

**The Regulation of Telomerase Reverse Transcriptase
(TERT) by CCAAT/Enhancer Binding Protein β (C/EBP β)
During Skeletal Muscle Differentiation**

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ABSTRACT

Our lab has identified the bZIP transcription factor CCAAT/Enhancer Binding Protein beta (C/EBP β) as a negative regulator of myogenic differentiation. C/EBP β is highly expressed in satellite cells and is downregulated during myogenic differentiation, a step that is critical for terminal differentiation, as ectopic C/EBP β expression blocks this process. Telomerase has been identified as a C/EBP β target gene in liver and other systems, and has been implicated in the regulation of muscle regenerative responses in models of Duchenne Muscular Dystrophy. Given that C/EBP β is overexpressed in models of muscle wasting, and high levels of telomerase inhibit differentiation, I hypothesized that C/EBP β inhibits myogenic differentiation through upregulation of TERT (telomerase reverse transcriptase) expression. I demonstrate that overexpression of C/EBP β in myoblasts increases mTERT expression under both growth and differentiation conditions. Conversely, loss of C/EBP β expression in myoblasts using shRNA technology or after isolation of primary myoblasts from conditional knockout mice, results in a downregulation of TERT expression and activity. When TERT was pharmacologically inhibited or knocked down using a shRNA, there was a significant improvement in differentiation and fusion in C2C12 myoblasts overexpressing C/EBP β as evidenced by an increase in the number of MHC⁺ fibers and expression of muscle-specific differentiation genes. Interestingly, I found that C/EBP β and TERT expression were increased in both embryonic and alveolar models of rhabdomyosarcoma. In response to this, a knockdown of C/EBP β in rhabdomyosarcoma cells decreased TERT expression and activity, and enhanced differentiation but not fusion in a model of embryonic rhabdomyosarcoma. These findings illustrate the novel regulation of TERT in skeletal muscle by C/EBP β , and reveal C/EBP β as an attractive therapeutic target for the treatment of muscle diseases such as rhabdomyosarcoma.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance
AML: acute myeloid leukemia
aRMS: alveolar rhabdomyosarcoma
bHLH: basic helix-loop-helix
BrdU: 5-Bromo-2'-deoxyuridine
bZIP: basic leucine zipper domain
C/EBP: CCAAT/Enhancer Binding Protein
C/EBP β : CCAAT/Enhancer Binding Protein beta
CD34: cluster of differentiation 34
CDK4: Cyclin-dependent kinase 4
cDNA: complimentary DNA
ChIP: chromatin immunoprecipitation
cKO: conditional knockout
CTED: carboxy-terminal extension domain
CUGBP1: CUG triplet repeat, RNA binding protein 1
CycloB: cyclophilin-B protein
DAPI: 4',6-diamidino-2-phenylindole
DIG: digoxigenin
DM: differentiation media
DMEM: Dulbecco's modified Eagle's medium
DNA: deoxyribonucleic acid
eIF2: Eukaryotic Initiation Factor 2
ELISA: enzyme-linked immunosorbent assay
eRMS: embryonic rhabdomyosarcoma
FGF: fibroblast growth factor
GM: growth media
GS: goat serum
HGF: hepatocyte growth factor
HI-FBS: heat-inactivated fetal bovine serum
HS: horse serum
HSMM: human skeletal muscle myoblasts
hTERT: human telomerase reverse transcriptase
IBMX: isobutyl methylxanthine

IL-6: Interleukin 6
LAP*: liver activating protein (full-length)
LAP: liver activating protein
LIP: liver inhibitory protein
MEF2: myocyte enhancer factor-2
MHC: myosin heavy chain
miR: microRNA
MRF: myogenic regulatory factor
MRF4: myogenic regulatory factor 4
mRNA: messenger RNA
mTERT: mouse telomerase reverse transcriptase
mTOR: mechanistic/mammalian target of rapamycin
NEAA: non-essential amino acids
P/S: penicillin/streptomycin
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PFA: paraformaldehyde
POT1: protection of telomeres 1
RAP1: repressor/activator protein 1
RBD: RNA-binding domain
RMS: rhabdomyosarcoma
RNA: ribonucleic acid
RTD: reverse transcriptase domain
RT-qPCR: reverse transcriptase quantitative PCR
SD: standard deviation
SEM: standard error of the mean
shRNA: short/small hairpin RNA
TERT: telomerase reverse transcriptase
TETA: Triethylenetetramine
TIN2: TRF1-interacting nuclear factor 2
TPP1: adrenocortical dysplasia protein homolog
TR: telomerase RNA subunit
TRAP: telomerase repeat amplification protocol

TRF1: telomeric repeat-binding factor 1

TRF2: telomeric repeat-binding factor 2

YY1: Yin Yang 1

1. INTRODUCTION

1.1 Satellite Cells and Myogenic Differentiation

Myogenic differentiation is a process by which committed muscle progenitor cells are activated from their quiescent state to proliferate and differentiate to become multinucleated myotubes. Myogenic differentiation is an important process in skeletal muscle that contributes to the growth and repair of muscle fibers after injury and exercise (Charge, 2004). The progenitor cells in adult muscle are referred to as satellite cells due to their satellite position on the muscle fiber, situated between the basal lamina and the sarcolemma of mature muscle fibers (Atsushi Asakura et al., 2007). Satellite cells have been shown to be critical for the regenerative capacity of skeletal muscle post-injury or exercise. As adult stem cells, satellite cells possess the capacity to self-renew, which is crucial for maintaining their population and long-term regenerative potential (McCullagh & Perlingeiro, 2015). Satellite cells can be characterized by the presence of specific protein markers such as CD34, Syndecan-3 and 4, m-Cadherin, and most notably, Pax7 (Motohashi & Asakura, 2014; Yin, Price, & Rudnicki, 2013).

Pax7 is a member of the paired box family of transcription factors that are involved in fetal development and tissue specification (Maroto et al., 1997; Strachan & Read, 1994). Pax7 is expressed in both quiescent and activated satellite cells. Studies have shown that *Pax7* transcripts are rapidly downregulated upon entry into the myogenic differentiation program (Seale et al., 2000). In addition, muscles undergoing extensive regeneration and repair, such as the *mdx* mouse model of muscular dystrophy, have a larger number of Pax7 positive cells due to increased numbers of activated satellite cells for chronic regenerative responses (Seale et al., 2000). In the *Pax7*(-/-) mice, satellite cells are absent suggesting an important role for Pax7 in satellite cell

survival and development (Relaix et al., 2006). In addition, mononuclear cells isolated from the muscle of *Pax7*^(-/-) mice do not form myoblasts but rather form a population of adipocytes and fibroblasts (Seale et al., 2000).

Once activated, satellite cells downregulate the expression of early markers, such as Pax7, and begin the differentiation process. Muscle cell differentiation is characterized by the sequential expression of the myogenic regulatory factors: MyoD, Myf5, myogenin, and MRF4 (Tapscott, 2005; Yin et al., 2013). The myogenic regulatory factors (MRFs) are a family of basic helix-loop-helix (bHLH) transcriptional factors that all have a conserved bHLH domain that is involved in DNA binding and dimerization (Hu, Geles, Paik, DePinho, & Tjian, 2008; Murre et al., 1989). The function and role of each MRF is apparent when studying the characteristics of the specific knockout mouse models. MyoD was once considered the “master regulator” of myogenesis (Atsushi Asakura et al., 2007; Charge, 2004). Expression of MyoD alone is able to drive myogenic differentiation of various cell types into muscle cells (Atsushi Asakura et al., 2007). MyoD deficiency results in the upregulation of a subset of stem cell markers, and in turn a downregulation of muscle-specific genes, resulting in a delay of muscle regeneration (A. Asakura et al., 2007; Megeney, Kablar, Garrett, Anderson, & Rudnicki, 1996). *MyoD*^{-/-} myoblasts were also better at populating the satellite cell niche (found beneath the basal lamina of muscle fibers) than myoblasts that expressed *Myod1* (A. Asakura et al., 2007). Transplantation of *MyoD*^{-/-} myoblasts into injured muscle resulted in significantly higher engraftment efficiency compared with wild-type myoblasts, though contributions to repair were reduced (Atsushi Asakura et al., 2007). Initially, MyoD and Myf5 were believed to be functionally redundant and able to compensate for loss of each other’s expression. In fact, loss of both MyoD and Myf5 results in a complete loss of skeletal muscle (Rudnicki et al., 1993). However, *MyoD*^{-/-} mice have impaired satellite cell

differentiation notwithstanding their elevated expression of Myf5 (Megeney et al., 1996; Sabourin, Girgis-Gabardo, Seale, Asakura, & Rudnicki, 1999; Yablonka-Reuveni et al., 1999). The *Myf5*^{-/-} mouse model appears mostly morphologically normal, except for abnormal rib development causing perinatal death, though the expression of MyoD and the other MRFs does not change (Braun, Rudnicki, Arnold, & Jaenisch, 1992). Subsequent studies instead suggest that MyoD and Myf5 have distinct functions in skeletal muscle specification. A *Myf5*^{-/-} mouse was bred into the *mdx* background (*mdx/Myf5*^{-/-}) to determine the role of Myf5 in adult skeletal muscle regeneration and a significant decrease in satellite cell-derived myoblast proliferation was observed (Ustanina, Carvajal, Rigby, & Braun, 2007). This was accompanied by a delay in the transition from proliferation to differentiation leading to a reduction in the number of myotube nuclei (Ustanina et al., 2007). Myf5 has thus been associated with transient myoblast amplification leading to robust muscle regeneration. Both MyoD and Myf5 are considered markers of early myogenic differentiation, deemed commitment factors. However, Myf5 is thought to regulate proliferation of muscle progenitors, whereas MyoD is required for entry of these proliferating cells into the muscle differentiation program (Megeney et al., 1996).

The other two MRFs, Myogenin and MRF4, are markers of late muscle differentiation and fusion. The *Myog*^{-/-} mouse has severe skeletal muscle deficiency despite the formation of myoblasts remains intact, the myoblasts are incapable of fusing to form myotubes, leading to perinatal death (Hasty et al., 1993; Nabeshima et al., 1993). MRF4 (also known as Myf6) is the most highly expressed MRF in adult skeletal muscle though the exact role remains unclear. There have been three models of MRF4 knockout using different alleles, with phenotypes ranging from complete viability to lethality (Braun, Bober, Rudnicki, Jaenisch, & Arnold, 1994; Patapoutian et al., 1995; W. Zhang, Behringer, & Olson, 1995). The variability of the phenotype has been

attributed to cis-regulatory interactions with *Myf5* since *MRF4* is found in very close proximity to *Myf5* on mouse chromosome 10 (Yoon, Olson, Arnold, & Wold, 1997). In fact, one of the MRF4 knockout models very closely resembles the *Myf5* knockout mouse (W. Zhang et al., 1995). MRF4 has recently been shown to be a negative regulator of adult skeletal muscle growth by repressing MEF2 activity, which is known to induce myofiber hypertrophy (Moretti et al., 2016). This could implicate MRF4 in the regulation of muscular atrophy and could make it an attractive target for potential therapies for cancer cachexia, for example.

1.2 CCAAT/Enhancer Binding Protein Beta (C/EBP β)

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors in which there are 6 characterized isoforms (α - ζ) named based on the chronological order of their discovery (Tsukada, Yoshida, Kominato, & Auron, 2011). C/EBPs are known to be involved in regulation of many different processes such as energy metabolism, inflammation, hematopoiesis, cell cycle regulation, adipogenesis, and osteoblastogenesis (Ramji & Foka, 2002; J.-W. Zhang, 2003). All of the members of this family share >90% sequence identity in the C-terminal 55-65 amino acid residues where the highly conserved basic-leucine zipper (bZIP) domain resides (Ramji & Foka, 2002; Tsukada et al., 2011). The bZIP domain consists of a heptad repeat of a minimum of four hydrophobic amino acids (usually leucines) which form an α -helical “coiled-coil” configuration (Ramji & Foka, 2002). The leucine zipper is involved in homo and hetero-dimerization; a prerequisite to DNA binding, while a basic sequence interacts with DNA. The N-termini of the C/EBP proteins, which contains the activation domain, are structurally distinct from one another and show <20% sequence identity (Ramji & Foka, 2002).

CCAAT/Enhancer Binding Protein Beta (*Cebpb*) is an intronless gene of the C/EBP family and was first identified based on its ability to regulate gene transcription in response to IL-6 (Ramji & Foka, 2002). There are three documented protein isoforms of C/EBP β generated by leaky ribosome scanning of the *Cebpb* transcript: LAP*, LAP, and LIP (Ramji & Foka, 2002). LAP* (liver-enriched activating protein*) is the full-length isoform whereas the LAP (liver-enriched activating protein) isoform lacks the first 21 amino acids. The shortest isoform, LIP (liver-enriched inhibitory protein), lacks the activation domain and acts through heterodimerization to antagonize the actions of the LAP and LAP* isoforms in many systems, such as in the liver (Descombes & Schibler, 1991; Luedde et al., 2004). The translational control and expression of C/EBP β isoforms have been shown to be dependent on mammalian target of rapamycin (mTOR) signaling and RNA-binding proteins. Specifically, high mTOR activity initiates preferential translation of the truncated LIP isoform (Bégay et al., 2015). CUGBP1, an RNA-binding protein, has also been shown to preferentially induce translation the LIP isoform. Briefly, it was shown that after a partial hepatectomy, CUGBP1 is activated by hyperphosphorylation. This in turn causes the activated CUGBP1 to interact with the alpha and beta subunits of initiation factor eIF2, enhancing the recruitment of ribosomes which facilitate the translation of the LIP isoform (Timchenko, Wang, & Timchenko, 2005).

Expression of *Cebpb* is highest in the liver, spleen, kidney, and myelomonocytic cells and it is known to be involved in immune and inflammatory responses (Ramji & Foka, 2002). *Cebpb* expression has also been implicated in a number of cancers such as breast and gastric cancers (Sankpal, Moskaluk, Hampton, & Powell, 2005; Cynthia A Zahnow, 2009). Alterations in the LIP to LAP ratio of C/EBP β isoforms, which is critical for normal proliferation and development, can

lead to some of the most aggressive forms of breast cancer when the LIP isoform is predominant (C A Zahnow, Cardiff, Laucirica, Medina, & Rosen, 2001).

1.3 The Role of C/EBP β in Skeletal Muscle

Prior to the discoveries made in our lab, knowledge about the role of C/EBP β in skeletal muscle was limited. *Cebpb* (-/-) mice have increased insulin signalling in the skeletal muscle without changes in insulin response in the liver or adipose tissue (L. Wang et al., 2000). In models of muscle wasting such as glucocorticoid-induced sepsis and sarcopenia, C/EBP β expression is increased, and C/EBP β has been implicated in the regulation of atrogen-1, a protein involved in the degradation of muscle proteins during atrophy (Giresi et al., 2005; Penner, Gang, Sun, Wray, & Hasselgren, 2002). Our lab has more recently demonstrated the involvement of C/EBP β in the regulation of myogenic differentiation and satellite cell maintenance, showing that C/EBP β is highly expressed in satellite cells of healthy muscle and acts to maintain the undifferentiated state (François Marchildon et al., 2012). C/EBP β expression is significantly downregulated with the onset of differentiation, in parallel with Pax7 expression (François Marchildon et al., 2012). Indeed, C/EBP β directly regulates Pax7 expression such that ectopic expression of C/EBP β in C2C12 and primary myoblasts promotes high levels of expression of Pax7 compared to controls. This in turn correlates with an increase in self-renewal of satellite cells and the inhibition of differentiation (François Marchildon et al., 2012). In addition to stimulation of Pax7, C/EBP β also inhibits MyoD protein expression and activity (François Marchildon et al., 2012). High levels of C/EBP β expression, promoted by IL-1 β expression, was also shown to promote satellite cell survival in a model of cancer cachexia (F Marchildon, Fu, Lala-Tabbert, & Wiper-Bergeron, 2016). Transient pharmacological induction of C/EBP β expression by a phosphodiesterase

inhibitor (IBMX) was able to increase myoblast expansion in culture as well as satellite cell marker expression (Lala-Tabbert, Fu, & Wiper-Bergeron, 2016). When these cells were transplanted into *mdx* mice, they were able to more efficiently contribute to muscle repair and improve engraftment efficiency compared to vehicle-treated cells (Lala-Tabbert et al., 2016).

In order to study the effects of loss of *C/EBPβ* in muscle, our lab generated a *C/EBPβ* conditional knockout (cKO) mouse model in which *C/EBPβ* is excised from skeletal muscle satellite cells. This is accomplished by crossing mice with a *C/EBPβ*-floxed allele with mice bearing the *Pax7-CreER* allele. Loss of *C/EBPβ* in muscle satellite cells resulted in precocious differentiation in growth conditions and increased cell fusion under differentiation conditions (François Marchildon et al., 2012). The enhancement of differentiation and fusion was accompanied by an increase in *MyoD* and myogenin expression, and a decrease in *Pax7* expression. In addition, conditional null animals had larger muscle fibers which was attributed to increased differentiation and fusion. *C/EBPβ* cKO mice also showed enhanced repair after a single injury which is also likely due to enhanced differentiation and fusion mechanisms (François Marchildon et al., 2012). After sequential injury however, a reduction in the population of satellite cells and regenerative capacity was noted which was attributed to a significant decrease in the *Pax7+* population (François Marchildon et al., 2012).

1.4 Telomerase Structure and Function

The Hayflick limit, which was first characterized by Leonard Hayflick in 1961, is the number of cell divisions that a particular somatic cell will experience before becoming apoptotic or senescent (Hayflick & Moorhead, 1961). To slow down this process, cells use telomeres which

are specialized structures that are present on the ends of linear chromosomes (Blackburn, 1991). These structures are made up of specific repeat nucleotide sequences that are added to the ends of chromosomes by the enzyme telomerase (Blackburn, 1991; Olovnikov, 1996). Telomeres act as a protective mechanism against end-to-end fusion, chromosomal degradation, and other detrimental reactions due to continual cycles of DNA replication (Blackburn, 1991). The presence of telomeres thus enhances cell survival and longevity. Numerous studies report an inverse correlation between telomere length and age (Blasco, 2005; Cawthon, Smith, O'Brien, Sivatchenko, & Kerber, 2003; Espejel et al., 2004; Harley, Futcher, & Greider, 1990). Telomeres are, however, subjected to shortening during repeated cycles of cell division due to the end-replication problem, which is caused by the incomplete synthesis of the lagging strand during DNA replication because of the inability of DNA polymerase to completely replicate the ends of chromosomal DNA (Gilson & Géli, 2007; Stewart, Chaiken, Wang, & Price, 2012). Critically shortened telomeres have decreased cellular proliferative potential and trigger apoptosis or senescence through a DNA damage response initiated by p53 (Blackburn, 1991).

Telomerase is the eukaryotic enzyme that functions to sustain chromosomal stability by maintaining the length of telomeres. This is accomplished by catalyzing the addition of specific repeat-nucleotide sequences (TTAGGG in vertebrates) to the ends of chromosomes (Blackburn, 1991; Meyne, Ratliff, & Moyzis, 1989). Telomeric DNA consists of TTAGGG repeats in an extended region of double-stranded DNA, and then ending with a single-stranded G-rich overhang (W E Wright, Tesmer, Huffman, Levene, & Shay, 1997). Working in conjunction with telomerase, a protein complex known as shelterin helps to regulate mammalian telomere length (Diotti & Loayza, 2011; Maciejowski & de Lange, 2017). This complex is made up of six subunits: telomeric repeat-binding factor 1 and 2 (TRF1 and TRF2), repressor/activator protein 1 (RAP1), TRF1-

interacting nuclear factor 2 (TIN2), adrenocortical dysplasia protein homolog (TPP1) and protection of telomeres 1 (POT1) (Diotti & Loayza, 2011; Maciejowski & de Lange, 2017). Shelterin is essential to the protective mechanism behind the capping of telomeres on the ends of chromosomes as the subunits of shelterin bind to the TTAGGG nucleotide sequences (Palm & de Lange, 2008). Specifically, TPP1 and POT1 bind to the single-stranded G-rich overhang. This binding then induces the formation of a “t-loop cap” that prevents DNA-damage-sensing machinery from accessing the telomeres (Griffith et al., 1999). When shelterin is absent, telomeres become “uncapped” and the DNA-damage response begins (Blackburn, 1991; Palm & de Lange, 2008; Rodriguez et al., 2008).

The enzyme telomerase is a ribonucleoprotein complex consisting of an RNA subunit (TR) which serves as a template to add new telomeric repeats, and a catalytic protein component, TERT (telomerase reverse transcriptase) (Blasco, 2005). The TERT component of telomerase is repressed in somatic tissues, rendering telomerase inactive, but is consequently reactivated by transcriptional upregulation in tumorigenesis, for example to promote proliferation (Ramlee, Wang, Toh, & Li, 2016). It has been shown that ectopic expression of TERT is sufficient to immortalize certain cell types (N. W. Kim et al., 1994; Meyerson et al., 1997; Olovnikov, 1996). The expression of TERT is strongly positively correlated with telomerase activity and is thus frequently used as a marker of enzyme activity (Nakamura et al., 1997; Olovnikov, 1996)

Despite differences in size and sequence of the RNA component of telomerase amongst different species, there exists common conserved motifs suggesting that telomere replication may share similar mechanisms amongst related organisms (Mitchell, Gillis, Futahashi, Fujiwara, & Skordalakes, 2010). It is however important to note some differences and limitations in studying TERT in mouse when modeling human disease. Mice and humans have been shown to have

significant differences in the length of their telomeres in that murine telomeres are 5-10 times longer than those of human origin, even though mice have significantly shorter lifespans (Calado & Dumitriu, 2013; Woodring E. Wright & Shay, 2000). It has been concluded that telomere length in mammals generally inversely correlates with lifespan, whereas telomerase expression inversely correlates with body mass (Gomes et al., 2011). Mice are thus useful to understand basic mechanisms of telomere biology, but may not be ideal to study the role of telomeres in human aging and disease.

The structure of TERT contains three different domains: an RNA-binding domain (RBD), the reverse transcriptase domain (RTD) and the carboxy-terminal extension domain (CTED) (Autexier & Lue, 2006). The RTD and the CTED represent the “fingers, palm and thumb” of a classic polymerase (Autexier & Lue, 2006). The domains that comprise the TERT structure are organized into a ring configuration that resembles the HIV reverse transcriptase (Mitchell et al., 2010). Though it may be structurally similar to other reverse transcriptases, TERT has two unique characteristics: its stable connection with the telomerase RNA and its ability to repetitively reverse transcribe the RNA template (Autexier & Lue, 2006).

1.5 TERT and Stem Cells

TERT is highly expressed in embryonic and stem cells as well as many cancerous cells and immortalized cell lines (such as C2C12s), but is generally not detectable or quite low in somatic cells (Hiyama & Hiyama, 2007; Meyerson et al., 1997; Tahara et al., 1999). The high expression of TERT expression and activity observed in stem cells significantly decreases upon induction to differentiate, concomitantly with cell cycle exit required for cellular differentiation (Armstrong et

al., 2005; Hiyama & Hiyama, 2007). This downregulation is correlated with histone deacetylation and DNA methylation of the *TERT* gene (L. Liu, Saldanha, Pate, Andrews, & Tollefsbol, 2004). Increased telomerase activity has also been shown to enhance the self-renewal ability of embryonic stem cells that overexpress TERT (Armstrong et al., 2005).

Though skeletal muscle is considered a post-mitotic tissue and contains mostly somatic cells, it was found that murine quiescent and activated satellite cells have high telomerase activity that can be maintained for several days of culturing after isolation (O'Connor, Carlson, & Conboy, 2009). On the other hand, primary myoblasts were shown to quickly and dramatically downregulate telomerase activity upon differentiation, similar to what is seen in most embryonic and stem cells (O'Connor et al., 2009). The rapid downregulation of telomerase would theoretically be more dramatic in larger species with longer lifespans, such as humans, which have much shorter telomeres compared to mice. This is supported by the fact that DMD patients experience telomere shortening after satellite cell exhaustion. In fact, it has been reported that human DMD patients have a 14-fold increase in the shortening of their telomeres, likely due to high replication of stem cells to support chronic regeneration (Decary et al., 2000).

Telomerase knock out mice have significantly impaired progenitor cell function leading to severely reduced regenerative potential (Allsopp, 2003). When TR (RNA subunit of telomerase) knock out mice were crossed into the *mdx* mouse bearing a mutation in the dystrophin gene, these *mdx/mTR(-/-)* mice had a more severe disease course that better recapitulated the phenotypic characteristics of muscular dystrophy in humans. This included significant loss of muscle force, poor treadmill performance, elevated creatine kinase levels, muscle fibrosis, calcium deposits, kyphosis and reduced lifespan (Sacco et al., 2010). In addition, the muscle stem cells in these mice had significantly reduced proliferation, unresponsiveness to muscle injury, and reduced

engraftment upon transplantation (Sacco et al., 2010). The differences in telomerase expression and activity could therefore explain, at least in part, the differences in disease produced by the *mdx* mutation in mice and in humans.

1.6 The Function of TERT in Cancer

Though it appears that telomerase acts solely to save our cells from premature senescence and death, telomerase can also have cancer-promoting properties. A strong positive correlation has been established between telomerase expression and cancer. Between 85-90% of cancers are known to express high levels of telomerase that allows for unchecked proliferation (Blasco, 2005; N. W. Kim et al., 1994; Shay & Bacchetti, 1997). The mechanisms governing *hTERT* activation in cancerous cells remains unclear, but include mutations in the *hTERT* promoter, epigenetic alterations, and changes in alternative splicing of *hTERT* pre-mRNA (Jafri, Ansari, Alqahtani, & Shay, 2016; Shay, 2013). Despite this, telomerase represents an attractive biomarker and highly-specific target for the development of cancer therapeutics, especially since most somatic cells have very low levels of telomerase expression (N. W. Kim et al., 1994).

1.7 Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common type of childhood soft tissue sarcoma accounting for approximately 40% of cases (Arndt & Crist, 1999). RMS is believed to arise from skeletal muscle cells that have failed to fully differentiate causing spontaneous tumour formation anywhere on the body, though most commonly found in the head and neck area (Arndt & Crist, 1999). It is divided into two primary histological subtypes: embryonic RMS (eRMS) and alveolar

RMS (aRMS) (Parham & Barr, 2013). eRMS generally develops before the age of 5 and has a much better clinical prognosis, whereas aRMS is typically diagnosed later in childhood and is associated with more aggressive tumours and a poorer prognosis (Pappo et al., 1999). Both eRMS and aRMS are characterized by skeletal muscle tumours that have impaired terminal differentiation, even though they still express skeletal muscle differentiation markers such as myogenin (Keller & Guttridge, 2013; Sebire & Malone, 2003; Tonin, Scrabble, Shimada, & Cavenee, 1991). In fact, the expression of both MyoD and myogenin are used as markers for the diagnosis of RMS (Dias et al., 2000; Sebire & Malone, 2003; Tonin et al., 1991). Despite expression of late markers of differentiation, RMS cells remain proliferative and therefore cannot fuse to form mature myofibers (Tapscott, Thayer, & Weintraub, 1993). The mechanism behind the impairment of differentiation is unclear, but it has been suggested to be due to alterations in MyoD function, epigenetic modifications of myogenic promoters, and microRNA expression (Keller & Guttridge, 2013).

Alveolar RMS tumours, the more aggressive subtype, are classified as fusion-negative or fusion-positive. The fusion-positive subtype results from the presence of recurrent chromosomal translocations, the most common being t(2;13)(q35;q14). This particular translocation results in the expression of an oncogenic fusion protein which combines the Pax3 transcription factor with the transcriptional activation domain of a member of the forkhead family of transcription factors, FOXO1 (Douglass et al., 1987; Fredericks et al., 1995; Galili et al., 1993). The Pax3-FOXO1 fusion protein is present in approximately 55% of aRMS cases (Sorensen et al., 2002). A similar fusion protein resulting from a translocation of t(1;13)(p36;q14) is present in 22% of aRMS cases and combines the Pax7 DNA-binding domains to FOXO1 (Davis, D'Cruz, Lovell, Biegel, & Barr, 1994; Sorensen et al., 2002). Expression of the oncogenic Pax3/Pax7-FOXO1 fusion proteins

impairs differentiation of aRMS tumour cells via several mechanisms. Firstly, while Pax3 expression can inhibit differentiation of cultured myoblasts, it is normally rapidly degraded during early myogenic differentiation. However, the Pax3-FOXO1 fusion protein has a significantly longer half-life than Pax3 and can thus prevent the normal cell cycle exit required for complete differentiation (Epstein, Lam, Jepeal, Maas, & Shapiro, 1995; Miller & Hollenbach, 2007). Pax3/7-FOXO1 has also been shown to cause alterations in MyoD expression and activity (Calhabeu, Hayashi, Morgan, Relaix, & Zammit, 2013; Olguín, Patzlaff, & Olwin, 2011). The Pax7-FOXO1 protein was shown to repress MyoD-dependent myogenesis (Olguín et al., 2011). In addition, both the Pax3 and the Pax7-FOXO1 proteins were found to suppress the transcriptional activation of MyoD target genes such as myogenin, muscle creatine kinase, and p21; all of which are known to be markers of myogenic differentiation (Calhabeu et al., 2013). Pax7-FOXO1 expression can also induce NFκB signaling, which is known to inhibit myogenesis through multiple mechanisms including activating the cyclin D1/CDK4 complexes and silencing miR-29 (Charytonowicz et al., 2012; H. Wang et al., 2008). Cyclin D1 has been shown to act as an inhibitor of myogenesis as forced cyclin D1 expression inhibits the transactivation of muscle-specific genes by MyoD, which was correlated with MyoD phosphorylation (Skapek, Rhee, Spicer, & Lassar, 1995). During differentiation of normal myoblasts, NFκB is downregulated which causes an upregulation in miR-29 thereby accelerating myogenic differentiation (H. Wang et al., 2008). In RMS cells however, which have a significant impairment in differentiation, miR-29 is epigenetically silenced by the improper activation of the NFκB-YY1 pathway. miR-29 overexpression in mice with RMS can inhibit the growth of tumours and stimulate differentiation which suggests that miR-29 may act as a tumour suppressor through the activation of the NFκB-YY1 pathway (H. Wang et al., 2008).

Tumour samples from patients with eRMS and aRMS revealed that most of these tumours also exhibited high telomerase activity (Ohali et al., 2008). One study examined RD cells, a commonly used human eRMS cell line, which have a very limited myogenic differentiation program. It was found that *hTERT* transcription and telomerase activity were repressed when these cells were induced to differentiate (Ma, Urquidi, Wong, Kleeman, & Goodison, 2003).

1.8 Regulation of TERT by C/EBP β

The relationship between C/EBP β and TERT has been previously established in other systems. In mammary carcinomas for example, C/EBP β and TERT were both shown to be upregulated during tumorigenesis in *hTERT_{p-lacZxWAP}-T* mice (Kumar et al., 2013). ChIP experiments revealed the binding of C/EBP β to two different regions within the *hTERT* promoter. This effect was seen in C/EBP β overexpression studies but also with endogenous C/EBP β in MCF7 breast cancer cells (Kumar et al., 2013). Reporter gene assays also revealed that co-transfection with C/EBP β induced a three-fold increase of luciferase gene expression and this effect was abrogated when the C/EBP binding sites were mutated (Kumar et al., 2013). C/EBP β thus acts as a transcriptional activator of *hTERT* gene expression and activity.

In the liver, it was found that after injury of old mice with CCl₄, the expression of the truncated C/EBP β -LIP isoform was increased compared to young littermate controls (Hong et al., 2014). This isoform is a positive regulator of liver proliferation by activating cell cycle protein promoters (Orellana et al., 2010). A ChIP assay was performed and found that C/EBP β -LIP occupies and represses the *mTERT* promoter after injury in old mice (Hong et al., 2014). However, it was also found that full-length C/EBP β binds to and causes activation of the *mTERT* promoter

in the livers of young WT mice (Hong et al., 2014). These findings suggest potential age-related changes in C/EBP β expression and their effect on binding to the *TERT* promoter and place C/EBP β as a direct transcriptional regulator of *hTERT* expression.

1.9 Rationale

The regulation of skeletal muscle differentiation is an important process due to its role in regeneration after injury, fiber development, and muscle tissue formation. Thus, uncovering the mechanisms behind muscle differentiation is critical to understanding diseases in which muscle differentiation is impaired such as rhabdomyosarcoma and muscular dystrophies. It was found that C/EBP β acts as a negative regulator of myogenic differentiation and is highly expressed in satellite cells (François Marchildon et al., 2012). Interestingly, in many stem cell populations, TERT expression also acts as a negative regulator of cellular aging, decreases upon differentiation and can be found highly expressed in muscle satellite cells (Hiyama & Hiyama, 2007; O'Connor et al., 2009). In addition, TERT is known to be upregulated in many types of cancer, as is C/EBP β , and C/EBP β is a transcriptional regulator of *TERT* expression (Hong et al., 2014; Kumar et al., 2013; Meyerson et al., 1997; Sankpal et al., 2005; Cynthia A Zahnow, 2009). While a link between C/EBP β and *TERT* expression has been described in certain cancers and in injured liver, the regulation of *TERT* expression has never been investigated in skeletal muscle.

1.10 Hypothesis

C/EBP β is a transcription factor involved in the regulation of myogenic differentiation and satellite cell maintenance. The expression of C/EBP β is significantly downregulated upon differentiation, which follows an expression pattern similar to that of TERT in stem cells. C/EBP β and TERT also exhibit similar expression patterns in model systems such as muscular dystrophy and cancer. Given that C/EBP β has been shown to bind to the *TERT* promoter and regulate its activity in other systems, I hypothesized that *C/EBP β acts as a regulator of TERT in skeletal muscle and that C/EBP β acts to inhibit myogenic differentiation, at least in part, via the stimulation of TERT expression.*

1.11 Objectives

- 1) Determine the expression of TERT in C/EBP β -overexpressing cells and in models of low or absent C/EBP β expression.
- 2) Evaluate the effects of TERT inhibition in control versus C/EBP β isoform-overexpressing myoblasts.
- 3) Evaluate occupancy of C/EBP β on the *TERT* promoter
- 4) Determine the effects of C/EBP β knockdown on myogenic differentiation and TERT expression/activity in models of rhabdomyosarcoma.

2. MATERIALS AND METHODS

2.1 Constructs

The C/EBP β expression plasmids have been previously described (Wiper-Bergeron, Wu, Pope, Schild-Poulter, & Haché, 2003). The C/EBP β Δ 21 LAP construct which lacks the first 21 amino acids in the N-terminus was originally generated by C. St-Louis using site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit - Agilent Technologies, Mississauga, Ontario, Canada) using the following primers: F (5'-GACGACGCGCCCGCCCTGGCGGCCGGTTTCC-3'), R (5'-GGAAACCGGCCGCCAGGGCCGGGCGCGTCGTC-3') and according to manufacturer's instructions. The pLXSN-C/EBP β LIP (*M. musculus*) construct was provided by Dr. Abdou-Salem (Abdou, Atlas, & Haché, 2011).

2.2 Cell culturing and differentiation

All cultured cell lines were kept in a humidified cell incubator at 37°C and 5% CO₂.

2.2.1 C2C12s

C2C12 myoblasts (ATCC, Manassas, VA, USA) were maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) (Wisent, Saint-Bruno, QC, Canada), 10% heat-inactivated fetal bovine serum (HI-FBS) (Invitrogen, Carlsbad, CA, USA) and 1% Non-Essential Amino Acids (NEAA) (Wisent, Saint-Bruno, QC, Canada). To induce differentiation of C2C12 myoblasts, cultures at 80% confluence were switched to differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum (HS) (Sigma-Aldrich, Oakville, ON, Canada).

2.2.2 Primary myoblast culture and isolation

Primary myoblasts were isolated from the mouse hindlimb muscle using the MACS Cell Separation protocol (Miltenyi Biotec Inc., Auburn, CA, USA) as previously described (Motohashi, Asakura, & Asakura, 2014). Cells were maintained in DMEM supplemented with 20% FBS, 10% HS, 1% penicillin/streptomycin (P/S) (ThermoFisher Scientific, Ontario, Canada), 10 ng/ml basic fibroblast growth factor (FGF) and 2ng/ml hepatocyte growth factor (HGF) (Peprotech, Rocky Hill, NJ, USA). Differentiation of primary myoblasts was achieved by switching confluent cultures to DMEM supplemented with 10% HS, 2% FBS, and 1% P/S. Primary myoblasts isolated from wild-type (*Cebpb*^{fl/fl}*Pax7*^{+/+}) and C/EBP β conditional knockout mice (*Cebpb*^{fl/fl}*Pax7*^{CreER/+}) were cultured in 2 μ M 4-OH tamoxifen (Sigma-Aldrich, Oakville, ON, Canada) to excise *Cebpb* in vitro.

2.2.3 Rhabdomyosarcoma (RMS) cells

Human skeletal muscle myoblasts (HSMM) (Lonza, NJ, USA) were cultured in skeletal muscle max growth media (Sk Max kit) according to the manufacturer's instructions (Wisent, Saint-Bruno, QC, Canada). To induce differentiation of HSMM cells, DMEM supplemented with 2% HS was added to cultures at high confluency. Embryonal rhabdomyosarcoma RD cells (CCL-136; ATCC, VA, USA) were cultured according to manufacturer's instructions in DMEM, 10% FBS, 1% L-glutamine (Wisent Bioproducts, Quebec, Canada), and 1% P/S. Alveolar rhabdomyosarcoma RH30 cells (CRL-2061; ATCC, VA, USA) were cultured in Multicell Roswell Park Memorial Institute medium (RPMI) 1640 1X (Wisent Bioproducts, Quebec, Canada) with 1% L-Glutamine, 15% HI-FBS and 1% P/S. For differentiation of RD and RH30 cells, the respective base media for each cell line (DMEM for RD cells and RPMI for RH30 cells) was supplemented with 2% HS, 1% L-glutamine and 1% P/S. HSMM, RD and RH30 cells were gifts from Dr. Bernard Jasmin (University of Ottawa).

2.3 Retroviral Transfection and Infection

Replication-incompetent pLXSN-based retroviruses (Clontech Laboratories, Inc., Mountain View, CA, USA) were created by calcium phosphate transfection of PhoenixTM Ampho packaging cells (ATCC, Manassas, VA, USA). Phoenix cells were grown in a 60mm dish and allowed to reach 70-80% confluency. Prior to transfection, Phoenix cells were switched to 3mL of C2C12 GM containing 25 μ M chloroquine diphosphate (Sigma-Aldrich, Oakville, ON, Canada). Plasmid DNA (10 μ g) was diluted in a final volume of 459 μ L of ddH₂O to which 31 μ L of 2M CaCl₂ and 500 μ L of 2X HBS (10mM KCl, 50mM HEPES pH 7.05, 12mM dextrose, 1.5mM Na₂HPO₄ and 280mM NaCl) were added. The DNA mixture was then added dropwise to the Phoenix cells. Phoenix cells were incubated overnight at 37°C and the following day cells were switched to C2C12 GM. The virus-containing media was collected 48 hours post-transfection and filtered through a 0.45 μ m syringe filter (Millipore, Billerica, MA, USA).

To create stable C2C12 myoblasts (pLXSN, pLXSN-C/EBP β , pLXSN-C/EBP β LAP and pLXSN-C/EBP β LIP), cells were grown in 60mm dishes (to 30% confluency) and infected with 1mL of viral media, 5mL of DMEM and 6 μ g/mL polybrene (Sigma-Aldrich, Oakville, ON, Canada). Cells were incubated for 15 minutes at 37°C and then the plates were wrapped with parafilm and centrifuged for 30 minutes at 1100g, after which the media was replaced with fresh C2C12 GM. Cells were selected with G418 (400 μ g/mL) (Sigma-Aldrich, Oakville, ON, Canada) 48 hours post-infection and was maintained for 5 days.

2.4 Lentiviral Infection

2.4.1 C2C12s

pLXSN and pLXSN-C/EBPβ C2C12 myoblasts were transduced with lentiviral particles targeting *mTERT* (shTERT, sc-36642-V) or a non-targeting control (shScr, sc-108080) (Santa Cruz Biotechnology) according to manufacturer's instructions. Briefly, cells were subjected to 20µl of lentiviral particles in 5ml of medium containing 1 µg/ml polybrene (Sigma-Aldrich, Oakville, ON, Canada). Cultures were selected in 2µm puromycin (Wisent, Saint-Bruno, QC, Canada) beginning 48 hours post-infection to create stable cell lines (pLXSN/shCTL, pLXSN/shTERT, C/EBPβ/shCTL, C/EBPβ/shTERT). Puromycin selection was continued for two days.

2.4.2 Primary myoblasts

Lentiviral particles containing an shRNA against mouse C/EBPβ (shC/EBPβ, sc-29862-V) or a scrambled shRNA control (shScr, sc-108080) (Santa Cruz Biotechnology) were added to isolated primary myoblasts at 30–40% confluency cultured in growth medium according to manufacturer's instructions as mentioned above. Cultures were selected in 2µm puromycin (Wisent, Saint-Bruno, QC, Canada) 48 hours post-infection to create stable cell lines (shCTL, shC/EBPβ). Puromycin selection was continued for two days.

2.4.3 RMS cells

Lentiviral particles containing an shRNA against human C/EBPβ (shC/EBPβ, sc-29229-V) or a scrambled shRNA control (shScr, sc-108080) (Santa Cruz Biotechnology) were added to HSMM myoblasts, RD and RH30 cells at 30–40% confluency in growth medium according to manufacturer's instructions as mentioned above. Cultures were selected in 2µm puromycin

(Wisent, Saint-Bruno, QC, Canada) 48 hours post-infection to create stable cell lines for each of HSMM, RD and RH30 (shCTL, shC/EBP β). Puromycin selection was continued for two days.

2.5 Western Blot Analysis

Cells were collected in 1mL PBS using a rubber policeman and centrifuged to pellet cells. The cell pellet was resuspended in IPH buffer (50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA, and 0.5% NP-40, 1x protease inhibitors, 1mM DTT) and kept on ice. Each sample was sonicated manually for 60 seconds and incubated on ice for 30 minutes. The cell suspension was centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatant was transferred to a new tube. A Bradford Assay was performed to determine the protein concentration of each sample. The protein sample (25 μ g) was loaded onto a 10%-15% SDS-PAGE gel and run at 200V for approximately 45 minutes after which it was transferred onto a PVDF transfer membrane (Bio-Rad, Mississauga, ON, Canada) at 100V for approximately 1.5 hours. After the transfer, the membrane was incubated in blocking solution (5% milk in 1X PBS-T (1X phosphate buffered saline with 0.05% Tween-20)) for 30 minutes. After blocking, the membrane was incubated overnight with primary antibody diluted in blocking solution (2% milk in PBS-T) at 4°C. For detection, the following antibodies were used: rabbit anti-mTERT (Santa Cruz-7212, 1:200), rabbit anti-C/EBP β (Abcam 32358, 1:500), mouse anti-MyoG (F5D, 1:500), mouse anti-Pax7 (DSHB, 1:1000), mouse anti-hTERT (Abcam 5181, 1:100), rabbit anti- C/EBP β (Santa Cruz-150, 1:1000), rabbit anti-cyclophilinB (Abcam 16045, 1:5000) and mouse anti- β -actin (Santa Cruz-47778, 1:5000). The next day, membranes were washed three times with 1X PBS-T for 5 minutes, after which horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:5000) or sheep anti-mouse IgG (1:2000) (GE Healthcare UK Limited, Buckinghamshire, UK) was added for 1 hour at room

temperature. Three additional PBS-T washes were performed before incubating membranes in ECL reagent (Bio-Rad, Mississauga, ON, Canada) for 5 mins. Chemiluminescence images were captured using the BioRad ChemiDoc MP Imaging System.

2.6 Real time quantitative PCR (RT-qPCR)

Total RNA was purified using the RNeasy kit (Qiagen, Germantown, MD, USA) as per manufacturer's instructions. 1µg of purified RNA was DNase treated (Ambion, Burlington, ON, Canada) for 30 minutes at 37°C and cDNA was synthesized using the iScript kit (Bio-Rad, Mississauga, ON, Canada) following manufacturer's instructions. cDNA was PCR-amplified on a Stratagene MX3005p real-time thermocycler (Agilent Technologies, Mississauga, Ontario, Canada) using an iTaq universal SYBR Green kit (Bio-Rad, Mississauga, ON, Canada). Primer sequences were as follows: C/EBPβ: F (5'- TCGAACCCGCGGACTGCAAG-3'), R (5'- CGACGACGACGTGGACAGGC-3'); mTERT: F (5'-ATGGCGTTCCTGAGTATG-3'), R (5'- TTCAACCGCAAGACCGACAG-3'); MyoG: F (5'-ATCGCGCTCCTCCTGGTTGA-3'), R (5'- CTGGGGACCCCTGAGCATTG-3'); Pax7: F (5'-GACGACGAGGAAGGAGACAA-3'), R (5'- CGGGTTCTGATTCCACATCT-3'); neoMHC: F (5'-TCGCTGGCTTTGAGATCTTT-3'), R (5'- ACGAACATGTGGTGGTTGAA-3'); and 18S: F (5'-CGCCGCTAGAGGTGAAATC-3'), R (5'- CCAGTCGGCATCGTTTATGG -3').

2.7 Immunocytochemistry

2.7.1 MHC Staining

Differentiated cells were washed twice with 1X PBS and fixed with ice-cold methanol for 10 minutes at room temperature. Cells were then washed again twice with 1X PBS and permeabilized for 15 minutes with 1X PBS/0.5% Triton X-100. Anti-myosin heavy chain primary antibody (MF-20, DSHB) was then added overnight at 4 C° at a 1:50 dilution in 1X PBS/0.1% Triton X-100. The next day, cells were washed three times with 1X PBS/0.3% Triton X-100 before incubating with Cy3-conjugated donkey anti-mouse IgG secondary antibody at a 1:1000 dilution (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour in the dark at room temperature. The wells were then washed with 1X PBS/0.1% Triton X-100 three times and counterstained with DAPI (0.5ug/mL) (ThermoFisher Scientific, Ontario, Canada) for 1 minute. Pictures were taken of five random fields of view at 10x magnification using the Leica DM 3000B Microscope and Infinity-3 Camera (Luminera). The differentiation index was calculated as the number of nuclei in myosin heavy chain positive cells divided by the total number of nuclei and the fusion index was counted as the number of myosin heavy chain positive nuclei divided by the number of myotubes. A myotube was defined as a fiber containing two or more nuclei.

2.7.2 Ki67

Ki67 staining was used to determine the percentage of proliferating cells in culture. Cells were seeded in 6-well plates and left in growth conditions for 24 hours. Cells were washed twice with 1X PBS and fixed in 2% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were then permeabilized with 1X PBS/0.3% Triton X-100 for 15 minutes at room temperature. 10% Goat Serum (GS)/0.3% Triton X-100 PBS was then used as a blocking solution for 30 minutes at room temperature, after which primary antibody (Ki67 – Abcam 15580; 1:100 dilution) diluted

in 2% GS/0.3% Triton X-100 PBS overnight at 4°C. The next day, cells were washed with 1X PBS/0.1% Triton X-100 three times and incubated with secondary antibody (594 conjugated donkey α -rabbit - Jackson ImmunoResearch, West Grove, PA, USA; 1:500) for 1 hour at room temperature. The wells were then washed with 1X PBS/0.1% Triton X-100 three times and counterstained with DAPI (0.5ug/mL) (ThermoFisher Scientific, Ontario, Canada) for 2 minutes. Pictures were taken of five random fields of view at 10x magnification using the Leica DM 3000B Microscope and Infinity-3 Camera (Luminera). Ki67-positive cells were scored and represented as a percentage of total nuclei.

2.7.3 BrdU

BrdU staining was used to determine the number of proliferating cells that have entered S-phase of the cell cycle. Cells were seeded in 6-well plates and left in growth conditions for 24 hours, after which they were pulsed with BrdU (10uM; Sigma-Aldrich, Oakville, ON, Canada) for 6 hours. The next day, cells were washed twice with 1X PBS and fixed in 2% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were then washed twice in PBS again and incubated with 95% methanol for 30 mins. Cells were washed twice with PBS and permeabilized with 1N HCl for 10 minutes on ice, followed by 2N HCl for 10 minutes at room temperature. Cells were incubated in sodium borate (pH 8.5) for 12 minutes at room temperature after which 5% goat Serum/0.1% Triton X-100 PBS was used as a blocking solution for 1 hour at room temperature. Primary antibody (BrdU – Abcam 2284; 1:250 dilution) diluted in 10% GS/0.1% Triton X-100 PBS was added and plates were incubated overnight at 4°C with agitation. The next day, cells were washed three times with PBS and incubated with secondary antibody (streptavidin Cy3- Sigma-Aldrich, Oakville, ON, Canada; 1:500) for 1 hour at room temperature. Cells were washed three times with PBS and counterstained with DAPI (0.5ug/mL) (ThermoFisher Scientific, Ontario,

Canada) for 1 minute. Pictures were taken of five random fields of view at 10x magnification using the Leica DM 3000B Microscope and Infinity-3 Camera (Luminera). BrdU-positive cells were scored as a percentage of total nuclei.

2.8 Telomerase Repeat Amplification Protocol

Telomerase activity was detected by the TRAP (Telomere repeat amplification protocol) assay using the TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's protocol. Briefly, 2×10^5 cells were harvested in which telomeric repeats (TTAGGG) were added to the 3' end of biotin-labelled primers. This was followed by product amplification by PCR using a thermocycler. For the ELISA reaction, the PCR product was denatured and hybridized using a digoxigenin (DIG)-labeled telomere repeat-specific detection probe. An anti-DIG antibody conjugated to peroxidase was then added to the hybridized PCR product. Peroxidase metabolizes 3,3',5,5' tetramethylbenzidine (TMB) which allows the detection probe to be visualized with a coloured reaction product. The absorbance was read at 450nm (with reference wavelength of 690nm) using a Synergy H1 multi-mode plate reader (BioTek Instruments Winooski, VT, USA).

2.9 Chromatin Immunoprecipitation (ChIP)

C2C12 myoblasts were retrovirally transduced to express C/EBP β or with empty virus (pLXSN) and grown under growth conditions. An equal number of cells (5×10^5) were plated and harvested two days later after crosslinking and ChIP was performed as previously described (F Marchildon et al., 2016) using 6ug of C/EBP β (C-19) (sc-150; Santa Cruz Biotechnology) and

normal rabbit IgG (Invitrogen, Carlsbad, CA, USA). Protein G conjugated Dynabeads (Invitrogen, Carlsbad, CA, USA) were used to precipitate immunoconjugates and DNA fragments were purified using the Qiaquick PCR purification kit (Qiagen Canada, Montreal, QC, Canada) as per manufacturer's protocol. DNA fragments were then subjected to RT-qPCR using primers to amplify the mouse *mTERT* promoter. The primer sequences used were: F (5'-AGGCGGCAGACCTCCTATAA -3') and R (5'-AGAGAGGGGCTGCTGAATTT-3').

2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). A two-tailed Student's t-test was used to compare a control and an experimental condition in one group. A one-way ANOVA followed by the software recommended post-hoc test was used to compare one factor in three or more groups. Statistical significance was always defined as having a p-value ≤ 0.05 .

3. RESULTS

3.1 The impact of C/EBP β -overexpression on mTERT expression in C2C12 myoblasts

C2C12 myoblasts were retrovirally transduced with an empty virus (pLX) or to overexpress full length C/EBP β and cultured in growth conditions for 24 hours. C/EBP β overexpression and mTERT protein expression were investigated by western blot. In four independent trials, C/EBP β overexpression resulted in the expression of both the LAP and LIP isoforms and an increase in mTERT protein expression as compared to empty vector controls (Fig 1A). Next, using RT-qPCR, the mRNA expression of *Tert* was investigated in cells overexpressing C/EBP β (Fig. 1B). Cells overexpressing *Cebpb* had approximately 3.5 times more *Tert* mRNA expression as compared to the empty virus controls (Fig 1B), consistent with a role for C/EBP β as a transcriptional regulator of *Tert* expression.

To determine whether C/EBP β could regulate *Tert* expression in differentiating myoblasts, C2C12 myoblasts overexpressing C/EBP β and empty vector controls were cultured in growth medium for 24 hours or in differentiation conditions for one day (differentiating myoblasts) or for four days (differentiated myotubes). As observed under growth conditions, *Tert* expression was significantly upregulated in C/EBP β -overexpressing cultures compared to controls both one day and four days after induction to differentiate (Fig 2).

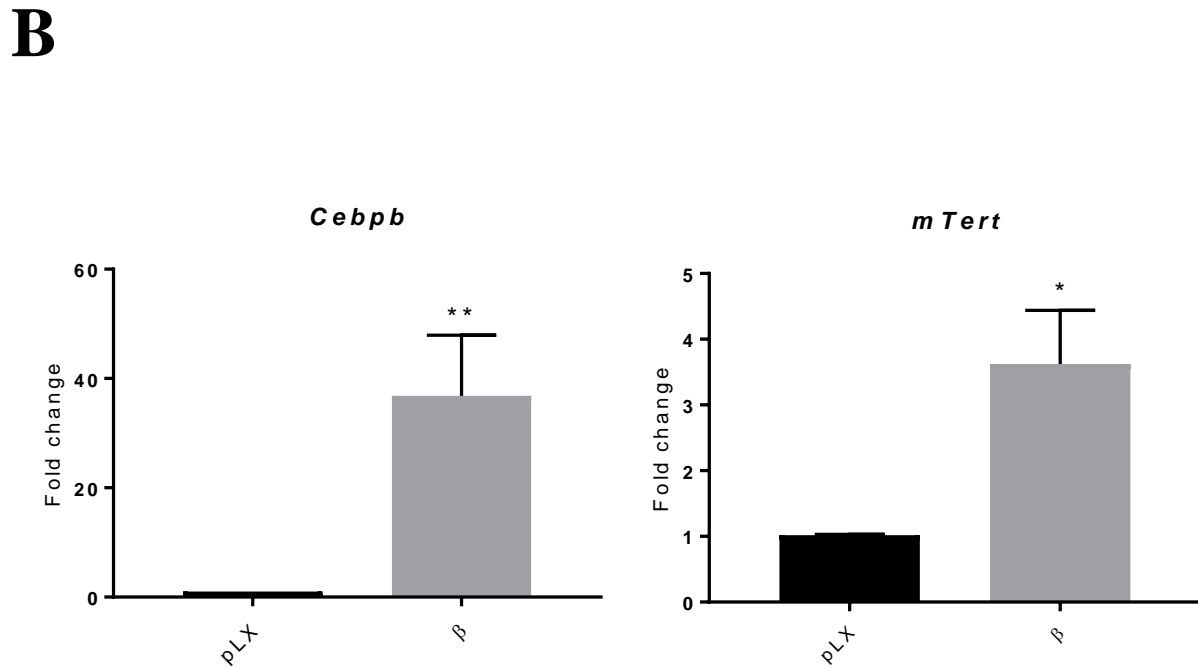
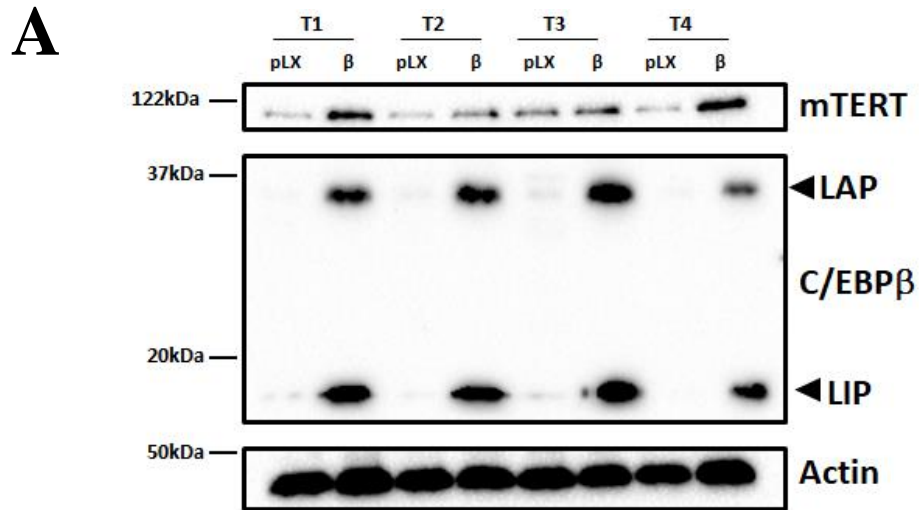


Figure 1: C/EBPβ upregulates mTERT expression. (A) Western blot of mTERT and C/EBPβ expression in C2C12 myoblasts retrovirally transduced to express C/EBPβ (β) or with empty virus (pLXSN). Cells were cultured in growth conditions (GM) for 24 hours and T1-T4 represent 4 independent trials. Actin is used as a loading control. (B) *Cebpb* and *Tert* mRNA expression as assessed by RT-qPCR in cells transduced and cultured as in (A). *p<0.05, n=6 (qPCR). Data is represented relative to pLX controls and error bars are the SEM.

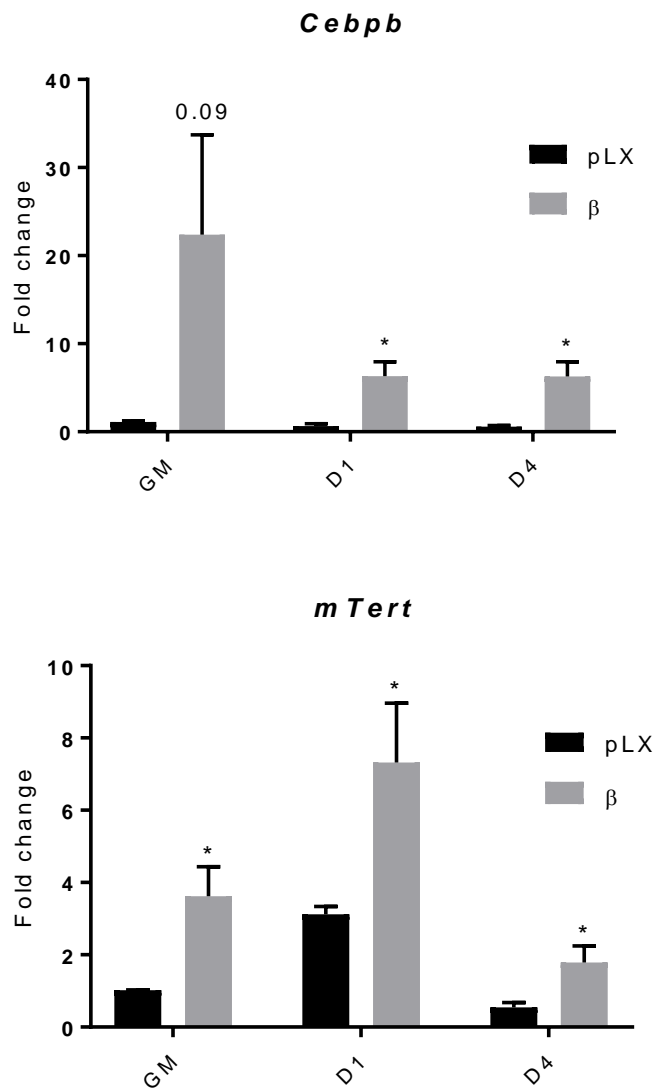


Figure 2: C/EBP β upregulates *Tert* mRNA expression under differentiation conditions. RT-qPCR analysis of *Tert* and *Cebpb* expression in C2C12 cells retrovirally transduced to express C/EBP β or with empty virus (pLXSN). Cells were cultured in growth conditions (GM) or differentiation conditions for 1 day (D1) or 4 days (D4). * $p < 0.05$, $n = 5$. Numbers on top of bars represent p values close to significance. Error bars represent SEM.

3.2 The effect of C/EBP β isoform overexpression on proliferation, TERT expression and activity

C2C12 myoblasts were retrovirally transduced to overexpress either the LAP or the LIP isoform of C/EBP β using two C/EBP β mutant vectors: pLXSN-C/EBP β Δ 21 (LAP) and pLXSN-C/EBP β _{LIP} (LIP). pLXSN-C/EBP β Δ 21, which drives overexpression the LAP isoform, by truncating the coding sequence to eliminate the first 21 amino acids. The pLXSN-LIP construct encodes a truncated *Cebpb* sequence coding for only the LIP isoform. When the LAP isoform (activating isoform) is overexpressed, an upregulation in *Tert* mRNA levels is observed, though this failed to achieve statistical significance (Fig. 3A). Overexpression of LIP, the dominant negative form of C/EBP β , did not affect *Tert* mRNA levels, which remained similar to the levels observed in the empty vector controls (Fig 3A). These trends were observed in both growth and differentiation conditions. As the C/EBP β LIP isoform is not detectable using the *Cebpb* primers used in RT-qPCR analysis to detect the LAP and full-length isoforms, a western blot was performed to confirm LIP overexpression (Fig 3B). While expression of the LAP isoform of C/EBP β results in an increase in mTERT protein expression, the expression of the LIP isoform decreased mTERT protein expression as compared to controls.

Next, to determine if overexpression of the LAP or LIP isoform influences cell growth characteristics, BrdU incorporation and Ki67 staining were performed. There was a significant decrease in the percentage of Ki67⁺ cells in LAP-overexpressing cultures as compared to empty vector controls, suggesting that C/EBP β -LAP can promote cell proliferation. Interestingly, expression of the LIP isoform also significantly decreased the percentage of Ki67⁺ cells, to a level significantly lower than that observed in LAP-overexpressing cultures (Fig 4A). While BrdU incorporation was not affected by expression of the LAP isoform, expression of the

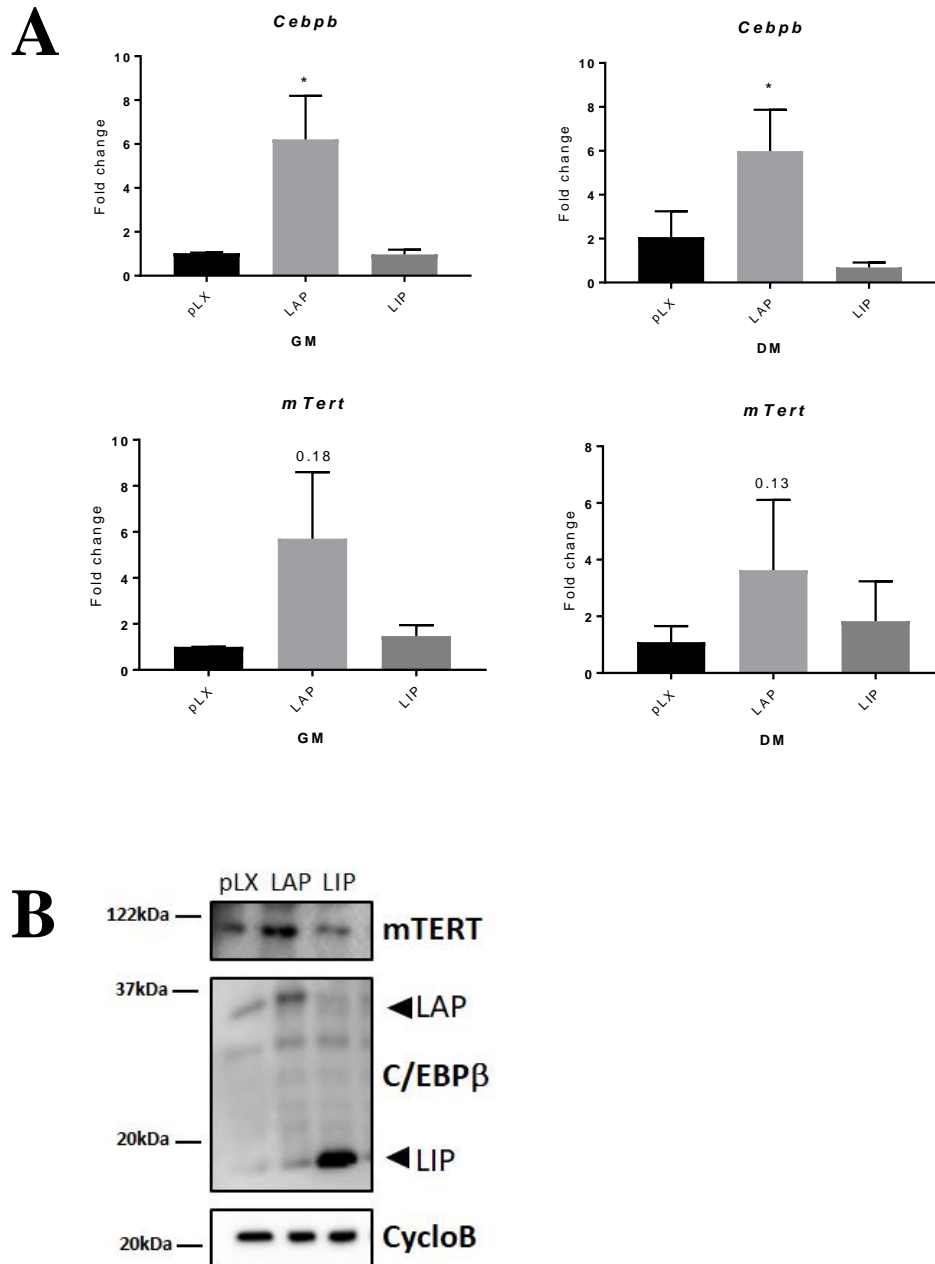


Figure 3: C/EBP β -LAP isoform increases mTERT expression whereas C/EBP β -LIP has no effect. (A) RT-qPCR analysis of *Cebpb* and *Tert* expression in C2C12 myoblasts retrovirally transduced to express the C/EBP β LAP or LIP isoform, or with empty virus (pLXSN). Cells were cultured in growth conditions (GM) or differentiation conditions (DM) for 24 hours. * $p < 0.05$, $n = 3$. Numbers on top of bars are p-values that are close to significance. Error bars represent SEM. (B) Western blot analysis of mTERT and C/EBP β protein expression levels in growth conditions (GM) in cells transduced as in (A). Cyclophilin-B (CycloB) is used as a loading control.

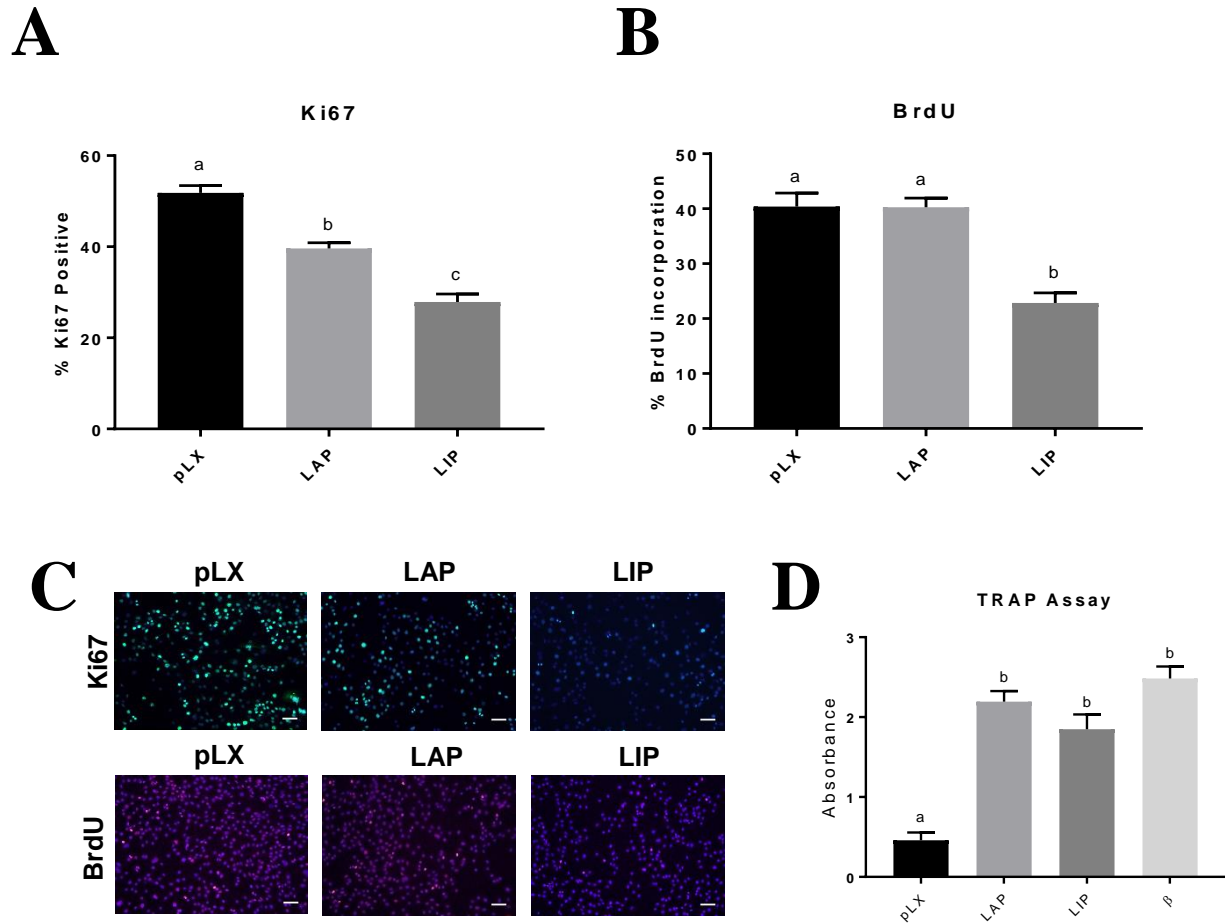


Figure 4: C/EBP β -LIP overexpressing myoblasts have significantly reduced proliferation but maintain high telomerase activity. (A) C2C12 myoblasts were retrovirally transduced with empty virus (pLXSN) or to express the LAP or LIP isoform. Cells cultured under growth conditions were immunostained with Ki67 antibody and counterstained with DAPI for visualization of nuclei. Data is represented as %Ki67+ cells relative to total nuclei, n=5. (B) Cells were plated and pulsed with BrdU for 6 hours overnight. The next morning, BrdU staining was performed on the same cells with DAPI as a counterstain. Bars are the %BrdU+ cells relative to total nuclei, n=5. (C) Representative pictures of Ki67 and BrdU from (A) and (B). Scale bars represent 100 μ m. (D) C2C12 myoblasts were retrovirally transduced with empty virus (pLXSN) or to express the LAP or LIP isoform or full-length C/EBP β . A TRAP (telomerase-repeat amplification protocol) assay was performed to detect telomerase activity by ELISA, n=3. Absorbance positively correlates with telomerase activity. For panels A, B, and D, bars marked with a different letter are significantly different from one another at a minimal p-value of <0.05. Error bars are the SEM.

LIP isoform resulted in significantly decreased BrdU incorporation, consistent with the decreased proliferative capacity of this culture (Fig 4B).

A TRAP assay was then performed on these cells to evaluate telomerase activity in the cultures. Expression of full length C/EBP β , the LAP isoform alone and the LIP isoform alone all resulted in a significant increase in telomerase activity compared to controls, despite very different levels of TERT protein expression (Fig. 4C).

3.3 The effects of C/EBP β knockdown on mTERT expression and activity

To evaluate the effects of C/EBP β knockdown on the expression and activity of TERT, primary myoblasts from C57BL/6 mice were isolated and lentivirally transduced to express a shRNA targeting mouse C/EBP β (shC/EBP β) or with a scrambled control sequence (shCTL). An RT-qPCR was performed to evaluate *Cebpb* and *Tert* expression levels (Fig. 5). There was a significant reduction in mRNA levels of *Cebpb* (**p<0.01) in the shC/EBP β cells compared to scrambled controls indicating that there was a robust knockdown in *Cebpb* expression (Fig 5). In cells expressing the shC/EBP β , *Tert* expression was significantly reduced compared to controls, suggesting that *Tert* expression requires C/EBP β (Fig 5).

To confirm the regulation of mTERT by C/EBP β in another model of C/EBP β knockdown in primary myoblasts, primary myoblasts from wild-type and C/EBP β conditional knockout mice (*Cebpb*^{fl/fl}*Pax7*^{CreER/+}) were isolated and *Tert* mRNA and mTERT protein expression was assessed (Fig 6). The C/EBP β conditional knockout mouse specifically excises *Cebpb* from muscle satellite cells by crossing mice with a *Cebpb*-floxed allele with mice bearing the Pax7-CreER allele. It was

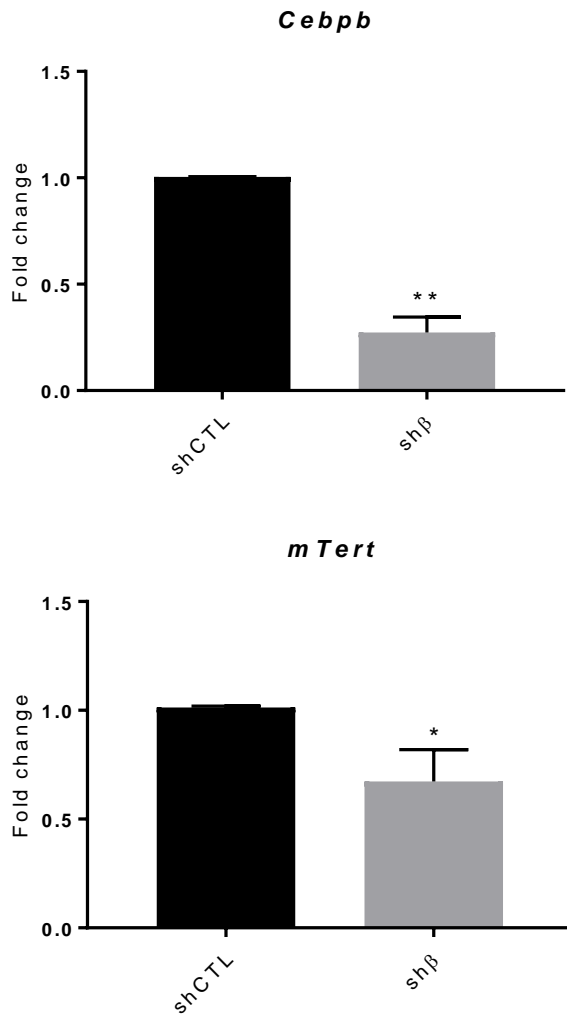


Figure 5: Knockdown of *Cebpb* leads to a significant decrease in *Tert* gene expression. RT-qPCR analysis of *Cebpb* and *mTert* expression in primary myoblasts lentivirally transduced to express shRNA against C/EBPβ (shβ) or a scrambled shRNA control (shCTL). Cells were cultured in growth conditions (GM) for 24 hours. *p<0.05, **p<0.01, n=4. Error bars represent SEM.

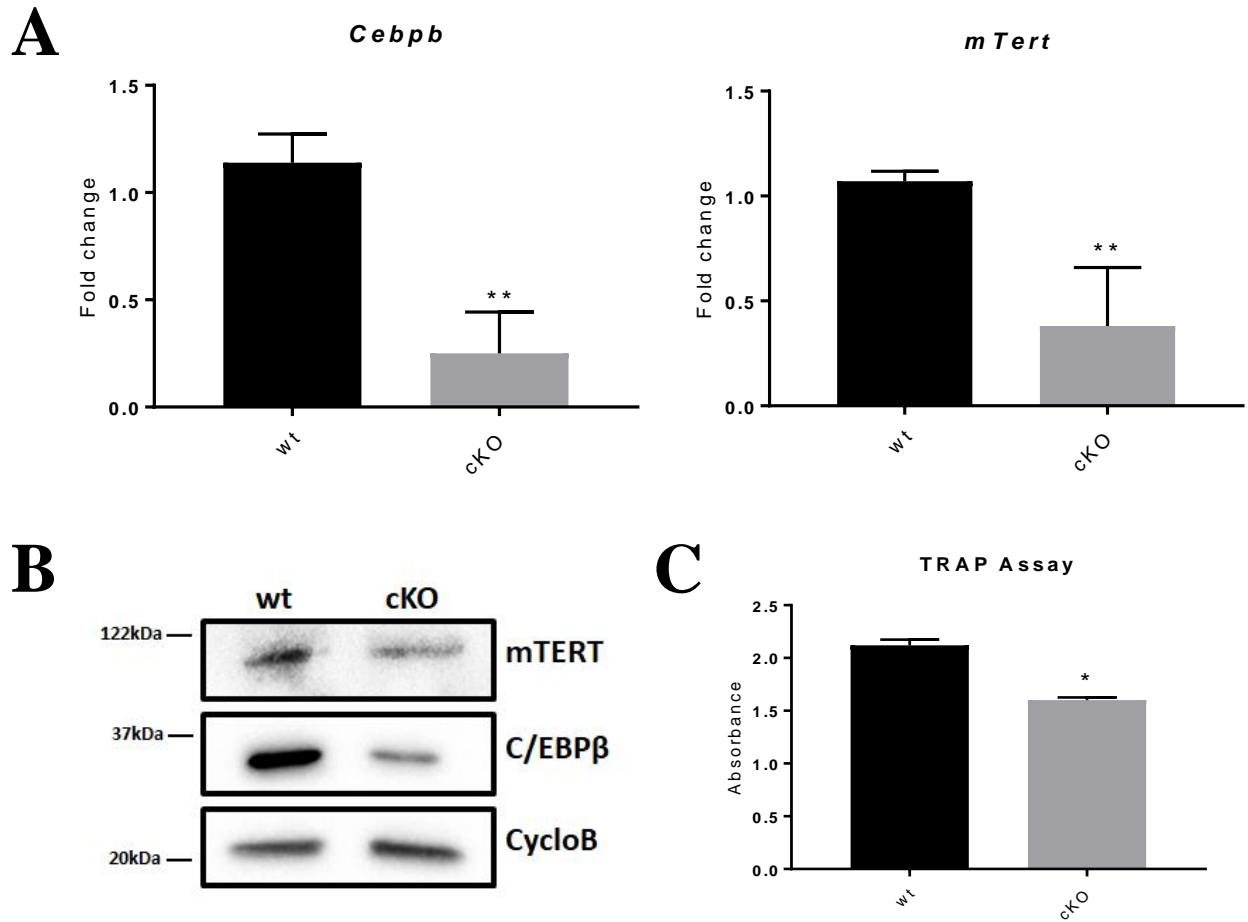


Figure 6: Conditional knockout of C/EBPβ in satellite cells leads to a significant decrease in mTERT expression and telomerase activity. (A) RT-qPCR and (B) Western blot analysis of mTERT and C/EBPβ expression from primary myoblasts isolated from wild-type (wt) or C/EBPβ conditional knockout mice (cKO). Cyclophilin-B (CyPB) is used as a loading control. ** $p < 0.01$, $n = 4$. Error bars represent SEM. (C) A TRAP (telomerase-repeat amplification protocol) assay was performed on isolated primary myoblasts from wild-type (wt) and C/EBPβ cKO mice to detect telomerase activity by ELISA. Absorbance positively correlates with telomerase activity. * $p < 0.05$, $n = 3$. Error bars represent SEM.

observed that in the *Cebpb*-deficient primary myoblasts, *Tert* mRNA expression was significantly reduced as compared to the wild-type controls (Fig 6A). Protein expression of mTERT was also significantly reduced in the *Cebpb*-deficient primary myoblasts as compared to the wild-type mouse controls (Fig 6B). *Cebpb*-deficient myoblasts also had significantly reduced telomerase activity compared to wild-type controls as evaluated using the TRAP assay (Fig 6C). Thus far, these results indicate that C/EBP β is a regulator of mTERT expression in skeletal muscle.

3.4 C/EBP β binds the *Tert* promoter

Chromatin Immunoprecipitation (ChIP) analysis was performed in C2C12 myoblasts retrovirally transduced to express C/EBP β or with empty virus (pLXSN) to determine if C/EBP β could occupy the *Tert* promoter. In the control cell line, C/EBP β occupancy was increased when specific pulldown was performed, but this failed to reach statistical significance. C/EBP β expression is known to be low in C2C12 myoblasts, and thus, the ChIP was also performed in myoblasts overexpressing C/EBP β . In C2C12 cells overexpressing C/EBP β , there was a significant increase in C/EBP β occupancy of the *mTert* promoter compared to pulldown with type-matched IgG control (Fig 7), suggesting that C/EBP β can occupy the *Tert* promoter and regulate transcription of *Tert* in muscle myoblasts.

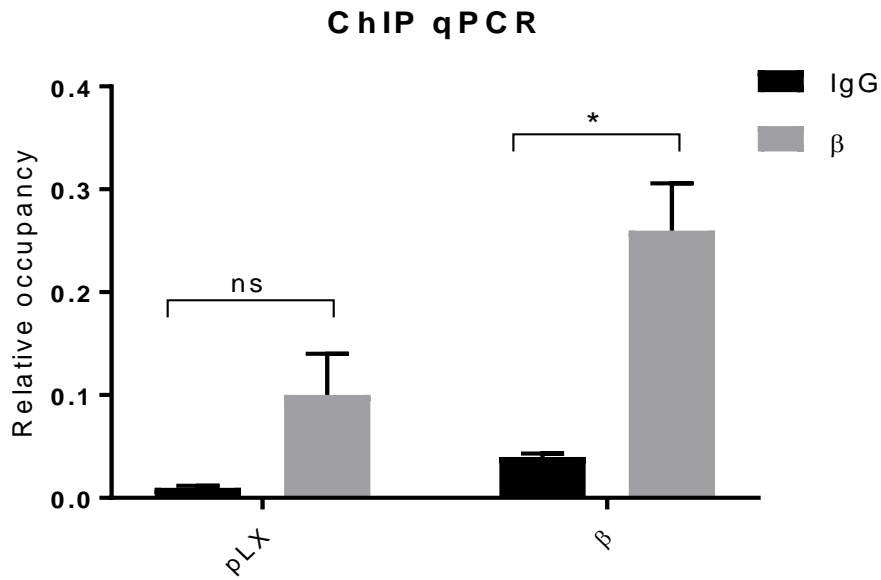


Figure 7: C/EBP β binds to the *Tert* promoter in skeletal muscle myoblasts. Chromatin immunoprecipitation analysis was performed in C2C12 myoblasts retrovirally transduced to express C/EBP β or with empty vector virus (pLX). ChIP analysis of C/EBP β occupancy on the *Tert* promoter is shown as the percent enrichment relative to 10% input used for immunoprecipitation for each condition. Cells were cultured in growth conditions (GM) for 24 hours and then collected for crosslinking. * $p < 0.05$, ns= no significance, $n=4$. Error bars represent the SEM.

3.5 TERT inhibition rescues myogenic differentiation and fusion in cells overexpressing C/EBP β

Given that overexpression of C/EBP β inhibits myogenic differentiation and results in higher mTERT expression and activity and that C/EBP β binds the mTERT promoter, it was hypothesized that C/EBP β blocks myogenic differentiation through the upregulation of mTERT expression. To determine if C/EBP β acts through mTERT to inhibit myogenic differentiation, a TERT inhibitor (TETA) was used to treat differentiating C2C12 cells that were retrovirally transduced with an empty virus (pLX) or to overexpress C/EBP β . While forced expression of C/EBP β inhibited myogenic differentiation and myoblast fusion, treatment with the telomerase inhibitor TETA resulted in a partial rescue of both, as evidenced by the development of large multinucleated myotubes expressing myosin heavy chain (Fig. 8A and B). This was further supported by RT-qPCR analysis of differentiation markers where a partial rescue of *Myh3* and complete rescue of *Myog* gene expression were observed in C/EBP β -overexpressing myoblasts treated with TETA (Fig. 8C). In addition, treatment of C/EBP β -overexpressing myoblasts with TETA did not compromise Pax7 expression levels as they remained significantly higher than pLX controls and did not statistically differ from C/EBP β myoblasts that were not treated with TETA (Fig 8C).

To confirm that C/EBP β acts, at least in part through mTERT to inhibit myogenic differentiation, control and C/EBP β -overexpressing C2C12 cells were lentivirally transduced to express a shRNA construct targeting mTERT (shTERT) or a scrambled control (shCTL). Again, while overexpression of C/EBP β significantly inhibited myogenic differentiation and fusion, the fusion defect was rescued with knock down of mTERT expression. While differentiation was also

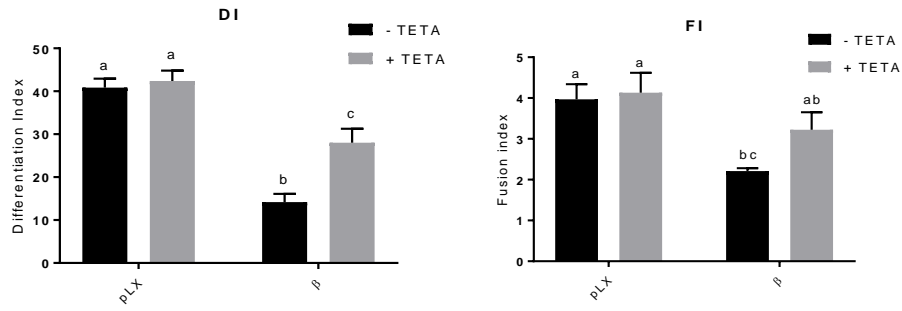
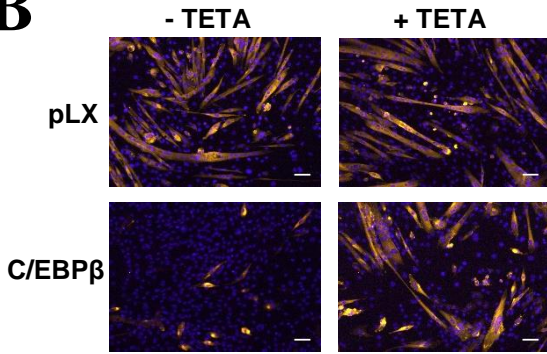
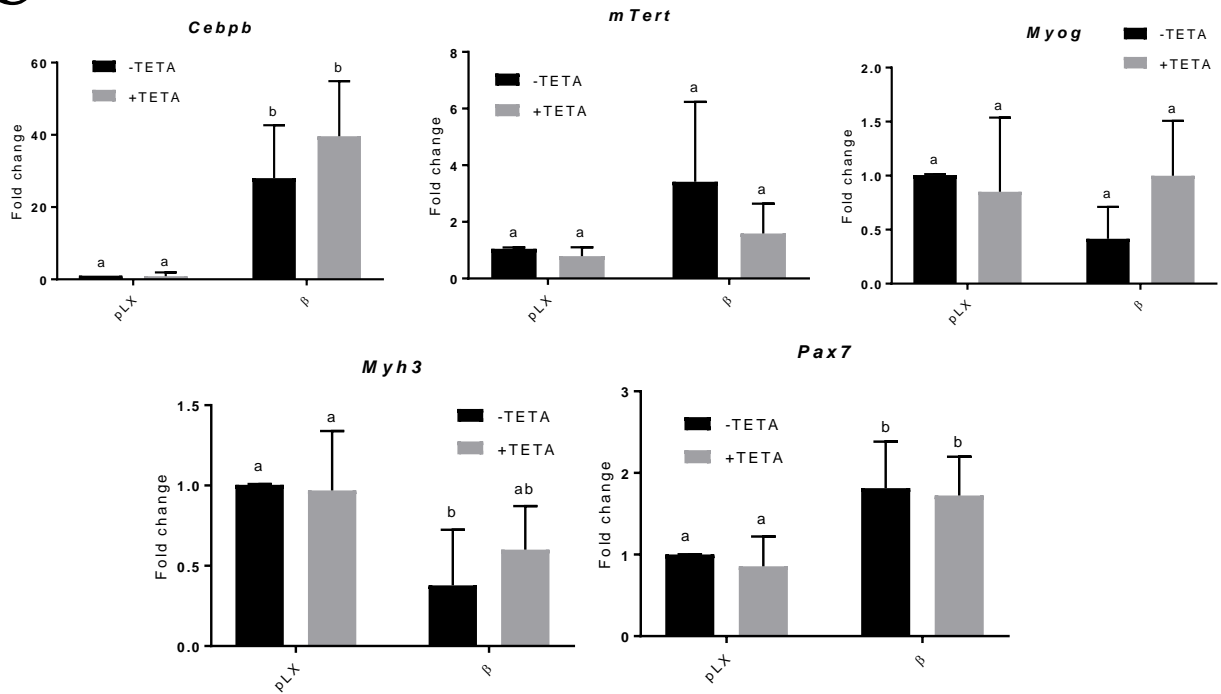
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Figure 8: Treatment of C/EBP β -overexpressing myoblasts with TETA partially rescues myogenic fusion and differentiation. (A) Differentiation and fusion index of differentiating C/EBP β -overexpressing C2C12 myoblasts (beta) and controls (pLX) treated with 100nM of TETA (TERT inhibitor) or vehicle control. (B) Representative pictures are shown where scale bars are 100um. (C) RT-qPCR analysis of *Cebpb*, *Tert*, *Myh3*, *Myog*, and *Pax7* expression in C2C12 cells retrovirally transduced and treated as in (A). Bars marked with different letter are significantly different from one another at a minimum significance of $p < 0.05$. Error bars are the SEM.

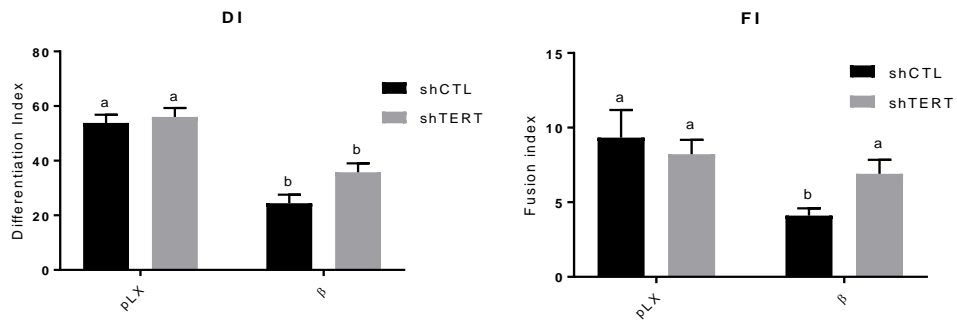
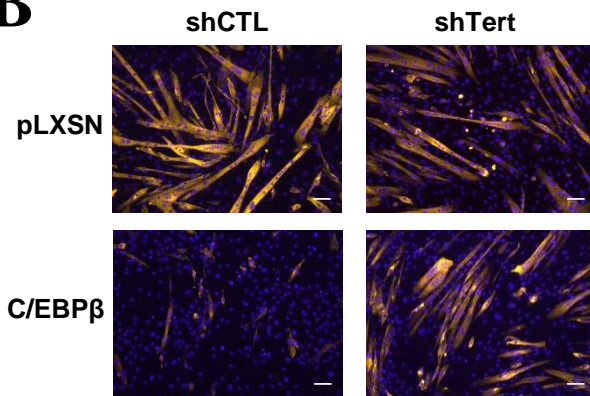
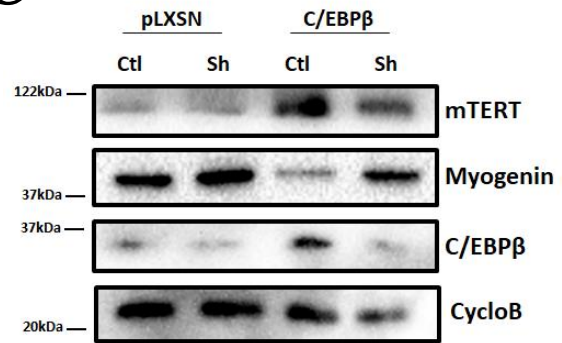
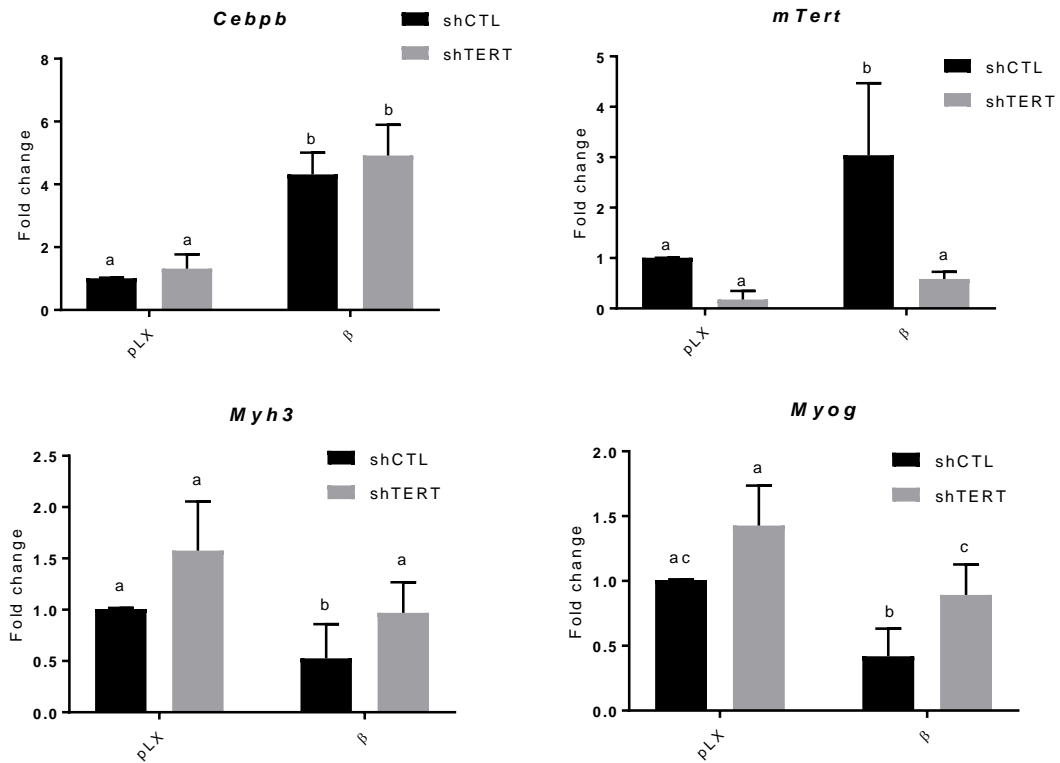
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Figure 9: Knockdown of *Tert* in C/EBP β -overexpressing myoblasts partially rescues myogenic differentiation and fully rescues fusion. (A) Differentiation and fusion index of differentiated C/EBP β -overexpressing and control (pLX) C2C12 myoblasts lentivirally transduced to express a shRNA against TERT (shTERT) or a scrambled shRNA control (shCTL). Bars marked with a different letter are significantly different from one another at a minimum significance of $p < 0.05$, $n = 3$. Error bars represent the SEM. (B) Representative pictures are shown and scale bars are 100 μ m. (C) Western blot analysis of the cells transduced and cultured as in (A). Cyclophilin-B (CycloB) is a loading control. (D) RT-qPCR analysis of *Cebpb*, *mTert*, *Myh3*, and *Myog* expression in the cells mentioned above. Cells were cultured in growth conditions (GM) and then induced to differentiate for 4 days. Bars marked with a different letter are significantly different from one another at a minimum significance of $p < 0.05$, $n = 4$.

increased in C/EBP β -overexpressing cultures when mTERT was knocked down, this failed to reach statistical significance (Fig. 9A and B). In support of this, western blot analysis revealed that myogenin protein expression was restored in C/EBP β -overexpressing cells that were mTERT-deficient (Fig. 9C). The knockdown of TERT was verified since C/EBP β /shTERT-expressing cells had higher levels of mTERT expression compared to pLX controls, but mTERT expression in this condition was attenuated compared to the C/EBP β /shCTL-expressing cells (Fig 9C). C/EBP β protein expression was very minimal in both the pLX/shCTL and pLX/shTERT controls as expected. C/EBP β was downregulated in the C/EBP β /shTERT-expressing cells compared to the C/EBP β /shCTL-expressing myoblasts (Fig 9C).

RT-qPCR analysis confirmed the overexpression of *Cebpb* and the knock down of *Tert* (Fig. 9D). *Myh3* gene expression was significantly downregulated in C/EBP β /shCTL cells compared to the pLX/shCTL, pLX/shTERT, and C/EBP β /shTERT cells which all did not statistically differ from each other (Fig 9D). *Myog* gene expression was also significantly downregulated in C/EBP β /shCTL cells, as seen in the Western blots. In the C/EBP β /shTERT cells, *Myog* expression was rescued to the same level as in the pLX/shCTL cells. *Myog* expression was highest in the pLX/shTERT cells, which showed a significant difference compared to both the C/EBP β /shCTL and C/EBP β /shTERT cells, but did not show a significant difference when compared to the pLX/shCTL cells (Fig 9D).

3.6 Knockdown of C/EBP β in rhabdomyosarcoma results in inhibition of hTERT and enhanced differentiation and fusion

To determine whether the C/EBP β -mTERT axis contributes to neuromuscular disease, the expression of C/EBP β and mTERT was assessed by western blot in two rhabdomyosarcoma cell

lines (RD and RH30) and normal human skeletal muscle myoblasts (HSMM). Both C/EBP β and hTERT protein expression were found to be higher in the cancer cell lines compared to the HSMM controls (Fig 10). The increase in C/EBP β and TERT is also correlated with higher Pax7 expression in the RD and RH30 cells (Fig 10).

Next, these cells were lentivirally transduced to express a shRNA against human C/EBP β (shC/EBP β) and subjected to differentiation conditions for 4 days to evaluate the effect of a reduction in C/EBP β expression on differentiation and TERT expression in the cancer cells compared to HSMM controls. Both the RD and the RH30 lines showed a decrease in hTERT expression and an increase in myogenin protein expression upon knockdown of C/EBP β (Fig. 11A), suggesting that loss of C/EBP β can promote myogenic differentiation in these cells.

Telomerase activity was also tested in these cells and the RD cell line demonstrated the most robust telomerase activity. In these cells, an inhibition of C/EBP β expression (RD sh β) leads to a loss of telomerase activity compared to the RD shCTLs, though this trend failed to reach statistical significance (Fig 11B). RH30 cells had very low telomerase activity and were comparable to the HSMM controls (Fig 11B), suggesting that telomerase is unlikely to be a mechanism driving cancerous growth in this model.

Myogenic differentiation in the RD cells demonstrated that knockdown of C/EBP β expression promoted myogenic differentiation without impacting fusion when compared to controls cells (Fig 11 B and C). This suggests that inhibition of C/EBP β expression in embryonic rhabdomyosarcoma cells leads to a loss of telomerase expression and activity and improved myogenic differentiation. By contrast, RH30 cells failed to form any MHC⁺ fibres in both control and treated conditions (data not shown). As mentioned above, this could mean that there is a mechanism independent of telomerase expression that causes these aRMS cells to be cancerous.

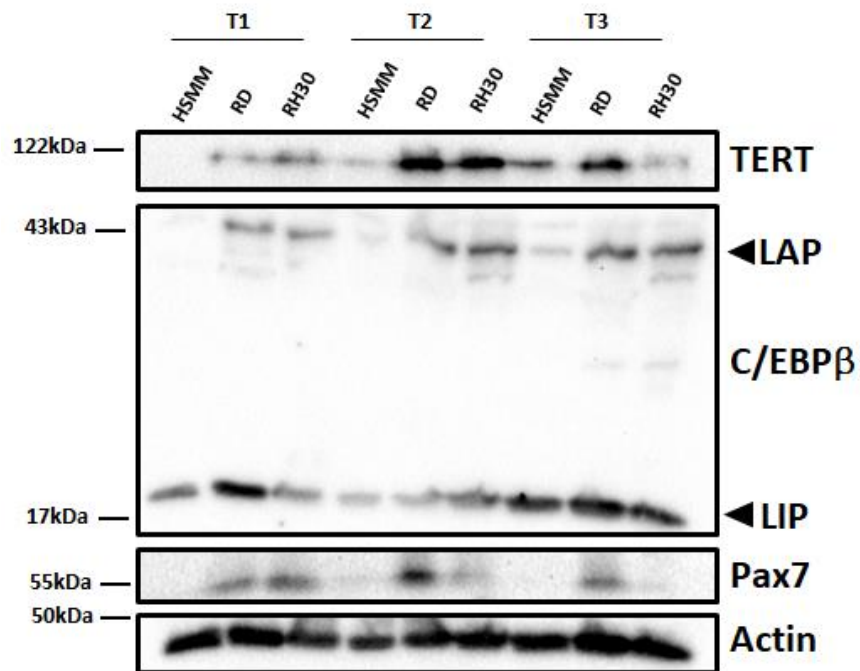


Figure 10: hTERT, C/EBP β , and Pax7 protein expression are increased in rhabdomyosarcoma cell lysates.

Western blot of three trials (T1-T3) of protein lysates extracted from human skeletal muscle myoblasts (HSM), RD cells (embryonic rhabdomyosarcoma cell line) and RH30 cells (alveolar rhabdomyosarcoma cell line). Actin is used as a loading control.

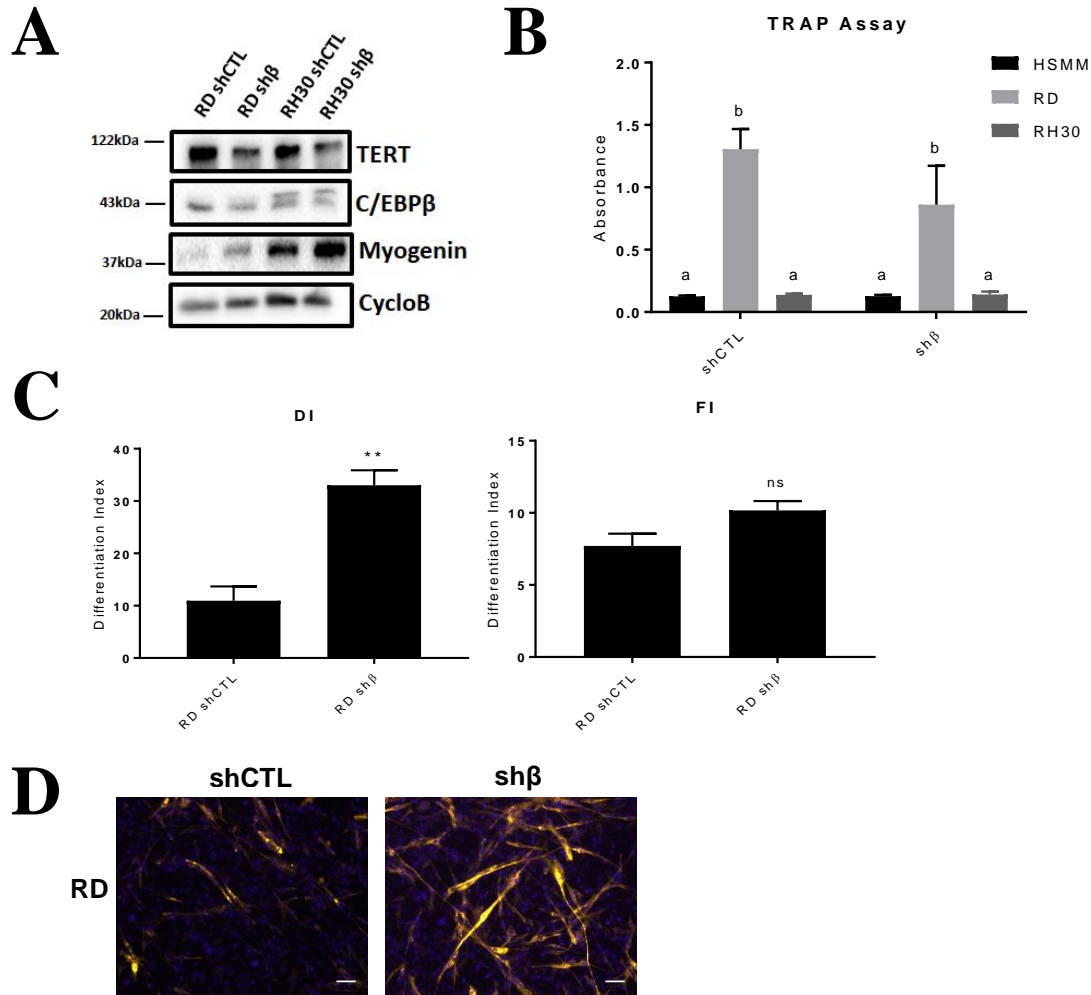


Figure 11: Knockdown of C/EBPβ in rhabdomyosarcoma cells enhances myogenic differentiation but not fusion. (A) Western blot analysis of protein lysates extracted from cultured RD and RH30 cells lentivirally transduced to express shRNA against human C/EBPβ (shβ) or a scrambled shRNA (shCTL). Cells were induced to differentiate for 4 days in low serum conditions. (B) Telomerase activity as measured by TRAP (telomerase-repeat amplification protocol) assay. Absorbance positively correlates with telomerase activity. Bars marked by different letters are different from one another at a minimum statistical significance of $p < 0.01$. Error bars are the SEM. (C) Differentiation and fusion index of RD cells lentivirally transduced to express shRNA against human C/EBPβ (shβ) or a scrambled shRNA (shCTL). Cells were cultured in growth conditions (GM) for 24 hours and then induced to differentiate for 4 days. (D) Representative pictures are shown and scale bars are 100μm.

4. DISCUSSION

4.1 Summary of findings

In this study, it was shown for the first time that C/EBP β is a regulator of TERT expression in skeletal muscle. In C/EBP β -deficient myoblasts, TERT expression and activity are downregulated and TERT deficiency can rescue differentiation in myoblasts overexpressing C/EBP β . In addition, knock-down of C/EBP β in rhabdomyosarcoma cells, where both C/EBP β and TERT expression are elevated, decreased TERT expression and activity, and enhanced differentiation. These findings describe the novel regulation of TERT expression in skeletal muscle and indicate that inhibition of C/EBP β could be an attractive therapeutic target for treatment of muscle diseases such as rhabdomyosarcoma.

4.2 Increasing C/EBP β and TERT expression could improve myoblast transplantation outcomes

The overexpression of C/EBP β in myoblasts promotes the expression of satellite cell markers and, when achieved pharmacologically using phosphodiesterase inhibitors, improves engraftment efficiency when transplanted into dystrophic muscle (Lala-Tabbert, Fu, & Wiperbergeron, 2016). Given that TERT is a downstream target of C/EBP β and that TERT expression is higher in stem cell populations, it is intriguing to speculate that some of the benefits of phosphodiesterase inhibitor treatment is the transient stimulation of TERT expression, promoting a reprogramming of myoblasts to a more stem cell-like state. Indeed, exogenous hTERT expression can immortalize muscle satellite cells without affecting their ability to normally differentiate (Di Donna et al., 2003). However, since hTERT expression is associated with various

types of cancers and can have oncogenic properties (Jafri, Ansari, Alqahtani, & Shay, 2016; Meyerson et al., 1997; Shay & Bacchetti, 1997; Wright & Shay, 2000), it cannot be used as an approach to promote satellite cell viability and differentiation potential in a therapeutic setting. Since it was found that increasing C/EBP β expression lead to an increase in TERT expression (Fig 1 and 2), it would be interesting to evaluate TERT expression in myoblasts after treatment with phosphodiesterase inhibitor and to determine the dependence of TERT expression on C/EBP β in this context. Further, as increased proliferation is a feature of phosphodiesterase inhibitor treatment of myoblasts, it would be interesting to assess whether this effect is dependent on TERT expression. If phosphodiesterase inhibitor treatment could transiently stimulate TERT expression through the upregulation of C/EBP β , this would provide a much safer treatment option for myoblasts compared to ectopic TERT.

4.3 The effects of C/EBP β isoforms LAP and LIP on TERT expression and activity

The LIP:LAP ratio of C/EBP β isoforms has been shown to be critical in many systems with an important role in growth and development (Diehl, 1998; Cynthia A Zahnow, 2009). Generally, LAP is known for its role as a transcriptional activator whereas LIP acts as a transcriptional repressor, since it lacks the activation domain. For example, it has been shown that an increase in the LIP:LAP ratio can lead to some aggressive forms of breast cancer (C A Zahnow et al., 2001; Cynthia A Zahnow, 2009). It was also found by a previous student that in *mdx* mice, the LIP:LAP ratio of C/EBP β isoforms increases compared to wild-type controls (Lee, unpublished data). In addition, the LIP:LAP ratio was found to increase in aged mice compared to younger controls (unpublished data). Interestingly, levels of TERT are also found to decrease with aging and in muscular dystrophy (Sacco et al., 2010; F. Zhang, Cheng, Wang, & Zhu, 2016). It would thus be

interesting to evaluate the correlation between TERT expression and activity and C/EBP β isoform expression in models such as muscular dystrophy, aged muscle, and cancer; all of which have been shown to have a misregulation of C/EBP β isoform expression. This would further solidify the relationship of C/EBP β and TERT in skeletal muscle and provide deeper insight into the specific roles of the protein isoforms of C/EBP β in these systems.

The role of the LAP and LIP isoforms of C/EBP β has been shown to be important in other systems. In bone for example, the LAP:LIP ratio is critical in the regulation of osteoclastogenesis and bone mass (Smink et al., 2009). It was shown that an increase in the LAP:LIP ratio, caused by inhibiting mTOR, induced the expression of MafB (Smink et al., 2009). MafB has previously been shown to be a negative regulator of osteoclast differentiation (K. Kim et al., 2007). In addition, LIP overexpression inhibited MafB and caused an increase in osteoclastogenesis (Smink et al., 2009). In skeletal muscle, a role for the individual C/EBP β isoforms remains to be elucidated. It was found that the LAP isoform increased *TERT* gene and protein expression, but the LIP isoform has no effect on either (Fig 3). In addition, LAP-expressing cells were more proliferative than cells expressing the LIP isoform as evidenced by BrdU incorporation and Ki67 analysis (Fig 4A and B). Taken together, this would support the fact that TERT expressing cells exhibit more proliferation. However, it was found that there was no difference in telomerase activity between cells overexpressing LAP and LIP and cells expressing full-length C/EBP β (Fig 4D). It would have been expected that LIP cells would have significantly reduced telomerase activity since the expression of TERT mRNA and protein are both reduced in these cells. This would be supported by the fact that LIP acts as a dominant negative transcription factor in many systems by binding and acting as a transcriptional repressor (Descombes & Schibler, 1991). These results, though

surprising, suggest that the regulation of TERT activity is complex, and does not necessarily correlate with expression of TERT protein as previously believed, at least in skeletal muscle.

4.4 Novel treatment options for rhabdomyosarcoma by pharmacological inhibition of C/EBP β and TERT

In this study, it was shown that TERT inhibition in C/EBP β -overexpressing cells via pharmacological treatment with TETA could rescue differentiation and fusion in these myoblasts (Fig 8). TETA treatment in C/EBP β -myoblasts was able to inhibit *mTERT* gene expression without affecting the expression of C/EBP β and Pax7 indicating that mTERT is downstream of C/EBP β and Pax7 (Fig 8). Though it was first used to treat Wilson's disease due to its copper-chelating properties (Lu, 2010), TETA has more recently been shown to inhibit telomerase activity by specifically decreasing the mRNA expression of TERT, consistent with what was seen in this study (Fig 8C) (Liu et al., 2008). Additionally, it can also induce senescence in some tumour cells (Liu et al., 2008). Though it has never been used in clinical trials, TETA has shown promising results in *in vivo* and *in vitro* pre-clinical studies by successfully inhibiting the growth of many tumour cells such as neuroblastomas, colorectal carcinomas, breast cancer cells (MCF-7), and fibrosarcomas (Kadowaki, Endoh, Okui, & Hayashi, 2009; J. Liu et al., 2008; Lixia, Fei, Jiajia, & Jianhui, 2007; Yoshii et al., 2001; Yoshiji et al., 2005). This inhibition of growth of the cancer cells was achieved through anti-angiogenic mechanisms, telomerase inhibition, and apoptosis. In C2C12 myoblasts that overexpress C/EBP β , TETA treatment caused a rescue in differentiation and fusion as evidenced by an increase in MHC⁺ fibers and *Myog* and *Myh3* gene expression (Fig 8). Since it can enhance differentiation and fusion and inhibit telomerase activity, it would be interesting to evaluate the effects of TETA treatment on the tumorigenicity of rhabdomyosarcoma

cells using a tumour xenograft mouse model. RMS cells would be treated *in vitro* with TETA or a vehicle control and then subsequently transplanted into immunocompromised NOD.CB17-*Prkdc^{scid}/J* mice using a subcutaneous injection. Tumour size and number would then be evaluated in both the vehicle and TETA treated xenografts to determine if TETA treatment resulted in decreased tumour size and/or number.

Rhabdomyosarcoma cells are unable to terminally differentiate and are stuck in a proliferative phase in which tumours develop throughout the skeletal muscle of the body (Arndt & Crist, 1999). The most common current treatment option for rhabdomyosarcoma is a multi-disciplinary approach involving surgery to remove the tumours, chemotherapy and radiation (Egas-Bejar & Huh, 2014). The chemotherapy consists of a combination drug known as VAC which is composed of three drugs: vincristine, actinomycin D, and cyclophosphamide (Egas-Bejar & Huh, 2014). These drugs inhibit cell growth by binding tubulin and preventing the separation of chromosomes during metaphase (vincristine), inhibiting transcription by competitively binding to DNA (actinomycin D), and forming irreversible DNA crosslinks and inducing apoptosis (cyclophosphamide). The five-year survival rate for RMS patients is around 82% for children, however, patients with aggressive forms of the cancer such as aRMS, patients in the late stages of the disease, or patients diagnosed in adulthood have a significantly lower five-year survival rates (Egas-Bejar & Huh, 2014; Sultan, Qaddoumi, Yaser, Rodriguez-Galindo, & Ferrari, 2009; Van Gaal, De Bont, Kaal, Versleijen-Jonkers, & van der Graaf, 2012). This reveals the need for novel, more specific treatment options. TETA treatment of RMS cells could potentially lead to the rescue of differentiation and fusion, as it did in the C/EBP β -overexpressing C2C12s. Though it has been used to treat various other types of tumours in pre-clinical studies, TETA would represent a novel treatment option for rhabdomyosarcoma. Thus, *in vitro* and *in vivo* studies involving the treatment

of RMS cells with TETA could provide more information regarding the possibility of the telomerase-mediated inhibition of growth in these cancer cells. TETA treatment did not however reduce the expression of C/EBP β as evidenced through RT-qPCR analysis of *Cebpb* mRNA expression (Fig 8). Similarly, knockdown of TERT using shRNA produced similar effects (Fig 9). In many cancers, C/EBP β expression is increased which leads to a more aggressive phenotype as seen in breast cancer, for example (Cynthia A Zahnow, 2009). Thus, pharmacologically inhibiting C/EBP β is an attractive option as it could directly inhibit C/EBP β while also theoretically indirectly inhibiting TERT expression.

TERT expression was shown to decrease when C/EBP β expression was knocked down using shRNA and in primary myoblasts isolated from a C/EBP β conditional knockout model (Fig 5, 6). In addition, in two models of rhabdomyosarcoma, knockdown of C/EBP β expression reduced TERT expression (Fig 11). This has large implications in cancer since telomerase-mediated cancer growth has been shown to be highly aggressive and difficult to treat (Balcom et al., 2001; Ceja-Rangel et al., 2016; C. Zhang et al., 2015). Knockdown of C/EBP β in the embryonic RMS cell line showed an increase in MHC⁺ fibers compared to RD cell controls which was correlated with a significant increase in the differentiation index, but not the fusion index (Fig 11C). RD cells are a commonly used model for embryonic rhabdomyosarcoma and have a very limited differentiation program. Since C/EBP β inhibition lead to an improvement in differentiation and a knockdown of TERT expression, pharmacological inhibition of C/EBP β is an appealing approach. It has recently been shown that helenalin acetate can act as a potent small-molecule inhibitor of C/EBP β in an acute myeloid leukemia (AML) cell line (Jakobs et al., 2016). AML is known to express high levels of C/EBP β , specifically the LAP* isoform. In this case, the LAP* isoform has a pro-proliferative role which is significantly inhibited when treated with helenalin acetate (Jakobs et al.,

2016). C/EBP β has a pro-oncogenic role in many other forms of cancer and it is interesting to speculate that part of this effect is mediated through TERT (Sankpal, Moskaluk, Hampton, & Powell, 2005; Xia, Zhang, & Ge, 2015; Zahnow, 2009). Both C/EBP β and TERT expression were shown to be elevated in both cell lines of RMS as compared to normal human myoblasts (Fig 10). Treatment of these cells with helenalin acetate and thereby reducing C/EBP β expression, could reduce TERT expression and promote cell cycle exit. This would lead to an improvement in differentiation and thus a potentially less aggressive cancer phenotype which would be of clinical benefit. Since the mechanisms of the inhibition of C/EBP β using this drug have not fully been elucidated, further studies would be beneficial to better understand this process. For example, since C/EBP β is expressed in several normal tissues, systemic delivery of a drug inhibiting C/EBP β could have various off-target effects. Indeed, it has been shown that a loss of C/EBP β promotes a loss of body fat, an increase in skeletal muscle insulin sensitivity, and impaired carbohydrate metabolism leading to hypoglycemia, for example (Croniger et al., 2001; Rahman et al., 2012; L. Wang et al., 2000). That being said, *in vitro* studies using helenalin acetate treatment in cell lines such as RD and RH30 could provide preliminary evidence that this therapeutic approach could be of benefit for the treatment of rhabdomyosarcoma.

4.5 Limitations

Though this study provides important information into the role of C/EBP β and TERT in myogenic differentiation, there are several study limitations to consider. Firstly, as mentioned in the introduction, one cannot neglect the fundamental differences in mouse and human telomere biology that could impact this study. The regulation of telomere length and proliferative senescence are mechanisms that lack evolutionary conservation, even between mice and humans

(Calado & Dumitriu, 2013; F. Zhang, Cheng, Wang, & Zhu, 2016). It has been found that many small mammals with short lifespans, such as rodents, have significantly longer telomeres in both somatic cells and stem cell populations compared to larger mammals with longer lifespans (Calado & Dumitriu, 2013). Mice are thus useful to understand basic mechanisms of telomere biology, but fail to provide in-depth insight into the role of telomeres in the human model of aging and disease. Though this may be the case, they do provide some important information into human disease for future studies. For example, *mdx* mice, which are a widely used mouse model for Duchenne muscular dystrophy, are known to have significantly shorter telomeres compared to wild-type littermates. These *mdx* mice have also been scrutinized for not properly modeling human DMD, since they experience a much milder phenotype that resolves after the crisis period. When *TR* (-/-) mice were crossed into the *mdx* background, these mice had a more severe disease phenotype that closely mirrored the characteristics of muscular dystrophy in humans. This suggests that telomere shortening may be critical in the DMD disease progression in humans and reveals the importance of telomeres in skeletal muscle proliferation and regeneration. An alternative to studying the *TERT* gene in mice to be applicable to human disease is creating transgenic mice that carry a reporter gene driven by the *hTERT* promoter. This has been performed and it was found to better recapitulate the expression and function of hTERT in human tissues, compared to studying mTERT and attempting to relate this to human disease (Horikawa et al., 2005).

C/EBP β was found to bind to the *mTERT* promoter in C2C12 myoblasts over-expressing C/EBP β (Fig 7). Though there was significant binding of C/EBP β to the *mTERT* promoter in C/EBP β -overexpressing C2C12s, there was also a difference seen in pLXSN control C2C12 cells where C/EBP β binding was increased compared to IgG controls (Fig 7). These results indicate that even endogenous levels of C/EBP β can bind to the *mTERT* promoter in skeletal muscle. However,

these experiments were performed in C2C12 myoblasts which are an immortalized cell line. Immortalized cell lines such as C2C12s are known to express higher levels of TERT compared to primary cells which could account for this effect (Holt, Wright, & Shay, 1996). ChIP analysis using primary myoblasts overexpressing C/EBP β would better recapitulate the endogenous regulation of this promoter *in vivo*.

Another limitation was found when studying the role of C/EBP β and TERT in rhabdomyosarcoma. RD and RH30 cells are commonly used rhabdomyosarcoma cell lines from an embryonic and alveolar origin, respectively (Hinson et al., 2013). Though these immortalized cell lines provide important information regarding the effects of knockdown of C/EBP β in myogenic differentiation, it would be useful to study the contribution of C/EBP β in human cells derived from rhabdomyosarcoma patient tissue samples. This would provide a more clinically relevant model for a better understanding of the potential *in vivo* effects of the knockdown.

4.6 Future directions

In order to better understand the relationship between C/EBP β and TERT, a C/EBP β ChIP analysis could be performed in RD and RH30 cells (human RMS cell lines) to confirm C/EBP β occupancy on the regulatory regions of *TERT* in human cells. The ChIP assay presented in this study was performed in the mouse which could significantly differ from the binding in human cells, especially since the regulatory regions of *TERT* are not conserved among mice and humans. Since C/EBP β and TERT expression were both elevated in RMS cells (Fig 10), they represent a useful model system to explore this relationship.

It was also found in this study that RD cells have improved differentiation when C/EBP β is knocked down via shRNA (Fig 11). RH30 cells were also evaluated but there was no appearance of MHC⁺ fibers in either the control or the shC/EBP β -expressing cells (data not shown). To better investigate loss of C/EBP β as a therapeutic avenue for rhabdomyosarcoma, experiments evaluating cell cycle exit could be performed on the RD and RH30 cell lines as a method of evaluating tumorigenicity. For myoblasts to commit to the differentiation program, they require permanent withdrawal from the cell cycle (Andrés & Walsh, 1996). Markers such as p21 and p57 could be evaluated by RT-qPCR and Western analysis as these genes are cell cycle inhibitors (P. Zhang et al., 1999). In addition, flow cytometry could be performed to evaluate the stage at which these cells are at in the cell cycle. Cell growth assays could also be performed to evaluate whether a loss of C/EBP β leads to a growth inhibition in the cancer cells which would be critical to clinical success.

4.7 Conclusions

This study provides evidence that C/EBP β is a regulator of TERT expression and activity in skeletal muscle. C/EBP β overexpression induced the upregulation of TERT expression and similarly, when C/EBP β is knocked down, TERT expression and activity are also reduced. In addition, TERT inhibition in C/EBP β -overexpressing C2C12s lead to a significant improvement in myogenic differentiation and fusion. C/EBP β and TERT expression were both found to be increased in embryonic and alveolar models of rhabdomyosarcoma. In response to this, a knockdown of C/EBP β in rhabdomyosarcoma cells was performed leading to a decrease in TERT expression and activity, and an enhancement of differentiation but not fusion in the embryonic cell

line. These findings reveal C/EBP β and TERT as attractive targets for future studies in treating muscle diseases such as muscular dystrophy and rhabdomyosarcoma.

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