with 1X PBS containing 0.5% Triton X-100. Cells were incubated with MF-20 primary antibody 1:100 dilution in 1X PBS containing 0.1% Triton X-100 overnight at 4 C° followed by incubation with Cy3-conjugated donkey anti-mouse IgG secondary antibody at 1:1000 dilution (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at room temperature. The cells were washed with 1X PBS containing 0.1% Triton X-100 three times and counterstained with DAPI at a concentration of 0.5ug/mL or 1 minute. Pictures were taken of a minimum of five random fields of view at 10x magnification. The differentiation index is the

number of myosin heavy chain positive nuclei divided by the total number of nuclei and the fusion index is the number of myosin heavy chain positive nuclei in myotubes divided by the total number of myotubes. To compare the percentages of proliferating cells between C2C12 myoblasts retrovially transduced to express C/EBPβ-LIP and LAP, the expression of the Ki67 protein was determined by the Ki67 immunofluorescence staining. Cells were washed twice with 1X PBS and fixed in 2% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were permeabilized with 0.3% Triton X-100 in 1X PBS for 15 minutes at room temperature. 10% Goat Serum (GS) in 0.3% Triton X-100 PBS (PBS-T) was used to block for 30 minutes at room temperature. Cells were then incubated with primary antibodies diluted in 2% GS and 0.3 PBS-T overnight at 4C°. Cell were washed with 0.1% PBS-T three times and incubated with secondary antibodies for 1 hour at room temperature. Ki67 (Abcam Inc. Cambridge, MA, USA) was used at 1:500 dilution as primary antibody and 594 conjugated donkey α-rabbit (1:500) was used as secondary antibody. The cells were washed with 1X PBS containing 0.1% Triton X-100 three times and counterstained with DAPI at a concentration of 0.5ug/mL or 1 minute.

4.6 Flow Cytometry

Cell cycle analysis by flow cytometry was performed using approximately 10^6 cells maintained in sub-confluent cultures. The cells were harvested by trypsinization and centrifuged at 1200 RPM for 2 min. The collected cells then washed in phosphate-buffered saline (PBS) and counted using a hemocytometer. The cells were fixed in 75% ethanol for 5 minutes on ice then washed and rehydrated in PBS for 15 minutes at room temperature. The cells were resuspended in 1mL of propidium iodide staining solution containing 100mM Tris, pH 7.4, 150mM NaCl, 1mM $CaCl_2$, 0.5mM $MgCl_2$ and 0.1% Nonidet P-40, and stained with 2ug/mL propidium iodide. C2C12 cells retrovially transduced to express C/EBPβ-LIP or C/EBPβ-LAP, or with empty virus

(pLXSN) were cultured in growth medium. To arrest C2C12 cells in G1, they were kept under deprivation condition using culture medium without L-methionine. The percentages of cells in the G0/G1, S, and G2/M phases were determined.

4.7 Senescence β-Galactosidase Staining

C2C12 myoblasts retrovially transduced to express C/EBPβ-LIP were tested for senescence using the Senescence β-Galactosidase Staining Kit from Cell Signaling Technology (Danvers, MA, USA), which detects β-galactosidase activity at pH 6, a widely known characteristics of senescent cells, according to manufacturer's instructions. The staining was verified under the microscope and pictures were taken of a minimum of 3 random field of view at 10X and 20X magnification per well.

4.8 Caspase 3/7

Caspase3/7 activity was assessed using the Apo-ONE ® Homogeneous Caspase-3/7 Assay Kit (Promega) according to manufacturer's instructions. Cells were trypsinized and diluted to $2x10^5$ cells per mL and 50uL of the cell suspension was added to each well of a 96 well-plate and incubated at 37C° overnight. Fluorescence was detected at the excitation wavelength of 499nm with emission wavelength of 521nm.

4.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). The student's *t* test was used to compare a control and an experimental condition in one group. One-way ANOVA followed by Tukey's post-hoc test was used to compare one factor in three or more groups.

5. RESULTS

5.1 The impact of LAP and LIP isoform expression on myogenesis in overexpression studies

Two different C/EBPβ mutants, pLXSN-C/EBPβΔ21$_{M131L}$ (LAP) and pLXSN-C/EBPβ$_{LIP}$ (LIP) were used to determine the impact of each C/EBPβ isoform on myogenesis. The retroviral vector pLXSN-C/EBPβΔ21$_{M131L}$ encoding LAP was generated using site-directed mutagenesis (QuikChange site-directed mutagenesis kit) by introducing a point mutation into the AUG codon that drives expression of LIP in a plasmid coding for a N-terminally truncated C/EBPβ construct (LAP) lacking the first 21 amino acids (Fig. 2). pLXSN-LIP was kindly provided by Dr. Abdou Salaam, and encodes the sequence for expression of mouse LIP. To determine the impact of each C/EBPβ isoform on myogenesis, C2C12 myoblasts were retrovirally transduced with empty vector (pLXSN) as a control or with either pLXSN-C/EBPβΔ21$_{M131L}$ or pLXSN-C/EBPβ$_{LIP}$ for the overexpression of C/EBPβ isoforms.

Pooled stable cell lines generated were cultured in growth medium or induced to differentiate into myotubes for 4 days and the level of construct overexpression was determined. Western analysis of LAP isoform expression in C2C12 cells transduced to express C/EBPβΔ21$_{M131L}$ compared to empty vector controls confirmed the overexpression of the LAP isoform by 6-fold in growth medium and 4-fold after 96 hours in differentiation medium (Fig. 3A). In cells transduced to express the LIP isoform, LIP expression was increased 33-fold in growth medium and 20-fold after 96 hours in differentiation medium as compared to empty vector controls (Fig. 3B).

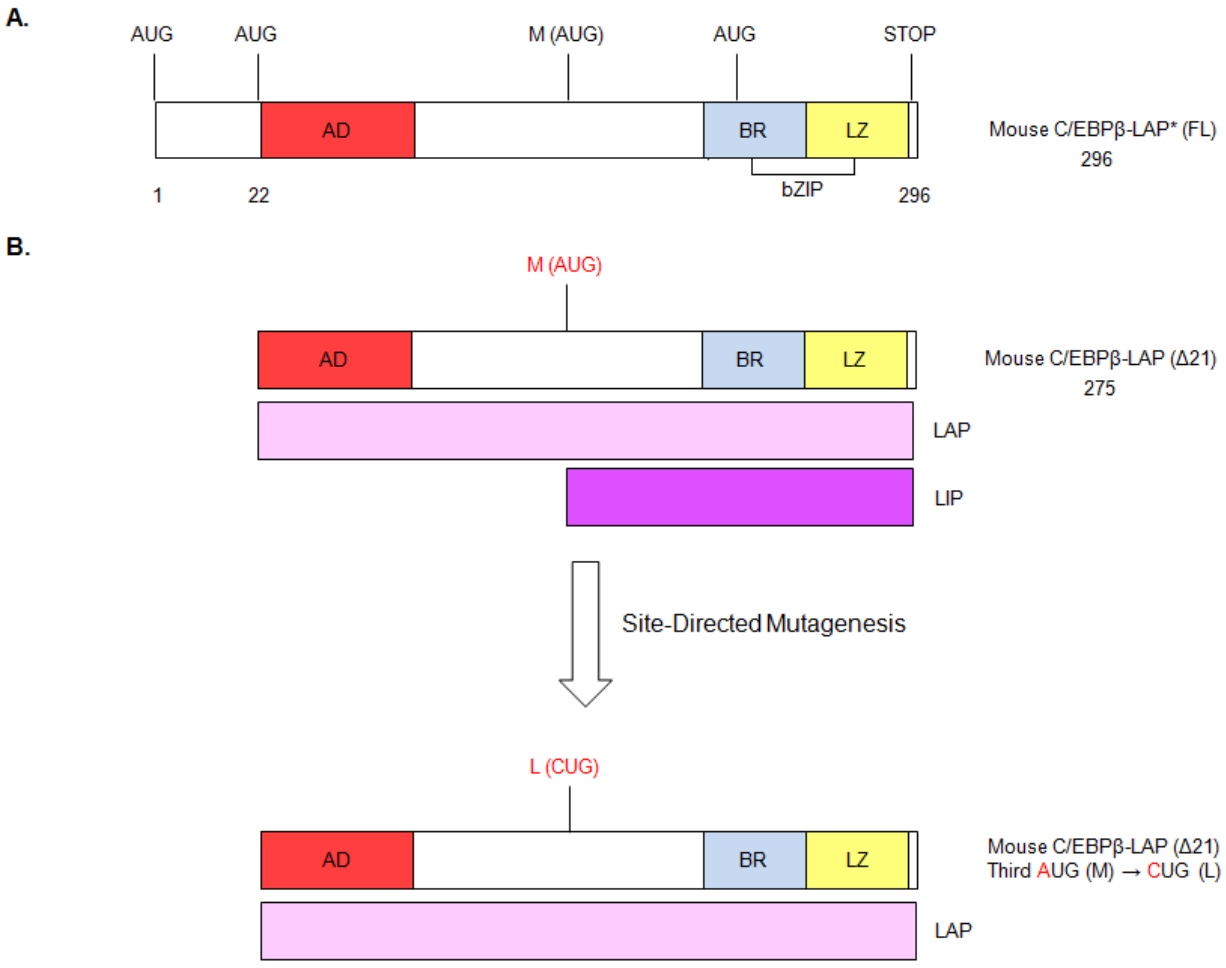


FIGURE 2: GENERATION OF C/EBPβ-LIP AND C/EBPβ-LAP CONSTRUCTS USING SITE-DIRECTED MUTAGENESIS.

(A) Full length LAP* (FL) and LAP (Δ21) differ by 21 amino acids. **(B)** To generate a plasmid driving the expression of only the LAP (Δ21) isoform, a point mutation (A to C) was introduced in the third AUG codon changing methionine (M) to leucine (L), which prevents the expression of LIP. Abbreviations: Activation domain (AD), Leucine zipper (LZ), and DNA basic binding region (BR).

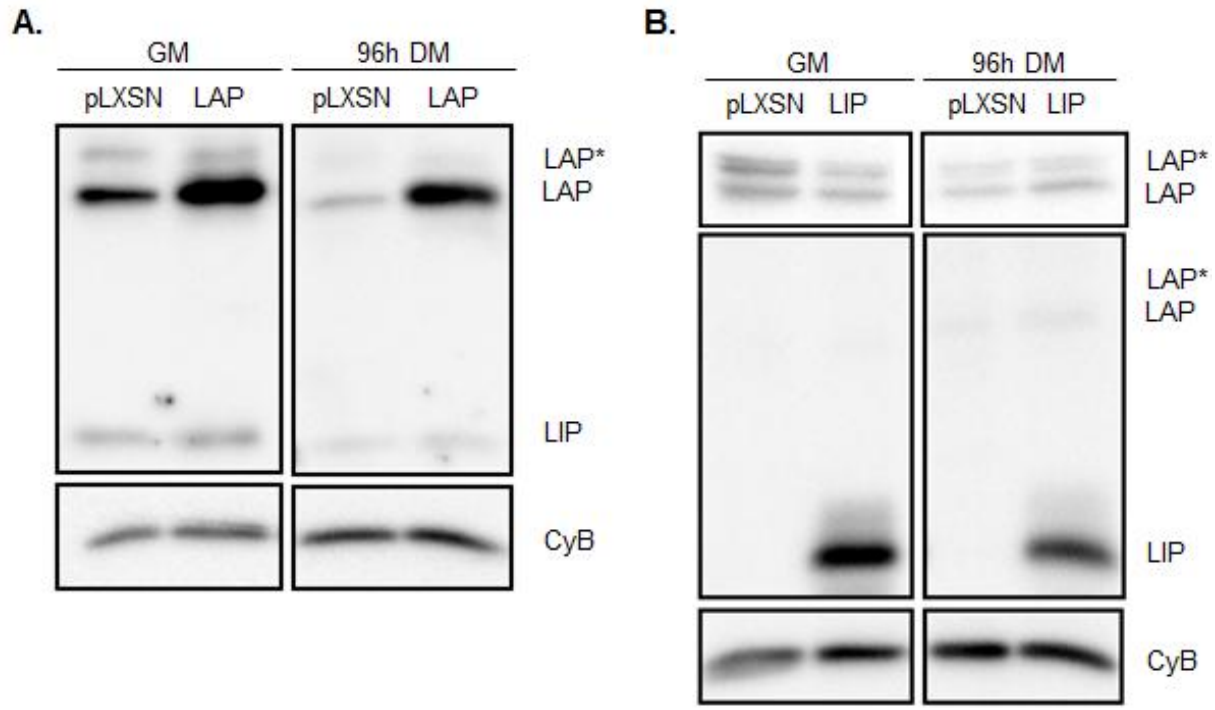


FIGURE 3: C/EBPβ ISOFORM EXPRESSION IN C2C12 MYOBLASTS.

(A) Western analysis of C/EBPβ isoform expression in C2C12 myoblasts retrovirally transduced with empty vector (pLXSN) or to express the C/EBPβ LAP isoform cultured in growth medium (GM) or induced to differentiate in low serum conditions for 96 hours (DM 96h). Cyclophilin B (CyPB) is used as a loading control. **(B)** Western analysis of C/EBPβ isoform expression in C2C12 myoblasts retrovirally transduced with empty vector (pQCXIP) as a control or to express the C/EBPβ-LIP isoform (pQCXIP-LIP) cultured as in (A). Top panel shows LAP* and LAP expression in a higher exposure image. Lower panel is a shorter exposure to show LIP expression. Abbreviations: CyB, cyclophilin B; LAP, liver activating protein; LIP, liver inhibitory protein; pLX, pLXSN retroviral vector; C, control pQCXIP retroviral vector.

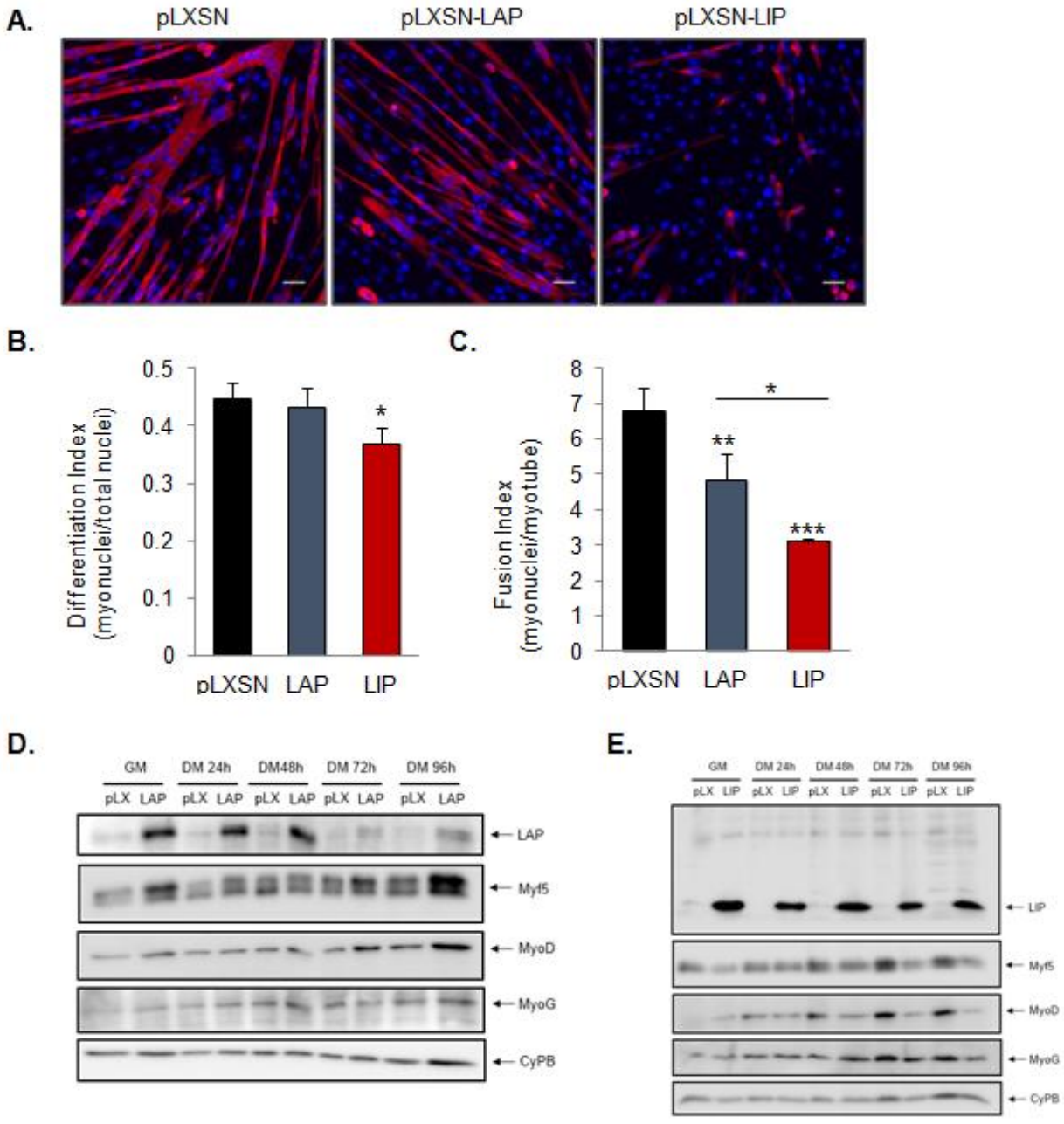
The pooled stable cell lines expressing LAP or LIP and their empty virus controls were then induced to differentiate for 4 days in low serum conditions at approximately 70% confluency to examine the effects of LAP and LIP overexpression on myogenesis. After 4 days in differentiation medium, cultures were subjected to indirect immunocytochemistry for myosin heavy chain (MHC) expression (Fig. 4A). While empty vector controls produced large multinucleated myotubes with high efficiency, LAP expressing cells produced smaller myotubes (Fig. 4A). Both differentiation and fusion were inhibited in LIP-expressing cultures (Fig. 4A).

To quantify the efficacy of differentiation and fusion into myotubes, the fusion index (FI, #myonuclei/myotube) and the differentiation index (DI, #myonuclei/total nuclei) for each stable cell line were calculated (Fig. 4B,C). The fusion index was reduced approximately 30% in cells ectopically expressing LAP as compared to empty virus controls (Fig. 4B). Ectopic expression of LIP also reduced myotube size by approximately 60% as compared to controls (Fig. 4C). While ectopic expression of the LIP isoform also caused a modest by significant reduction in the differentiation index (approximately 18%), ectopic expression of the LAP isoform did not affect the DI when compared to controls, suggesting that the LAP isoform does not negatively affect differentiation but rather acts to limit cell fusion. To characterize the observed defects, myogenic regulatory factor expression in growth medium and throughout differentiation was quantified by western blot (Fig. 4D-H). While LIP isoform expression in transduced C2C12 pooled stable cell lines remained constant throughout differentiation, it was noted that LAP expression in the LAP-overexpressing cultures was variable, and decreased after 48 hours in differentiation medium (Fig. 4D,E). Expression of the LAP isoform in growth medium resulted in an increase in Myf5 expression, without affecting the expression of MyoD. With induction to differentiate, the effect of LAP expression on Myf5 became more variable, and no changes were noted in MyoD or

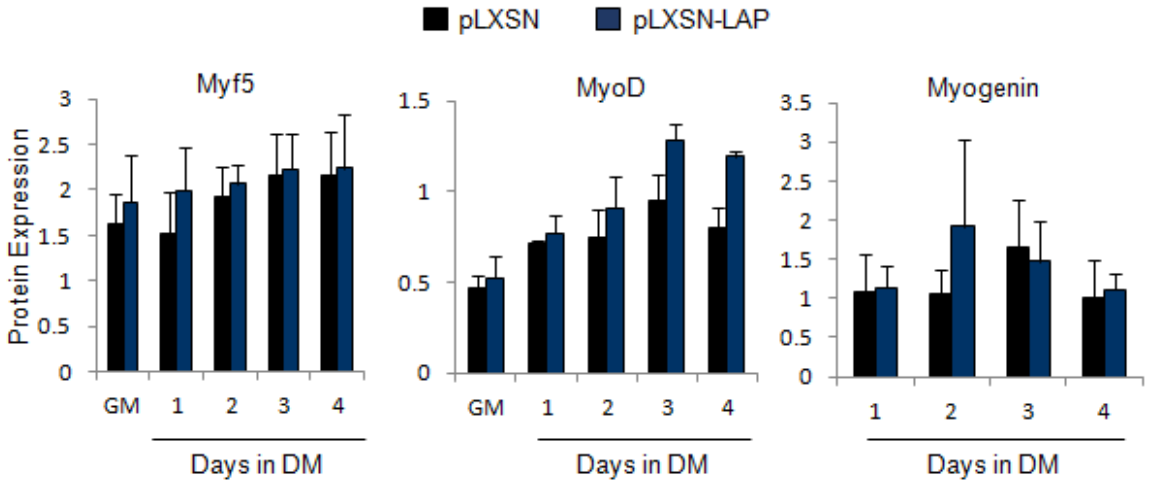
myogenin expression when compared to controls (Fig. 4D,F). C/EBPβ-LIP overexpression did not affect Myf-5 expression, but significantly decreased both MyoD and myogenin expression after 4 days in differentiation medium (Fig. 4E,G) consistent with a decrease in differentiation.

To confirm that the inhibitory effect of LIP on myogenesis is not due to non-specific effects due to high levels of protein overexpression, a LIP mutant where point mutations were introduced into the DNA-binding domain to abolish interaction with DNA was generated (C/EBPβ LIP dbd-). The C/EBPβ LIP dbd- cannot bind to DNA but it still has the ability to dimerize via its leucine zipper. C2C12 cells were retrovirally transduced to express LIP, LIP dbd- or with empty virus. Overexpression of the LIP and dbd- constructs was verified by western blotting (Fig. 5A). Cells were differentiated for 4 days and stained for myosin heavy chain (MHC) expression for visualization of myotube formation (Fig. 5B).

In contrast to LIP-expressing cells, C2C12 cells ectopically expressing C/EBPβ LIP dbd- had efficient differentiation and fusion comparable to the pLXSN controls, suggesting that LIP must contact DNA to exert its effects on differentiation (Fig. 5A). While LIP overexpression decreased the DI by approximately 40%, the LIP dbd- increased the DI by approximately 25% as compared to controls (Fig. 5C). Similarly, while LIP expression decreased the fusion index by approximately 70%, expression of the LIP dbd- construct resulted in myotube size comparable to empty virus controls (Fig. 5D). Western analysis confirmed that while LIP expression reduced the expression of Myf5, MyoD and myogenin 4 days after induction to differentiate, the expression of these myogenic regulatory was comparable to controls or higher in LIP dbd- expressing cells (Fig. 5E).


A.
pLXSN
pLXSN-LAP
pLXSN-LIP
B.
Differentiation Index
(myonuclei/total nuclei)
0.5
0.4
0.3
0.2
0.1
0
*
pLXSN
LAP
LIP
C.
Fusion Index
(myonuclei/myotube)
8
7
6
5
4
3
2
1
0
*
**
***
pLXSN
LAP
LIP
D.
GM
DM 24h
DM48h
DM 72h
DM 96h
pLX LAP pLX LAP pLX LAP pLX LAP pLX LAP
LAP
Myf5
MyoD
MyoG
CyPB
E.
GM
DM 24h
DM 48h
DM 72h
DM 96h
pLX LIP pLX LIP pLX LIP pLX LIP pLX LIP
LIP
Myf5
MyoD
MyoG
CyPB

F.


pLXSN
pLXSN-LAP
Myf5
MyoD
Myogenin
Protein Expression
GM
1
2
3
4
Days in DM

G.

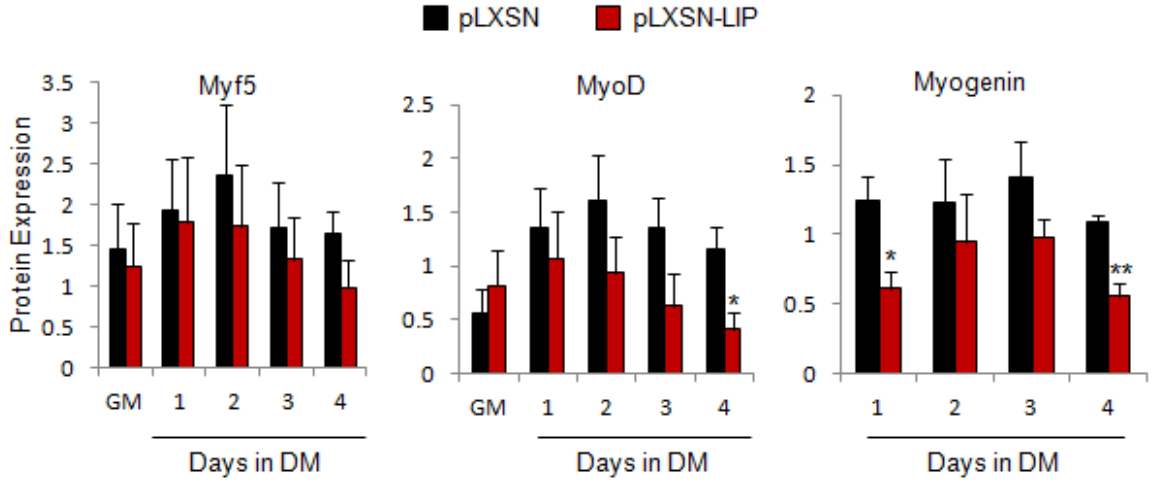

pLXSN
pLXSN-LIP
Myf5
MyoD
Myogenin
Protein Expression
GM
1
2
3
4
Days in DM
*
**

FIGURE 4: EFFECT OF ECTOPIC C/EBPβ LIP AND LAP (M. musculus) ISOFORMS. **(A)** Indirect immunofluorescence of MyHC expression in C2C12 cells. Cells were induced to differentiate in low serum (DM) for 4 days (96h). DAPI marks the nuclei. Representative pictures were taken at 10x magnification. Scale bar: 50um. **(B)** Fusion Index (#myonuclei/#myotubes), *p<0.05, **p<0.01, ***p<0.001, n=4 **(C)** Differentiation Index (#myonuclei/#total nuclei). *p<0.05, n=4. Data are expressed as a mean ± standard error. **(D)** Western analysis of C/EBPβ (LAP) and myogenic regulatory factor (Myf5, MyoD, and MyoG) expression in C2C12 cells retrovirally transduced with empty vector (pLXSN) or to express C/EBPβ LAP. Cells were cultured under growth conditions for 24h then collected or induced to differentiate in low serum for 4 days (DM1-DM4). Cells were harvested every 24h after switching to differentiation media. Cyclophilin B (CyPB) expression is a loading control. **(E)** Western analysis of C/EBPβ (LIP) and myogenic regulatory factors (Myf5, MyoD, and MyoG) expression in C2C12 cells retrovirally transduced with empty vector (pLXSN) or to express C/EBPβ-LIP. Cells were cultured under growth conditions for 24h then collected or induced to differentiate in low serum for 4 days (96h). Cells were harvested every 24h after switching to differentiation media. CyPB expression is a loading control. **(F)** Quantification of Myf5, MyoD, and myogenin expression from **(D)** relative to CyPB expression. **(G)** Quantification of Myf5, MyoD, and myogenin expression from **(E)** relative to CyPB expression. *p<0.05, **p<0.01, n=3. Data are expressed as a mean ± standard error.

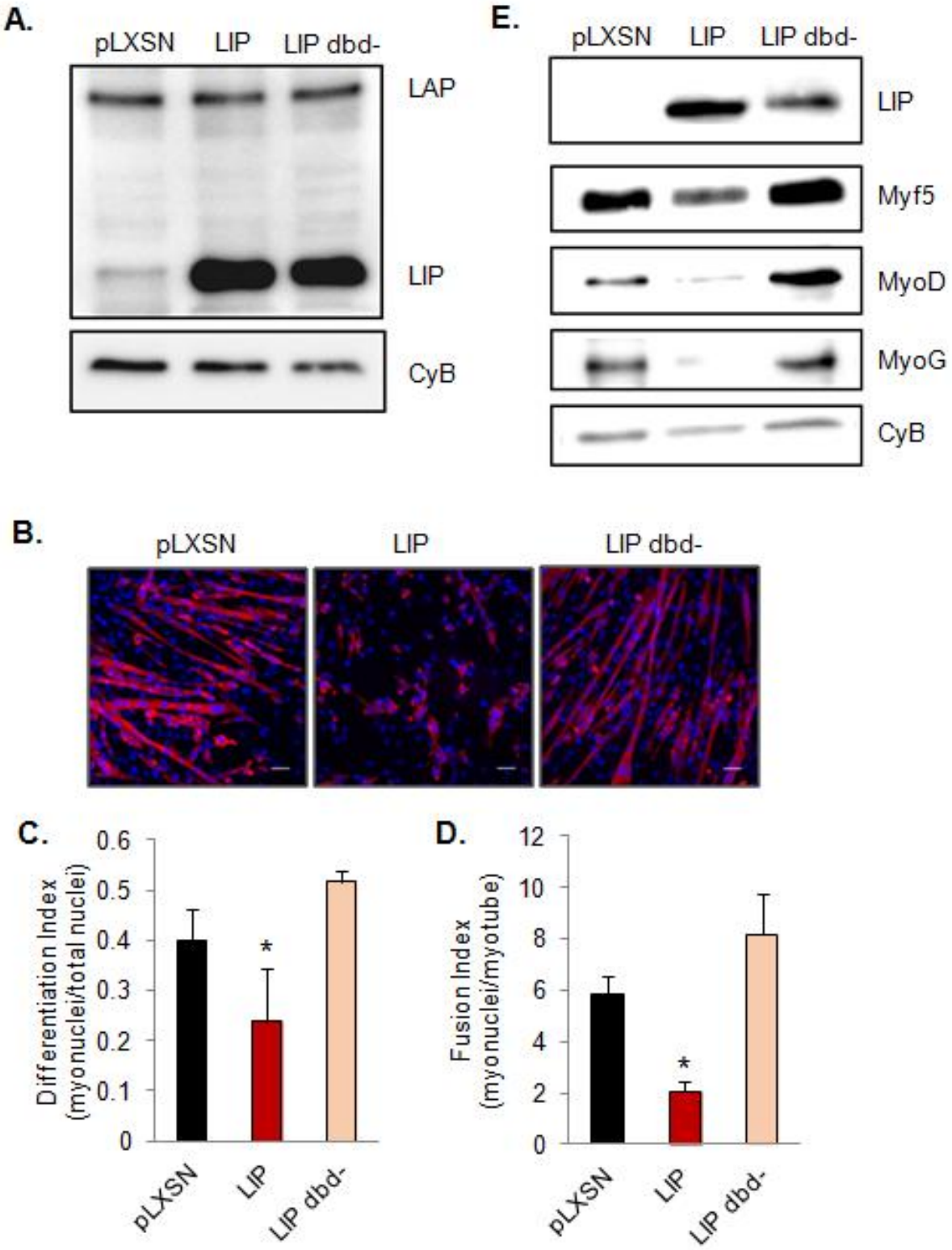

A.
pLXSN
LIP
LIP dbd-
LAP
LIP
CyB
E.
pLXSN
LIP
LIP dbd-
LIP
Myf5
MyoD
MyoG
CyB
B.
pLXSN
LIP
LIP dbd-
C.
Differentiation Index
(myonuclei/total nuclei)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
pLXSN
LIP
LIP dbd-
D.
Fusion Index
(myonuclei/myotube)
12
10
8
6
4
2
0
*
pLXSN
LIP
LIP dbd-

FIGURE 5: ECTOPIC C/EBPβ LIP dbd- (M. musculus) RESCUES DIFFERENTIATION AND FUSION DEFECTS.

(A) Western analysis of C/EBPβ (LIP and LIP dbd-) expression in C2C12 cells retrovirally transduced to express the LIP constructs and cultured under growth conditions for 24h then collected for analysis. Cyclophilin B (CyB) expression is used as a loading control. **(B)** Indirect immunofluorescence of MyHC expression (MF-20 antibody) in C2C12 cells. Cells were induced to differentiate in low serum (DM) for 4 days (96h). **(C)** Differentiation Index (#myonuclei/#total nuclei) **(D)** Fusion Index (#myonuclei/#myotubes) **(E)** Western Analysis of the myogenic regulatory factors (Myf5, MyoD, and MyoG) in C2C12 retrovirally transduced with empty vector (pLXSN) or to express C/EBPβ LIP or LIP dbd mutant. Cells were cultured under growth conditions for 24h then induced to differentiate in low serum for 4 days (96h). CyB expression is used as a loading control. Abbreviations: dbd, DNA binding domain; pLXSN, empty virus control; MyHC, myosin heavy chain; CyB, Cyclophilin B.

5.2 The effect of LIP and LAP on the proliferation of C2C12

Despite equal numbers of cells plated, cultures ectopically expressing LIP grew more slowly resulting in culture densities inferior to controls (Fig. 6A). Culture density can have an impact on the efficiency of myogenesis (Tanaka *et al.*, 2011), so the defect was quantified by counting the total number of DAPI stained nuclei in C2C12 cells expressing LIP as compared to controls. There were approximately 1.8-fold fewer cells in the LIP-expressing cultures than in the control cultures (Fig. 6B). Cell proliferation and cell death were next examined. Immunohistochemistry was performed on C2C12 retrovirally transduced to express LIP or LAP for the nuclear antigen Ki67 in growing cultures (Fig. 6C). The percentage of cells that were positive for Ki67 was determined as a percentage of total nuclei (Fig.6D). While 55% of vector control cells were positive for Ki67 stain and thus proliferating, only 33% of C2C12 cells expressing LIP were cycling (Fig. 6D). By contrast, overexpression of the LAP isoform did not significantly change the percentage of Ki67+ cells from that of controls (Fig. 6D). As such, these results suggest that the LIP isoform induces a growth defect that may contribute to the defect in differentiation observed.

To further characterize the growth defect observed in LIP-expressing cells, cellular DNA content was analyzed using flow cytometry. Propidium iodide staining of nucleic acids allowed for the distribution of cells into three phases of the cell cycle, based on DNA content (G1, S and G2/M). C2C12 cells expressing LIP and LAP were compared to empty vector controls and G1 arrested cells (Fig. 7A). While empty vector controls and LAP expressing cells had comparable levels of cells in G1 (59% and 61% respectively), 75% of LIP-expressing cells were in G1 (Fig. 7B), significantly increased when compared to empty vector controls and comparable to G1 arrested cells.

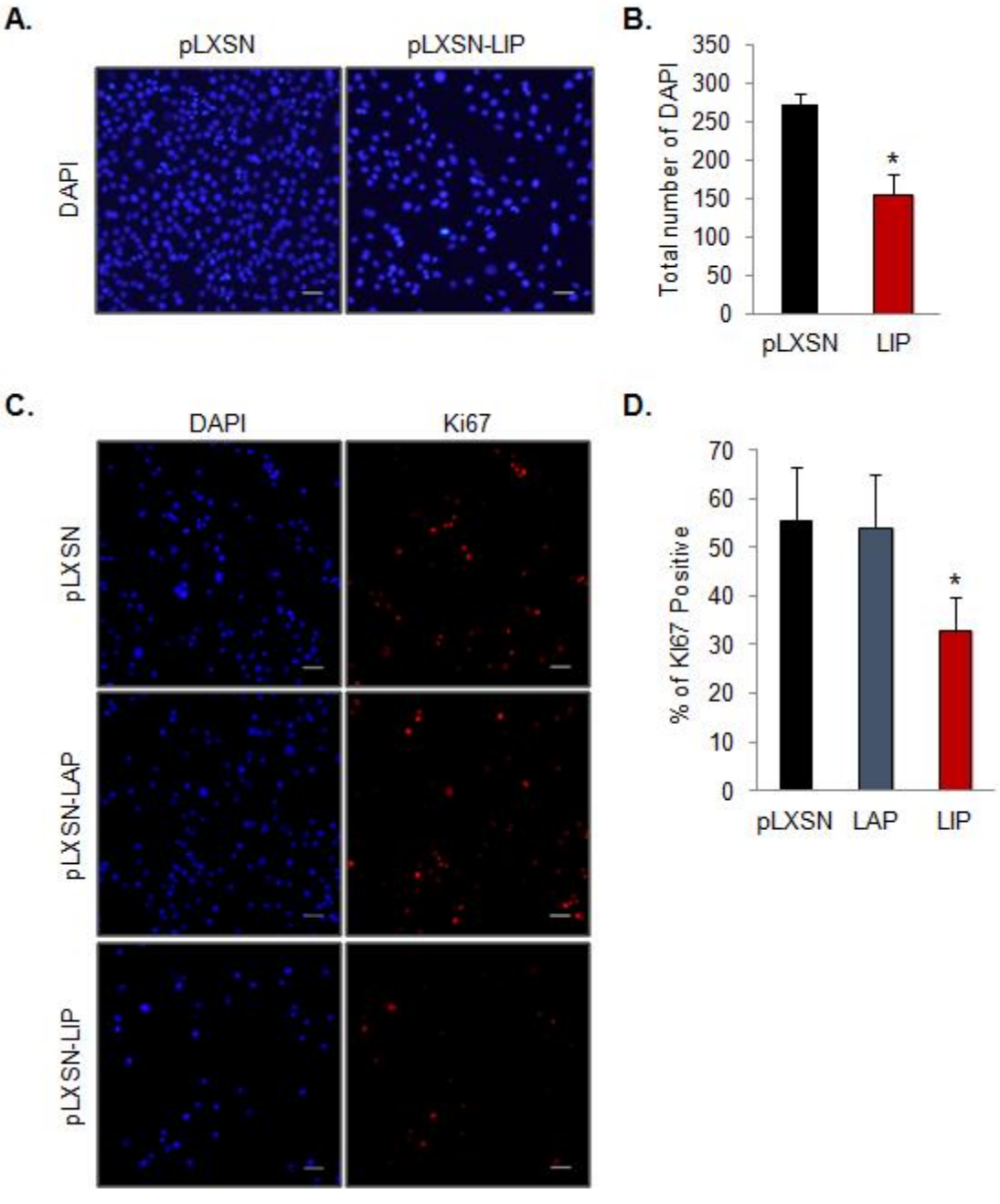
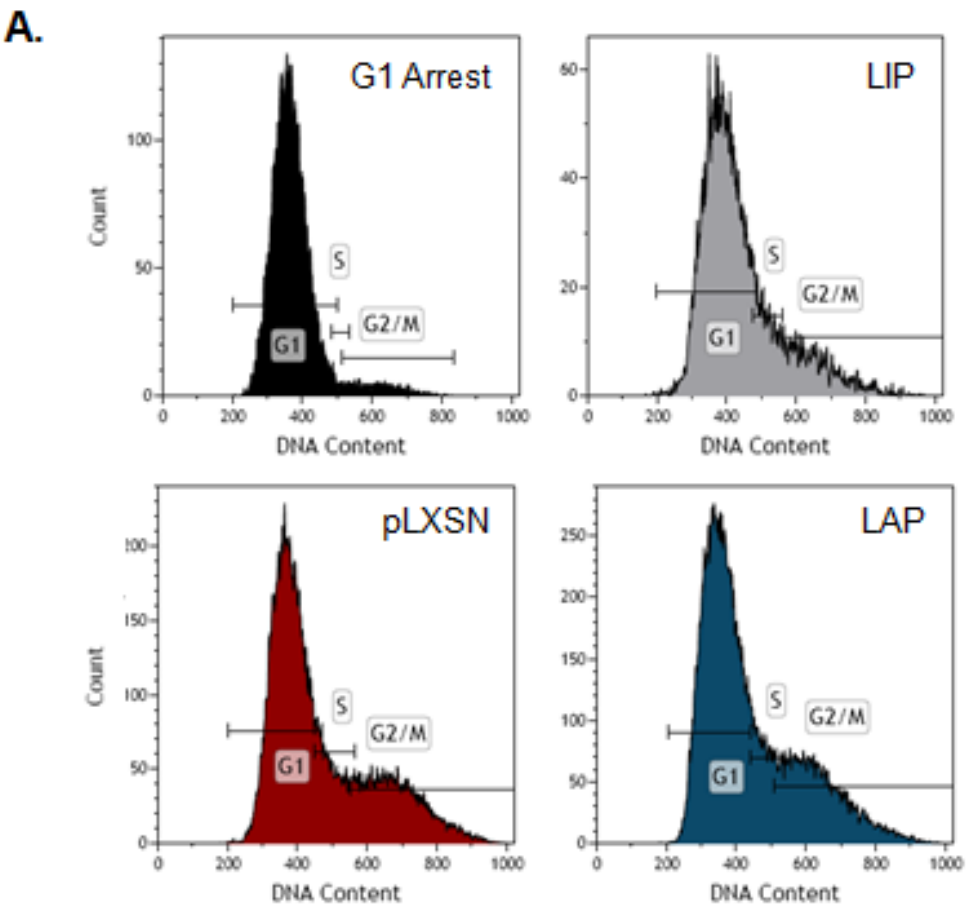

A.
pLXSN
pLXSN-LIP
DAPI
B.
Total number of DAPI
350
300
250
200
150
100
50
0
*
pLXSN
LIP
C.
DAPI
Ki67
pLXSN
pLXSN-LAP
pLXSN-LIP
D.
% of KI67 Positive
70
60
50
40
30
20
10
0
*
pLXSN
LAP
LIP

FIGURE 6: EXPRESSION OF THE PROLIFERATION MARKER Ki67 IS REDUCED BY LIP OVEREXPRESSION.

(A) C2C12 retrovirally transduced with empty virus (pLXSN) or to express LIP were immunostained with DAPI for visualization of nuclei in growing cultures. Representative pictures were taken at 10x magnification. Scale bar: 50um. **(B)** Quantification of DAPI staining from **(A)**. **(C)** Indirect immunofluorescence of Ki67 expression in pLXSN (control), LAP and LIP expressing cultures. Representative pictures were taken at 10x magnification. Scale bar: 50um. **(D)** Quantification of Ki67 positive cells relative to total nuclei. *$p<0.05$, $n=4$. Abbreviations: DAPI, 4',6-diamidino-2-phenylindone.


A.
G1 Arrest
LIP
pLXSN
LAP
Count
DNA Content
S
G2/M
G1

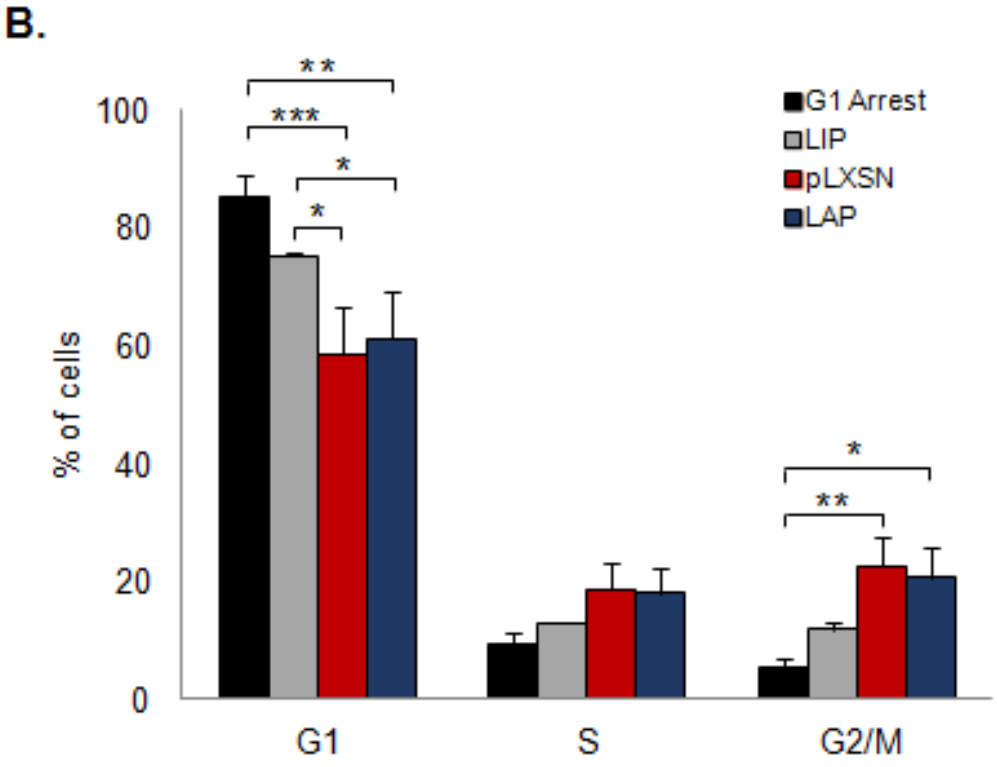

B.
G1 Arrest
LIP
pLXSN
LAP
% of cells
G1
S
G2/M

FIGURE 7: EFFECTS OF LIP AND LAP ON PROLIFERATION AND THE CELL CYCLE USING FLOW CYTOMETRIC CELL CYCLE ANALYSIS.

(A) Representative flow cytometry histograms of cells counts *versus* propidium iodide staining as a measure of DNA content for G1-arrested cells, LIP-expressing, empty vector controls (pLXSN), and LAP-expressing C2C12 myoblasts. **(B)** Graphical representation of the percentage of cells in G1, S, G2/M phases determined by gating the area of parameter histogram. *$p<0.05$, **$p<0.01$, ***$p<0.001$, n=3.

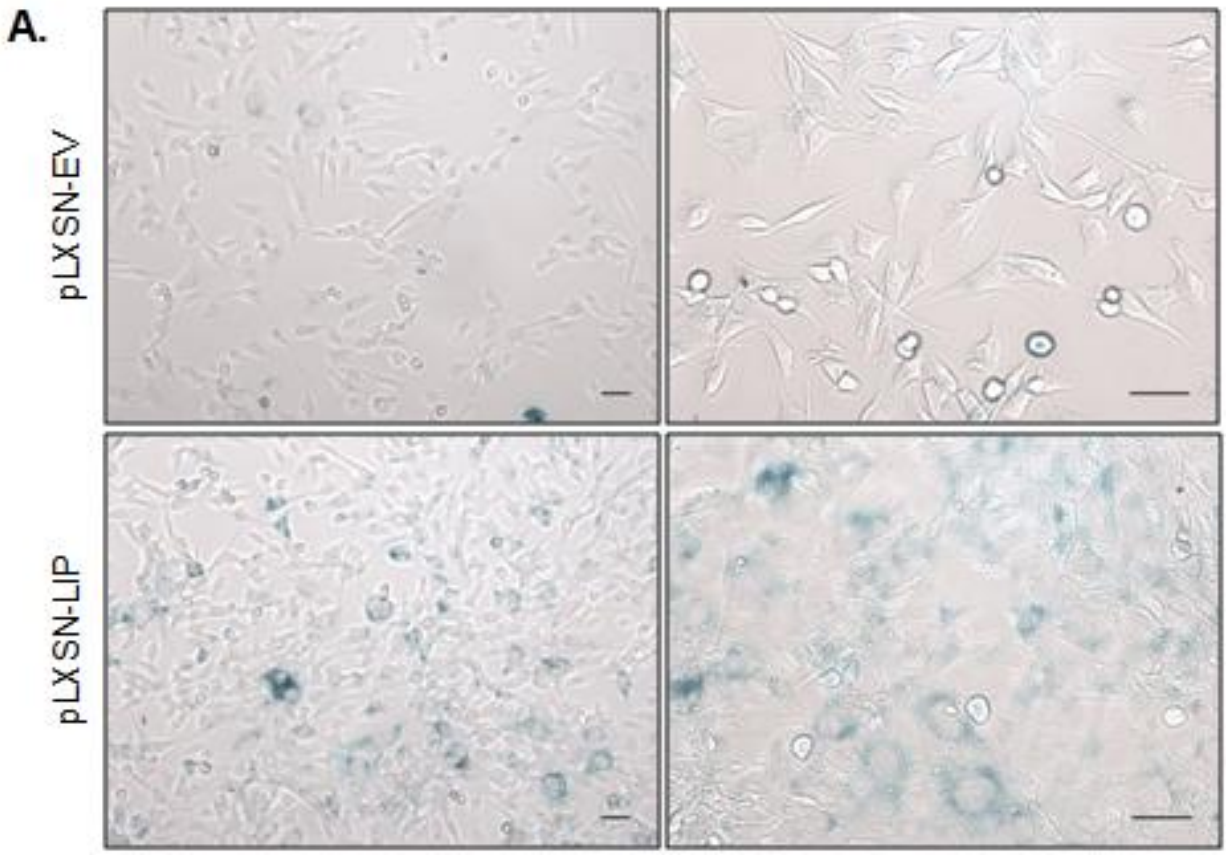
Further, while the percentage of cells in S phase was not significantly different between cell lines, the percentage of cells in G2/M was lowest in G1 arrested cells, and while failing to achieve statistical significance in this experiment, trended towards a decrease in LIP-expressing cells when compared to pLXSN controls and LAP-expressing cells. Taken together, these results suggest that overexpression of LIP induces a G1 cell cycle arrest.

To further understand if the LIP isoform can induce a permanent state of cell cycle arrest, SA-β-gal staining, one of the most widely used biomarkers for senescence, was performed. Qualitative senescence-associated β-galactosidase activity was assessed in C2C12 cells overexpressing LIP and empty vector controls (Fig. 8A). Whereas less than 10% of controls cells were positive for SA-β-galactosidase activity, greater than 45% of the LIP-expressing cells were positive, suggesting that LIP expression can induce an irreversible withdrawal from the cell cycle in myoblasts (Fig. 8B).

Low cell numbers in a culture can also be caused by increased cell death. C/EBPβ expression has been implicated in the regulation of apoptosis such that C/EBPβ knockout mice are more sensitive to apoptosis (Yoon *et al.*, 2007). To determine whether apoptosis also contributes to the low numbers of LIP-expressing cells, caspase3/7 activity was measured in C2C12 cells expressing the LIP isoform and control cultures (pLX) (Fig. 9). In untreated cells, caspase 3/7 activity was comparable in both cultures. Next, apoptosis was induced with thapsigargin and the caspase 3/7 activity was again measured. In cells induced to apoptose, caspase activity was increased 5-fold over untreated cells, but was not changed by overexpression of LIP (Fig. 9). Previous studies have suggested that senescent cells are resistant to apoptosis even though senescence and apoptosis follow two different cellular pathways

(Marcotte *et al*., 2004; Chaturvedi *et al*., 1999). In the cell model studied, the increased senescent phenotype did not provide protection from apoptosis induced by thapsigargin.

Expression of the LIP isoform is very low in C2C12 cells and is undetectable in primary myoblasts (Marchildon *et al.*, 2012). The absence of the LIP isoform in wild type primary myoblasts raised important questions about the physiological and pathological role of C/EBPβ LIP isoform. Duchenne muscular dystrophy is a progressive neuromuscular disorder associated with muscle wasting because of mutations in the dystrophin gene, which is one of important structural proteins in the muscle fiber. Satellite cell defects have been observed in dystrophic muscle, in particular dysregulation of the cell cycle (Heslop *et al*., 2000). The mdx mouse is an animal model for Duchenne muscular dystrophy and the mdx mouse is studied extensively to understand muscle regeneration and pathology of this disease. Primary myoblasts were isolated from the hindlimb muscle of C57BL/6 and mdx mice and western blotting was performed to assess C/EBPβ isoform expression. While primary myoblasts from C57BL/6 cells expressed the LAP isoform, the LIP isoform was undetectable, consistent with previous observations (Fig. 10A). However, in primary myoblasts isolated from mdx mice, higher levels of both LAP and LIP were detected (Fig. 10A, B). The appearance of the LIP isoform in the satellite cells of mdx mice suggests a possible mechanism regulating the activation and expansion of muscle stem cells.


A.
pLXSN-EV
pLXSN-LIP

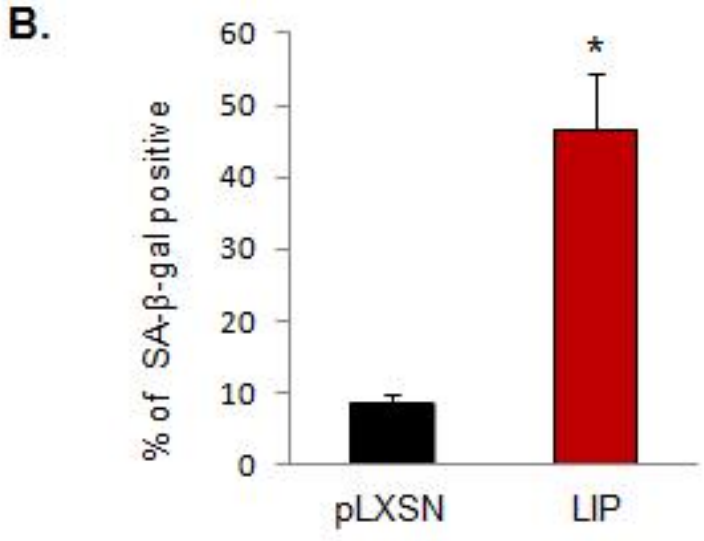

B.
% of SA-β-gal positive
60
50
40
30
20
10
0
*
pLXSN
LIP

FIGURE 8: LIP OVEREXRESSION INCREASES SA-β-GAL ACTIVITY.

(A) Senescence-associated β-galactosidase activity in C2C12 retrovirally transduced to express C/EBPβ-LIP (bottom) or with empty virus (EV) pLXSN (top) cultured in growth medium. Representative pictures were taken at both 10x and 20x magnification. Scale bar: 50um. **(B)** Quantification of SA-β-gal positive cells relative to total cells. *$p<0.05$, n=3.

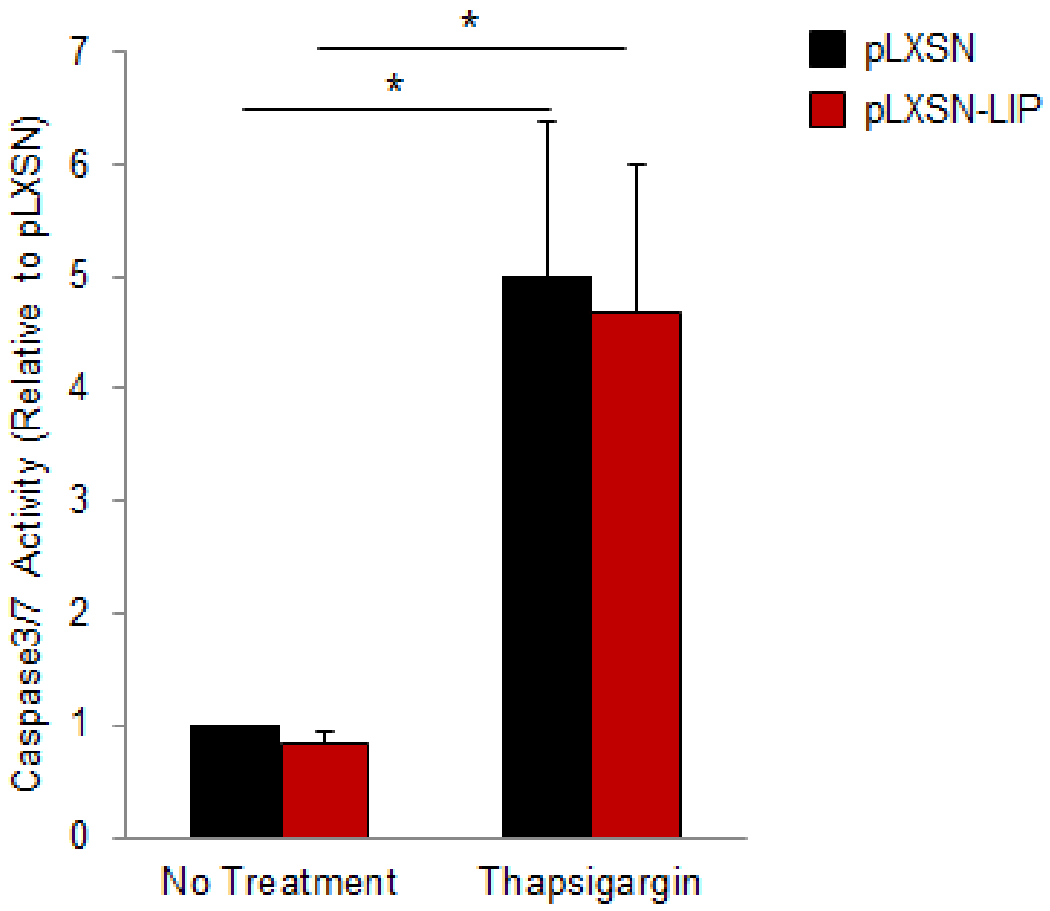


FIGURE 9: LIP OVEREXPRESSION DOES NOT INCREASE MYOBLAST CASPASE ACTIVATION.

Caspase3/7 activity measured in C2C12 retrovirally transduced to express C/EBPβ LIP (pLXSN-LIP) or with empty virus (pLXSN) in the presence or absence of thapsigargin as indicated. *p<0.05, n=5.

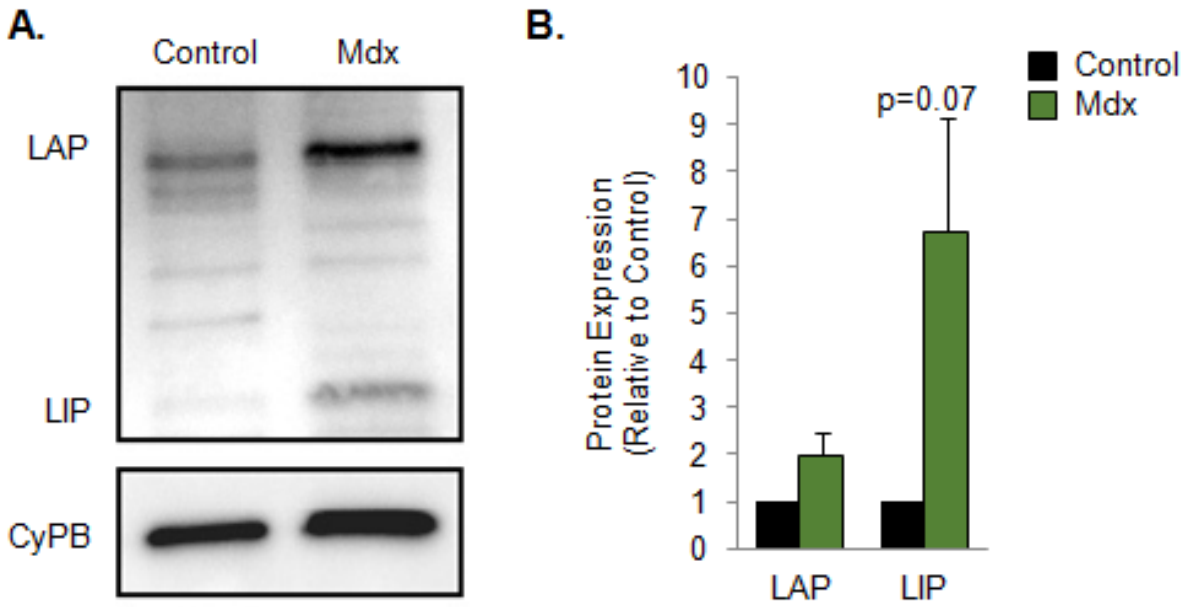


FIGURE 10: EXPRESSION OF C/EBPβ LAP and LIP ISOFORM IN PRIMARY MYOBLASTS ISOLATED FROM DYSTROPHIC MUSCLE.

(A) Western analysis of C/EBPβ isoform expression in primary myoblasts isolated from the hindlimb muscle of male C57BL/6 mice or mdx mice, aged 5-8 weeks and cultured under growth conditions. Cyclophilin B (CyPB) is a loading control. **(B)** Quantification of C/EBPβ LAP and LIP isoform expression from (A) relative to CyPB expression, where protein expression of LAP and LIP in satellite cells of wild type mice are set arbitrarily to 1. Error bars represent the standard error of the mean of four independent trials, p=0.07.

6. DISCUSSION

The regulation of C/EBP transcription factors is tightly controlled by several mechanisms including dimerization, either homo- and heterodimerization among family members, and the production of protein isoforms (Ramji and Foka 2002; Cao *et al*., 1991; Descombes and Schibler, 1991). The investigation on the functional differences between the LAP* and LAP isoform of C/EBPβ has revealed that only the full length LAP* can interact with ATPase/helicase-type chromatin remodeling complex SWI/SNF (Kwenz-Leutz *et al*., 1999). This suggests that LAP* can recruit nucleosome-remodeling complexes to promoters allowing gene expression, emphasizing that LAP* might play important roles in gene activation that is distinct from that of LAP (Kwenz-Leutz *et al*., 1999). Therefore, it would be interesting to investigate the impact of LAP* isoform expression on myogenesis and proliferation of myoblasts.

The competition between stimulatory and inhibitory isoforms can influence the transcriptional activation of target genes. As such, the regulation of LIP and LAP isoform expression must be tightly controlled during cellular differentiation processes. During myogenesis, C/EBPβ levels are highest in the undifferentiated cell and decrease as cells begin to express higher levels of MyoD and progress through differentiation. While both LIP and LAP isoform expression was noted in C2C12 cells, primary myoblasts isolated from mouse hindlimb express predominantly the LAP isoform, though the LAP* isoform is also detectable. The LIP isoform is absent in primary myoblasts (Marchildon *et al*., 2012). The production of the three isoforms of C/EBPβ is regulated by numerous pathways including eukaryotic translation initiation factors (eIFs), the kinases PKR and the mammalian target of rapamycin (mTOR) (Hershey, 1991; Morris, 1995; Calkhoven *et al*. 2000). PKR and mTOR signaling pathways modulate the activity of eukaryotic translation initiation factors such as eIF-2 and eIF-4E, which

can change the expression of LIP (Calkhoven *et al*. 2000). The mTOR signaling pathway is extensively involved in regulation of skeletal myogenesis and is required for muscle growth (Erbay and Chen, 2001). Rapamycin, the inhibitor of mTOR, inhibits differentiation of mouse C2C12 myoblasts (Erbay and Chen, 2001). Increasing mTOR signalling pathway enhances the level of eIF activities which in turn promotes the expression of LIP in other systems, leading to the prediction that differentiation would be inhibited. This prediction contradicts the findings of this thesis. However, the study involving eIF factors regulating C/EBPβ isoforms was performed using 3T3-L1 adipocytes, where increased LIP expression prevented cell cycle arrest, an effect opposite of what we observe in C2C12 myoblasts (Calkhoven *et al*. 2000; Fig. 6,7 and 8). Indeed, C/EBPβ has been shown to have opposing effects based in different cell types.

Recent work by our lab has demonstrated that C/EBPβ protein is regulated by the E3 ubiquitin ligase Mouse double minute 2 homolog (Mdm2) during myogenic differentiation. Mdm2 binds to and ubiquitylates C/EBPβ, leading to its degradation early in myogenic differentiation, allowing myogenesis to occur (Fu *et al*., 2015). Interestingly, Mdm2 interacts with C/EBPβ through a domain that is not expressed in the LIP isoform, suggesting an additional mechanism by which the LIP:LAP ratio could be increased through selective degradation of the LAP isoform. Given that C/EBPβ LIP is not expressed in satellite cells from healthy muscle, the effects of Mdm2 on the relative levels of LIP in muscle will likely be restricted to the pathological expression of the LIP isoform in conditions such as Duchenne Muscular Dystrophy.

While the ectopic expression of C/EBPβ LIP remained stable throughout differentiation of C2C12 myoblasts, the expression of ectopically expressed LAP decreased 2 days after induction to differentiate (Fig. 4D). Given that the expression of the LAP isoform was driven by a viral promoter and that *Cebpb* mRNA expression is fairly stable in C2C12 cells (Fu *et al*., 2015), this

observation suggests that the protein is subject to degradation by an E3 ligase other than Mdm2, which is not expressed in late differentiation, or alternatively that the translation of the *Cebpb* mRNA is regulated. Regardless of the mechanism driving the loss of LAP expression in late differentiation, reduced LAP expression in this experimental system may negatively impact the interpretation of the effects of LAP on myogenesis. Indeed, later in differentiation, it is clear that LAP is no longer overexpressed, resulting in a trend towards inhibited differentiation, without achieving statistical significance. By achieving stable overexpression of the LAP isoform comparable to the LIP isoform throughout differentiation, I anticipate that the negative impact of LAP isoform on myogenesis would be more severe and statistically significant.

While it was known that C/EBPβ expression inhibits MyoD protein expression, it was surprising to observe that the LIP isoform alone was also able to inhibit MyoD expression. To further understand MyoD regulation by the LIP isoform, the mRNA expression of MyoD in C2C12 cultures expressing the LIP isoform should be determined to assess if MyoD is regulated at the transcriptional level. However, overexpression of C/EBPβ in C2C12 myoblasts did not result in changes in *Myod1* mRNA expression, suggesting that regulation is occurring at the level of translation or protein stability. We have also failed to rescue MyoD protein expression using inhibitors of the 26S proteasome (Marchildon *et al.,* 2012; Fu *et al*., 2015), suggesting that the regulation of MyoD expression may occur at the level of protein translation. The profile of miRNA expression during myogenesis has revealed several miRNAs that are upregulated during myoblast differentiation and a recent study has profiled miRNA expression in skeletal muscles of old and young mice (Chen *et al.,* 2006; Kim *et al.,* 2014). Many potential miRNAs that might be controlling MyoD gene expression during differentiation have been discovered using a blast search (Yan *et al*., 2012). Among these, miR-203b expression is inversely correlated with MyoD

expression in the fish Nile tilapia (Yan *et al*., 2012). Furthermore, miR-203b has been shown to directly target MyoD expression by interacting with the MyoD 3'UTR using a luciferase reporter assay (Yan *et al*., 2012). miR-203 is, in turn, regulated by C/EBPβ LIP which represses miR-203 expression through a direct binding on two C/EBPβ binding motifs present in miR-203 demonstrating the importance of the C/EBPβ isoform LAP:LIP ratio (Li *et al*., 2014). It would be interesting to explore whether miR-203b also targets MyoD in mammals.

The inhibition of differentiation and fusion by the LIP isoform raises another interesting question. The combined results from cell cycle analysis and proliferation assay strongly suggest that low levels of differentiation may stem from a failure of proliferation. Indeed, low density cells that do not proliferate once activated to differentiate will produce fewer cells competent to fuse into myotubes. This result was surprising given that loss of C/EBPβ expression in a mouse model does not impair myoblast proliferation nor adversely affect myogenesis. Indeed, primary myoblasts lacking C/EBPβ precociously differentiate (Marchildon *et al*., 2012). We thus expected that overexpression of the LIP isoform would interfere with C/EBPβ activity and promote precocious differentiation similar to the null model. However, given that C/EBP family members can homo- and heterodimerize and can interact with other bzip transcription factors, the effects of LIP overexpression may be due to dysregulation of other pathways, rather than the activities of C/EBPβ specifically. The effects are dependent on DNA binding however as expression of the LIP isoform that was unable to bind to DNA (C/EBPβ LIP dbd-) did not affect differentiation (Fig 5). The crucial question to be addressed is whether C/EBPβ LIP dbd- affects cell proliferation and cellular senescence detected in cultured C2C12 myoblasts.

The transcription regulators of G1-S phase include the E2F transcription factors and partner proteins p107, 130 and Rb (Hurford *et al*., 1997). The findings from this thesis study suggest that

C/EBPβ-LIP is anti-proliferative and it is interesting to speculate that LIP acts on the Rb-E2F complex to target genes that regulate the G1/S transition or by silencing genes that regulate the S phase entry. Interestingly, C/EBPβ is regulated by pRb. Earlier studies have shown the physical interaction between all three isoforms of C/EBPβ and the hypophosphorylated form Rb (Chen *et al*., 1996). Further investigation into the specific regions of interaction within C/EBPβ using deletion mutants reveals that both the N-terminal and the C-terminal half of C/EBPβ were important for binding to Rb. The N-terminal part of C/EBPβ has sequences similar to that of E2F-1 that bind Rb; however, the C-terminal part of C/EBPβ that binds Rb remains unknown (Chen *et al*., 1996). Using transiently transfected cells, Rb has been shown to increase the transcriptional activity of C/EBPβ (Chen *et al*., 1996). In addition, C/EBPβ can also specifically decrease the expression of several E2F target genes such as c-myc, DHFR, cyclinA2, and PCNA, suggesting a mechanism for C/EBPβ-mediated cell cycle arrest (Sebastian *et al*., 2005).

In addition, because many mutations in genes that regulate G1 contribute to oncogenesis, understanding progression through the G1 phase of the cell cycle by C/EBPβ isoforms might significantly contribute to the field of cancer biology. Increased level of the LIP isoform was previously observed in breast cancer (Zahnow *et al*., 1997). Forced expression of the LAP isoform into human hepatoma HepG2 cells inhibited the proliferation and entry into S phase (Buck *et al*., 1994). Immunohistochemical staining revealed that protein expression of C/EBPβ is detected in many of cancer tissues suggesting C/EBPβ might be an important therapeutic target.

Despite evidence of a growth defect in LIP overexpressing cells, increased apoptosis could also contribute to the low cell numbers observed. Looking only at caspase3/7 activity as a marker for apoptosis, LIP expressing cells were no different in compared to the control. However, caspase activity can occur independent of cell death (Lee *et al*., 2008), and thus alternative

markers such as AIFs (Apoptosis inducing factors) and γH2AX, which are independent of the caspase pathway should be investigated for apoptosis (Antonsson 2004; Rogakou *et al*., 2000).

The ability of adult skeletal muscles to regenerate new muscle fibers is largely due to quiescent cells known as satellite cells. The number of satellite cells is generally stable due to asymmetric divisions that maintain the quiescent cell pool. Many rounds of regeneration and degeneration can eventually decrease the number of satellite cells because of the normal aging process (Snow, 1977). In Duchenne muscular dystrophy caused by mutations in the dystrophin gene, the regenerative potential of satellite cells is diminished. This prevents effective repair of muscle, eventually leading to wasting and weakness. Dystrophin is also deficient in the mdx mouse, which is an animal model of Duchenne muscular dystrophy. The satellite cell proliferation and number have shown to be altered in Duchenne muscular dystrophy (Webster and Blau, 1990; Blau *et al*., 1983). The quiescent satellite cells, marked as Pax7+MyoD-, are reduced by half in mdx mice compared to the wild-type (Jiang *et al.*, 2014). A 14-fold increase in telomeric DNA loss has been observed in dystrophic muscles compared to the healthy muscles and the telomerase-knockout mouse shows more a severe phenotype, revealing the important role of telomeres in muscle cell proliferation and regeneration (Decary *et al*., 2000; Sacco *et al.*, 2005). I have documented that high levels of LIP expression in myoblasts leads to the induction of cell cycle arrest and cellular senescence. Indeed, I have observed increased LIP expression in satellite cells isolated from the mdx mouse. Given that hTERT expression is reduced in DMD as compared to healthy controls (Mouly *et al.*, 2205; Abdel *et al.*, 2007), and that active telomerase can protect from replicative senescence, it is an interesting therapeutic target. In mammary carcinomas, hTERT expression is regulated by CCAAT/Enhancer Binding Protein beta (C/EBPβ), and thus the regulation of hTERT expression in myoblasts may contribute to the

growth defect observed (Kumar *et al.*, 2013; Robinson *et al.*, 1998). The expression of LIP, which induces senescence and inhibits myogenesis, may therefore contribute to the defect in muscle regeneration that occurs in these pathological conditions, making the pathways that regulate LIP expression in muscle interesting therapeutic targets to preserve satellite cell stamina.

7. CONCLUSION

This thesis examines the individual effects of the LIP and LAP isoforms of C/EBPβ on myogenesis. C2C12 cells retrovirally transduced to express LAP only or LIP only were induced to differentiate for 4 days in low serum conditions. While control cultures differentiated efficiently and exhibited extensive fusion of myoblasts to multinucleated myotubes, cells ectopically expressing C/EBPβ-LIP inhibited both differentiation and fusion. Cells ectopically expressing C/EBPβ-LIP (*M. musculus*) demonstrated 18% and 60% reduction in DI and FI respectively, compared to that of pLXSN control. LAP expressing cultures demonstrated 30% reduction in FI. Western blot analysis showed that upregulation of C/EBPβ-LIP expression led to the loss of Myf5, MyoD, and myogenin expression in DM which suggest the potential role of C/EBPβ-LIP in regulating myogenic regulatory factors. LIP overexpression reduced cell numbers and the percentage of proliferating cells, and increased the percentage of cells in the G2/M phase of the cell cycle, suggesting a defect in cell growth. Based on the results shown here, these data indicate that LIP is an important regulator of cell cycle progression in myoblasts, and expressed in disease muscle from mdx mice.

REFERENCES

Abdel, S.E., I. Abdel-Meguid, and S. Korraa. 2007. Markers of oxidative stress and aging in Duchene muscular dystrophy patients and the possible ameliorating effect of He:Ne laser. *Acta Myol.* 26:14-21.

Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto. 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *Embo j.* 9:1897-1906.

Allen, D.L., E.R. Bandstra, B.C. Harrison, S. Thorng, L.S. Stodieck, P.J. Kostenuik, S. Morony, D.L. Lacey, T.G. Hammond, L.L. Leinwand, W.S. Argraves, T.A. Bateman, and J.L. Barth. 2009. Effects of spaceflight on murine skeletal muscle gene expression. *J.Appl.Physiol.(1985).* 106:582-595.

Andres, V., and K. Walsh. 1996. Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *J.Cell Biol.* 132:657-666.

Antonsson, B. 2004. Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. *Mol.Cell.Biochem.* 256-257:141-155.

Bertoli, C., J.M. Skotheim, and R.A. de Bruin. 2013. Control of cell cycle transcription during G1 and S phases. *Nat.Rev.Mol.Cell Biol.* 14:518-528.

Birkenmeier, E.H., B. Gwynn, S. Howard, J. Jerry, J.I. Gordon, W.H. Landschulz, and S.L. McKnight. 1989. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev.* 3:1146-1156.

Bischoff, R., and C. Heintz. 1994. Enhancement of skeletal muscle regeneration. *Dev.Dyn.* 201:41-54.

Blau, H.M., C. Webster, and G.K. Pavlath. 1983. Defective myoblasts identified in Duchenne muscular dystrophy. *Proc.Natl.Acad.Sci.U.S.A.* 80:4856-4860.

Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H.H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *Embo j.* 8:701-709.

Buck, M., H. Turler, and M. Chojkier. 1994. LAP (NF-IL-6), a tissue-specific transcriptional activator, is an inhibitor of hepatoma cell proliferation. *Embo j.* 13:851-860.

Bundy, L.M., and L. Sealy. 2003. CCAAT/enhancer binding protein beta (C/EBPbeta)-2 transforms normal mammary epithelial cells and induces epithelial to mesenchymal transition in culture. *Oncogene.* 22:869-883.

Calkhoven, C.F., C. Muller, and A. Leutz. 2000. Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev.* 14:1920-1932.

Calkhoven, C.F., L. Snippe, and G. Ab. 1997. Differential stimulation by CCAAT/enhancer-binding protein alpha isoforms of the estrogen-activated promoter of the very-low-density apolipoprotein II gene. *Eur.J.Biochem.* 249:113-120.

Canalis, E., V. Deregowski, R.C. Pereira, and E. Gazzerro. 2005. Signals that determine the fate of osteoblastic cells. *J.Endocrinol.Invest.* 28:3-7.

Cao, Z., R.M. Umek, and S.L. McKnight. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* 5:1538-1552.

Charge, S.B., and M.A. Rudnicki. 2004. Cellular and molecular regulation of muscle regeneration. *Physiol.Rev.* 84:209-238.

Chaturvedi, V., J.Z. Qin, M.F. Denning, D. Choubey, M.O. Diaz, and B.J. Nickoloff. 1999. Apoptosis in proliferating, senescent, and immortalized keratinocytes. *J.Biol.Chem.* 274:23358-23367.

Chellappan, S.P., S. Hiebert, M. Mudryj, J.M. Horowitz, and J.R. Nevins. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell.* 65:1053-1061.

Chen, J.F., E.M. Mandel, J.M. Thomson, Q. Wu, T.E. Callis, S.M. Hammond, F.L. Conlon, and D.Z. Wang. 2006. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat.Genet.* 38:228-233.

Chen, P.L., D.J. Riley, S. Chen-Kiang, and W.H. Lee. 1996. Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. *Proc.Natl.Acad.Sci.U.S.A.* 93:465-469.

Collins, C.A. 2006. Satellite cell self-renewal. *Curr.Opin.Pharmacol.* 6:301-306.

Cornelison, D.D., and B.J. Wold. 1997. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev.Biol.* 191:270-283.

Cornelison, D.D., B.B. Olwin, M.A. Rudnicki, and B.J. Wold. 2000. MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Dev.Biol.* 224:122-137.

d'Adda di Fagagna, F., P.M. Reaper, L. Clay-Farrace, H. Fiegler, P. Carr, T. Von Zglinicki, G. Saretzki, N.P. Carter, and S.P. Jackson. 2003. A DNA damage checkpoint response in telomere-initiated senescence. *Nature.* 426:194-198.

Dannenberg, J.H., A. van Rossum, L. Schuijff, and H. te Riele. 2000. Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. *Genes Dev.* 14:3051-3064.

Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell.* 51:987-1000.

Decary, S., C.B. Hamida, V. Mouly, J.P. Barbet, F. Hentati, and G.S. Butler-Browne. 2000. Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children. *Neuromuscul.Disord.* 10:113-120.

Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler. 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* 4:1541-1551.

Descombes, P., and U. Schibler. 1991. A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell.* 67:569-579.

Di Leonardo, A., S.P. Linke, K. Clarkin, and G.M. Wahl. 1994. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev.* 8:2540-2551.

Dimri, G.P., X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, and O. Pereira-Smith. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc.Natl.Acad.Sci.U.S.A.* 92:9363-9367.

Eaton, E.M., M. Hanlon, L. Bundy, and L. Sealy. 2001. Characterization of C/EBPbeta isoforms in normal versus neoplastic mammary epithelial cells. *J.Cell.Physiol.* 189:91-105.

Edmondson, D.G., and E.N. Olson. 1989. A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev.* 3:628-640.

Endo, T., and S. Goto. 1992. Retinoblastoma gene product Rb accumulates during myogenic differentiation and is deinduced by the expression of SV40 large T antigen. *J.Biochem.* 112:427-430.

Flemington, E.K., S.H. Speck, and W.G. Kaelin Jr. 1993. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. *Proc.Natl.Acad.Sci.U.S.A.* 90:6914-6918.

Flodby, P., C. Barlow, H. Kylefjord, L. Ahrlund-Richter, and K.G. Xanthopoulos. 1996. Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein alpha. *J.Biol.Chem.* 271:24753-24760.

Franceschi, R.T., C. Ge, G. Xiao, H. Roca, and D. Jiang. 2007. Transcriptional regulation of osteoblasts. *Ann.N.Y.Acad.Sci.* 1116:196-207.

Friend, S.H., R. Bernards, S. Rogelj, R.A. Weinberg, J.M. Rapaport, D.M. Albert, and T.P. Dryja. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature.* 323:643-646.

Fu, D., N. Lala-Tabbert, H. Lee, and N. Wiper-Bergeron. 2015. Mdm2 Promotes Myogenesis through the Ubiquitination and Degradation of CCAAT/Enhancer-binding Protein beta. *J.Biol.Chem.* 290:10200-10207.

Gigliotti, A.P., and J.W. DeWille. 1998. Lactation status influences expression of CCAAT/enhancer binding protein isoform mRNA in the mouse mammary gland. *J.Cell.Physiol.* 174:232-239.

Giresi, P.G., E.J. Stevenson, J. Theilhaber, A. Koncarevic, J. Parkington, R.A. Fielding, and S.C. Kandarian. 2005. Identification of a molecular signature of sarcopenia. *Physiol.Genomics.* 21:253-263.

Grana, X., J. Garriga, and X. Mayol. 1998. Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene.* 17:3365-3383.

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

Gu, W., J.W. Schneider, G. Condorelli, S. Kaushal, V. Mahdavi, and B. Nadal-Ginard. 1993. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell.* 72:309-324.

Guo, K., and K. Walsh. 1997. Inhibition of myogenesis by multiple cyclin-Cdk complexes. Coordinate regulation of myogenesis and cell cycle activity at the level of E2F. *J.Biol.Chem.* 272:791-797.

Guo, K., J. Wang, V. Andres, R.C. Smith, and K. Walsh. 1995. MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. *Mol.Cell.Biol.* 15:3823-3829.

Harley, C.B., A.B. Futcher, and C.W. Greider. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature.* 345:458-460.

Harrison, J.R., Y.F. Huang, K.A. Wilson, P.L. Kelly, D.J. Adams, G.A. Gronowicz, and S.H. Clark. 2005. Col1a1 promoter-targeted expression of p20 CCAAT enhancer-binding protein beta (C/EBPbeta), a truncated C/EBPbeta isoform, causes osteopenia in transgenic mice. *J.Biol.Chem.* 280:8117-8124.

Hasty, P., A. Bradley, J.H. Morris, D.G. Edmondson, J.M. Venuti, E.N. Olson, and W.H. Klein. 1993. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature.* 364:501-506.

Hata, K., R. Nishimura, M. Ueda, F. Ikeda, T. Matsubara, F. Ichida, K. Hisada, T. Nokubi, A. Yamaguchi, and T. Yoneda. 2005. A CCAAT/enhancer binding protein beta isoform, liver-enriched inhibitory protein, regulates commitment of osteoblasts and adipocytes. *Mol.Cell.Biol.* 25:1971-1979.

Hayflick, L. 1965. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp.Cell Res.* 37:614-636.

Helin, K., and H. Ed. 1993. The retinoblastoma protein as a transcriptional repressor. *Trends Cell Biol.* 3:43-46.

Hershey, J.W. 1991. Translational control in mammalian cells. *Annu.Rev.Biochem.* 60:717-755.

Heslop, L., J.E. Morgan, and T.A. Partridge. 2000. Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *J.Cell.Sci.* 113 (Pt 12):2299-2308.

Hu, P., K.G. Geles, J.H. Paik, R.A. DePinho, and R. Tjian. 2008. Codependent activators direct myoblast-specific MyoD transcription. *Dev.Cell.* 15:534-546.

Hurford, R.K.,Jr, D. Cobrinik, M.H. Lee, and N. Dyson. 1997. pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes. *Genes Dev.* 11:1447-1463.

Irintchev, A., M. Zeschnigk, A. Starzinski-Powitz, and A. Wernig. 1994. Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Dev.Dyn.* 199:326-337.

Itahana, K., J. Campisi, and G.P. Dimri. 2004. Mechanisms of cellular senescence in human and mouse cells. *Biogerontology.* 5:1-10.

Jiang, C., Y. Wen, K. Kuroda, K. Hannon, M.A. Rudnicki, and S. Kuang. 2014. Notch signaling deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy. *Dis.Model.Mech.* 7:997-1004.

Kim, J.Y., Y.K. Park, K.P. Lee, S.M. Lee, T.W. Kang, H.J. Kim, S.H. Dho, S.Y. Kim, and K.S. Kwon. 2014. Genome-wide profiling of the microRNA-mRNA regulatory network in skeletal muscle with aging. *Aging (Albany NY).* 6:524-544.

Konigsberg, I.R. 1963. Clonal analysis of myogenesis. *Science.* 140:1273-1284.

Kosar, M., J. Bartkova, S. Hubackova, Z. Hodny, J. Lukas, and J. Bartek. 2011. Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell

type- and insult-dependent manner and follow expression of p16(ink4a). *Cell.Cycle.* 10:457-468.

Kouzarides, T. 1995. Transcriptional control by the retinoblastoma protein. *Semin.Cancer Biol.* 6:91-98.

Kumar, D., J.L. Shadrach, A.J. Wagers, and A.B. Lassar. 2009. Id3 is a direct transcriptional target of Pax7 in quiescent satellite cells. *Mol.Biol.Cell.* 20:3170-3177.

Kumar, M., B. Witt, U. Knippschild, S. Koch, J.K. Meena, C. Heinlein, J.M. Weise, F. Krepulat, F. Kuchenbauer, S. Iben, K.L. Rudolph, W. Deppert, and C. Gunes. 2013. CEBP factors regulate telomerase reverse transcriptase promoter activity in whey acidic protein-T mice during mammary carcinogenesis. *Int.J.Cancer.* 132:2032-2043.

Landschulz, W.H., P.F. Johnson, E.Y. Adashi, B.J. Graves, and S.L. McKnight. 1988. Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.* 2:786-800.

Le Grand, F., and M.A. Rudnicki. 2007. Skeletal muscle satellite cells and adult myogenesis. *Curr.Opin.Cell Biol.* 19:628-633.

Lee, R.E., L.G. Puente, M. Kaern, and L.A. Megeney. 2008. A non-death role of the yeast metacaspase: Yca1p alters cell cycle dynamics. *PLoS One.* 3:e2956.

Lepper, C., S.J. Conway, and C.M. Fan. 2009. Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. *Nature.* 460:627-631.

Lepper, C., T.A. Partridge, and C.M. Fan. 2011. An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. *Development.* 138:3639-3646.

Li, J., F. Shan, G. Xiong, X. Chen, X. Guan, J.M. Wang, W.L. Wang, X. Xu, and Y. Bai. 2014. EGF-induced C/EBPbeta participates in EMT by decreasing the expression of miR-203 in esophageal squamous cell carcinoma cells. *J.Cell.Sci.* 127:3735-3744.

Luedde, T., M. Duderstadt, K.L. Streetz, F. Tacke, S. Kubicka, M.P. Manns, and C. Trautwein. 2004. C/EBP beta isoforms LIP and LAP modulate progression of the cell cycle in the regenerating mouse liver. *Hepatology.* 40:356-365.

Marchildon, F., N. Lala, G. Li, C. St-Louis, D. Lamothe, C. Keller, and N. Wiper-Bergeron. 2012. CCAAT/enhancer binding protein beta is expressed in satellite cells and controls myogenesis. *Stem Cells.* 30:2619-2630.

Marcotte, R., C. Lacelle, and E. Wang. 2004. Senescent fibroblasts resist apoptosis by downregulating caspase-3. *Mech.Ageing Dev.* 125:777-783.

Martelli, F., C. Cenciarelli, G. Santarelli, B. Polikar, A. Felsani, and M. Caruso. 1994. MyoD induces retinoblastoma gene expression during myogenic differentiation. *Oncogene.* 9:3579-3590.

Matsusaka, T., K. Fujikawa, Y. Nishio, N. Mukaida, K. Matsushima, T. Kishimoto, and S. Akira. 1993. Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc.Natl.Acad.Sci.U.S.A.* 90:10193-10197.

Megeney, L.A., B. Kablar, K. Garrett, J.E. Anderson, and M.A. Rudnicki. 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.* 10:1173-1183.

Morgan, J.E., and T.A. Partridge. 2003. Muscle satellite cells. *Int.J.Biochem.Cell Biol.* 35:1151-1156.

Morris, D.R. 1995. Growth control of translation in mammalian cells. *Prog.Nucleic Acid Res.Mol.Biol.* 51:339-363.

Motyl, K.J., M. Raetz, S.A. Tekalur, R.C. Schwartz, and L.R. McCabe. 2011. CCAAT/enhancer binding protein beta-deficiency enhances type 1 diabetic bone phenotype by increasing marrow adiposity and bone resorption. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 300:R1250-60.

Mouly, V., A. Aamiri, A. Bigot, R.N. Cooper, S. Di Donna, D. Furling, T. Gidaro, V. Jacquemin, K. Mamchaoui, E. Negroni, S. Perie, V. Renault, S.D. Silva-Barbosa, and G.S. Butler-Browne. 2005. The mitotic clock in skeletal muscle regeneration, disease and cell mediated gene therapy. *Acta Physiol.Scand.* 184:3-15.

Murre, C., P.S. McCaw, H. Vaessin, M. Caudy, L.Y. Jan, Y.N. Jan, C.V. Cabrera, J.N. Buskin, S.D. Hauschka, and A.B. Lassar. 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. *Cell.* 58:537-544.

Narita, M., S. Nunez, E. Heard, M. Narita, A.W. Lin, S.A. Hearn, D.L. Spector, G.J. Hannon, and S.W. Lowe. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell.* 113:703-716.

Nerlov, C. 2007. The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. *Trends Cell Biol.* 17:318-324.

O'Rourke, J.P., G.C. Newbound, J.A. Hutt, and J. DeWille. 1999. CCAAT/enhancer-binding protein delta regulates mammary epithelial cell G0 growth arrest and apoptosis. *J.Biol.Chem.* 274:16582-16589.

Ossipow, V., P. Descombes, and U. Schibler. 1993. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc.Natl.Acad.Sci.U.S.A.* 90:8219-8223.

Penner, G., G. Gang, X. Sun, C. Wray, and P.O. Hasselgren. 2002. C/EBP DNA-binding activity is upregulated by a glucocorticoid-dependent mechanism in septic muscle. *Am.J.Physiol.Regul.Integr.Comp.Physiol.* 282:R439-44.

Poli, V. 1998. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J.Biol.Chem.* 273:29279-29282.

Pope, R.M., A. Leutz, and S.A. Ness. 1994. C/EBP beta regulation of the tumor necrosis factor alpha gene. *J.Clin.Invest.* 94:1449-1455.

Ramji, D.P., and P. Foka. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem.J.* 365:561-575.

Raught, B., W.S. Liao, and J.M. Rosen. 1995. Developmentally and hormonally regulated CCAAT/enhancer-binding protein isoforms influence beta-casein gene expression. *Mol.Endocrinol.* 9:1223-1232.

Relaix, F., D. Montarras, S. Zaffran, B. Gayraud-Morel, D. Rocancourt, S. Tajbakhsh, A. Mansouri, A. Cumano, and M. Buckingham. 2006. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J.Cell Biol.* 172:91-102.

Rhodes, S.J., and S.F. Konieczny. 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev.* 3:2050-2061.

Robinson, G.W., P.F. Johnson, L. Hennighausen, and E. Sterneck. 1998. The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes Dev.* 12:1907-1916.

Rogakou, E.P., W. Nieves-Neira, C. Boon, Y. Pommier, and W.M. Bonner. 2000. Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. *J.Biol.Chem.* 275:9390-9395.

Roninson, I.B. 2003. Tumor cell senescence in cancer treatment. *Cancer Res.* 63:2705-2715.

Rosen, E.D., C.J. Walkey, P. Puigserver, and B.M. Spiegelman. 2000. Transcriptional regulation of adipogenesis. *Genes Dev.* 14:1293-1307.

Rudnicki, M.A., and R. Jaenisch. 1995. The MyoD family of transcription factors and skeletal myogenesis. *Bioessays.* 17:203-209.

Rudnicki, M.A., P.N. Schnegelsberg, R.H. Stead, T. Braun, H.H. Arnold, and R. Jaenisch. 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell.* 75:1351-1359.

Sacco, A., F. Mourkioti, R. Tran, J. Choi, M. Llewellyn, P. Kraft, M. Shkreli, S. Delp, J.H. Pomerantz, S.E. Artandi, and H.M. Blau. 2010. Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice. *Cell.* 143:1059-1071.

Sage, J., G.J. Mulligan, L.D. Attardi, A. Miller, S. Chen, B. Williams, E. Theodorou, and T. Jacks. 2000. Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev.* 14:3037-3050.

Schmalbruch, H., and U. Hellhammer. 1976. The number of satellite cells in normal human muscle. *Anat.Rec.* 185:279-287.

Screpanti, I., L. Romani, P. Musiani, A. Modesti, E. Fattori, D. Lazzaro, C. Sellitto, S. Scarpa, D. Bellavia, and G. Lattanzio. 1995. Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. *Embo j.* 14:1932-1941.

Seagroves, T.N., S. Krnacik, B. Raught, J. Gay, B. Burgess-Beusse, G.J. Darlington, and J.M. Rosen. 1998. C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. *Genes Dev.* 12:1917-1928.

Seale, P., L.A. Sabourin, A. Girgis-Gabardo, A. Mansouri, P. Gruss, and M.A. Rudnicki. 2000. Pax7 is required for the specification of myogenic satellite cells. *Cell.* 102:777-786.

Sebastian, T., R. Malik, S. Thomas, J. Sage, and P.F. Johnson. 2005. C/EBPbeta cooperates with RB:E2F to implement Ras(V12)-induced cellular senescence. *Embo j.* 24:3301-3312.

Slingerland, J., and M. Pagano. 1998. Regulation of the cell cycle by the ubiquitin pathway. *Results Probl.Cell Differ.* 22:133-147.

Smink, J.J., V. Begay, T. Schoenmaker, E. Sterneck, T.J. de Vries, and A. Leutz. 2009. Transcription factor C/EBPbeta isoform ratio regulates osteoclastogenesis through MafB. *Embo j.* 28:1769-1781.

Snow, M.H. 1978. An autoradiographic study of satellite cell differentiation into regenerating myotubes following transplantation of muscles in young rats. *Cell Tissue Res.* 186:535-540.

Snow, M.H. 1977. The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res.* 185:399-408.

Takai, H., A. Smogorzewska, and T. de Lange. 2003. DNA damage foci at dysfunctional telomeres. *Curr.Biol.* 13:1549-1556.

Tanaka, K., K. Sato, T. Yoshida, T. Fukuda, K. Hanamura, N. Kojima, T. Shirao, T. Yanagawa, and H. Watanabe. 2011. Evidence for cell density affecting C2C12 myogenesis: possible regulation of myogenesis by cell-cell communication. *Muscle Nerve.* 44:968-977.

Tanaka, T., N. Yoshida, T. Kishimoto, and S. Akira. 1997. Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. *Embo j.* 16:7432-7443.

Tang, Q.Q., T.C. Otto, and M.D. Lane. 2003. CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. *Proc.Natl.Acad.Sci.U.S.A.* 100:850-855.

Tedesco, F.S., A. Dellavalle, J. Diaz-Manera, G. Messina, and G. Cossu. 2010. Repairing skeletal muscle: regenerative potential of skeletal muscle stem cells. *J.Clin.Invest.* 120:11-19.

Tiainen, M., D. Pajalunga, F. Ferrantelli, S. Soddu, G. Salvatori, A. Sacchi, and M. Crescenzi. 1996. Terminally differentiated skeletal myotubes are not confined to G0 but can enter G1 upon growth factor stimulation. *Cell Growth Differ.* 7:1039-1050.

Timchenko, N.A., G.L. Wang, and L.T. Timchenko. 2005. RNA CUG-binding protein 1 increases translation of 20-kDa isoform of CCAAT/enhancer-binding protein beta by interacting with the alpha and beta subunits of eukaryotic initiation translation factor 2. *J.Biol.Chem.* 280:20549-20557.

Wang, J., and B. Nadal-Ginard. 1995. Regulation of cyclins and p34CDC2 expression during terminal differentiation of C2C12 myocytes. *Biochem.Biophys.Res.Commun.* 206:82-88.

Wang, J.Y., E.S. Knudsen, and P.J. Welch. 1994. The retinoblastoma tumor suppressor protein. *Adv.Cancer Res.* 64:25-85.

Wang, L., J. Shao, P. Muhlenkamp, S. Liu, P. Klepcyk, J. Ren, and J.E. Friedman. 2000. Increased insulin receptor substrate-1 and enhanced skeletal muscle insulin sensitivity in mice lacking CCAAT/enhancer-binding protein beta. *J.Biol.Chem.* 275:14173-14181.

Webster, C., and H.M. Blau. 1990. Accelerated age-related decline in replicative life-span of Duchenne muscular dystrophy myoblasts: implications for cell and gene therapy. *Somat.Cell Mol.Genet.* 16:557-565.

Welm, A.L., N.A. Timchenko, and G.J. Darlington. 1999. C/EBPalpha regulates generation of C/EBPbeta isoforms through activation of specific proteolytic cleavage. *Mol.Cell.Biol.* 19:1695-1704.

Wessells, J., S. Yakar, and P.F. Johnson. 2004. Critical prosurvival roles for C/EBP beta and insulin-like growth factor I in macrophage tumor cells. *Mol.Cell.Biol.* 24:3238-3250.

Williams, S.C., N.D. Angerer, and P.F. Johnson. 1997. C/EBP proteins contain nuclear localization signals imbedded in their basic regions. *Gene Expr.* 6:371-385.

Williams, S.C., M. Baer, A.J. Dillner, and P.F. Johnson. 1995. CRP2 (C/EBP beta) contains a bipartite regulatory domain that controls transcriptional activation, DNA binding and cell specificity. *Embo j.* 14:3170-3183.

Williams, S.C., C.A. Cantwell, and P.F. Johnson. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes Dev.* 5:1553-1567.

Winslow, M.M., M. Pan, M. Starbuck, E.M. Gallo, L. Deng, G. Karsenty, and G.R. Crabtree. 2006. Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev.Cell.* 10:771-782.

Wiper-Bergeron, N., D. Wu, L. Pope, C. Schild-Poulter, and R.J. Hache. 2003. Stimulation of preadipocyte differentiation by steroid through targeting of an HDAC1 complex. *Embo j.* 22:2135-2145.

Wright, W.E., D.A. Sassoon, and V.K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell.* 56:607-617.

Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature.* 270:725-727.

Yan, B., J.T. Guo, C.D. Zhu, L.H. Zhao, and J.L. Zhao. 2013. miR-203b: a novel regulator of MyoD expression in tilapia skeletal muscle. *J.Exp.Biol.* 216:447-451.

Yoon, K., S. Zhu, S.J. Ewing, and R.C. Smart. 2007. Decreased survival of C/EBP beta-deficient keratinocytes is due to aberrant regulation of p53 levels and function. *Oncogene.* 26:360-367.

Zahnow, C.A. 2009. CCAAT/enhancer-binding protein beta: its role in breast cancer and associations with receptor tyrosine kinases. *Expert Rev.Mol.Med.* 11:e12.

Zahnow, C.A. 2002. CCAAT/enhancer binding proteins in normal mammary development and breast cancer. *Breast Cancer Res.* 4:113-121.

Zahnow, C.A., P. Younes, R. Laucirica, and J.M. Rosen. 1997. Overexpression of C/EBPbeta-LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. *J.Natl.Cancer Inst.* 89:1887-1891.

Zammit, P., and J. Beauchamp. 2001. The skeletal muscle satellite cell: stem cell or son of stem cell? *Differentiation.* 68:193-204.

Zanotti, S., L. Stadmeyer, A. Smerdel-Ramoya, D. Durant, and E. Canalis. 2009. Misexpression of CCAAT/enhancer binding protein beta causes osteopenia. *J.Endocrinol.* 201:263-274.

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

Zhou, B.B., and S.J. Elledge. 2000. The DNA damage response: putting checkpoints in perspective. *Nature.* 408:433-439.

Zhu, S., H.S. Oh, M. Shim, E. Sterneck, P.F. Johnson, and R.C. Smart. 1999. C/EBPbeta modulates the early events of keratinocyte differentiation involving growth arrest and keratin 1 and keratin 10 expression. *Mol.Cell.Biol.* 19:7181-7190.