Ex vivo expansion of skeletal muscle stem cells with a novel inhibitor of eIF2α dephosphorylation

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Abstract

Regeneration of adult skeletal muscle depends on rare skeletal muscle stem cells (MuSCs) that reside in a quiescent state underneath the basal lamina of the myofibre. The study, manipulation and use of MuSCs for cell-based therapies is hindered by their scarcity and the inability to expand them *ex vivo* under current culture conditions. We have shown that a general repression of translation, mediated by the phosphorylation of translation initiation factor eIF2α at serine 51 (P-eIF2α), is essential for maintenance of MuSC quiescence and self-renewal. MuSCs unable to phosphorylate eIF2α exit quiescence, activate the myogenic program and contribute to muscle differentiation, but do not self-renew or return to their quiescent state underneath the basal lamina of the myofibre. Here we show that pharmacological inhibition of eIF2α dephosphorylation by the novel small compound C10 permitted the expansion of MuSCs retaining capacity to regenerate muscle and self-renew after engraftment into a preclinical mouse model of Duchenne muscular dystrophy. We optimized culture conditions with C10 to facilitate a) passaging of MuSCs retaining regenerative capacity and b) genome editing of MuSCs with CRISPR/Cas9.

Résumé

La régénération du muscle squelettique adulte dépend d'une population rare de cellules souches musculaires (CSMs) qui résident normalement dans un état de quiescence sous la lame basale des fibres musculaires. L'étude, la manipulation et l'utilisation des CSMs dans le cadre de thérapies cellulaires sont entravées par leur rareté et l'incapacité de les amplifier ex vivo dans des conditions de culture normales. Nous avons montré qu'une répression générale de la traduction, médiée par la phosphorylation sur la sérine 51 du facteur d'initiation de la traduction eIF2α(P-eIF2α), est essentielle au maintien de la quiescence et de l'auto-renouvellement des CSMs. Les CSMs incapables de phosphoryler eIF2α quittent leur état quiescent, activent le programme myogénique et contribuent à la différenciation musculaire ; cependant elles ne s'auto-renouvellent pas ni ne reviennent à leur état quiescent sous la lame basale des fibres musculaires. Ici, nous montrons que l'inhibition pharmacologique de la déphosphorylation de eIF2α par la nouvelle petite molécule C10 permet l'amplification de CSMs qui conservent leur capacité de régénérer le muscle et de s'auto-renouveler une fois greffées dans un modèle murin préclinique de la dystrophie musculaire de Duchenne. Nous optimisons l'utilisation de C10 dans les conditions de culture pour faciliter a) le passage des CSMs tout en conservant leur capacité de régénération et b) l'édition du génome de ces mêmes CSMs par le système CRISPR / Cas9.

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List of Abbreviations

2OMeAO: 2-O-methylated phosphorothionated antisense oligoribonucleotides

^{3}H: Tritium

AAV: Adeno-associated virus

ALP: Alkaline phosphatase

ATP: Adenosine triphosphate

CLP: Common lymphoid progenitors

CMP: Common myeloid progenitors

CRISPR: Clustered regularly interspaced short palindromic repeats

Cas9: CRISPR associated protein 9

DMD: Duchenne muscular dystrophy

DML: Dorsal medial lip

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

EGF: Epidermal growth factor

eIF: Eukaryotic initiation factor

ES: Embryonic stem

FACS: Fluorescence-activated cell sorting

FGF: Fibroblast growth factor

GMP: Granulocyte/macrophage progenitor

HDR: Homology-directed repair

HSC: Hematopoietic stem cell

IGF: Insulin-like growth factor

IL: Interleukin

iPSCs: Induced pluripotent stem cells

LacZ: Gene for β-galactosidase

$m^{7}G$ cap: 7-methyl-guanosine cap

MACS: Magenetic-activated cell sorting

MAPK: Mitogen-activated protein kinases

MEP: Megakaryocyte/erythrocyte progenitor

miRISC: miRNA-induced silencing complex

MRF: Muscle-specific Regulator Factor

mRNA: Messenger RNA

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NHEJ: Non-homologous end-joining

NK: Natural Killer

NSC: Neural stem cell

PAM: Protospacer adjacent motif

PIC: Pre-initiation complex

RBP: RNA-binding protein

RG: Radial glial

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

sFRP: Secreted Frizzled-related protein

sgRNA: Single guide RNA

TALENS: Transcription activator-like effector nuclease

TC: Ternary complex

uORF: Upstream open reading frame

UTR: Untranslated region

α-SG: Alpha sarcoglycan

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Preface to the Thesis

This thesis is written according to McGill University Department of Human Genetics guidelines. This thesis is composed of four chapters. Chapter I is a comprehensive review of the literature relevant to this thesis. Chapter II is a presentation of results in manuscript format, and is in preparation for submission as a short report to the journal *eLife*. Chapter III is a discussion of the data presented in this thesis, while Chapter IV is a conclusion discussing future research directions.

Contribution of Authors

The work presented in this thesis was designed by Dr. Colin Crist. Solene Jamet and Dr. Ryo Fujita assisted with cell culture. Small molecules were designed and synthesized by Dr. Jean-Philip Lumb and Matt Halloran. Christian Young assisted with FACS and flow cytometry analysis. Krum Asiev assisted with irradiation on mice prior to engraftments. Jean Duchaine provided his plate reader for the AlphaScreen assay. I completed all other experimental work. Dr. Colin Crist, and I, analyzed the data and wrote the manuscript in Chapter II.

Chapter I: Introduction

1.1 Skeletal Muscle

Skeletal muscle represents the largest, and one of the most dynamic organs in the human body accounting for approximately 40% of total mass in humans. While we typically think of skeletal muscle in terms of its contractile ability enabling movement, its role in a variety of biological systems is diverse. Skeletal muscle metabolism represents a key regulator for energy expenditure, which has implications for common morbidities, such as the development of obesity, type-2 diabetes and sarcopenia (Kelley et al., 2002; Zurlo et al., 1990). Further, skeletal muscle is an important source of hormones for endocrine signalling referred to as "Myokines" (Pedersen and Febbraio, 2012). Myokines such as IL-6, IL-8 and IL-15 function in a variety of processes including angiogenesis, skeletal muscle anabolism, inflammation, and changes in muscle, adipose and liver metabolism (Pedersen et al., 2007). Changes in myokine expression represent both an acute and chronic signalling mechanism, as myokine levels react rapidly due to external stimuli, such as exercise (Pedersen et al., 2007).

Skeletal muscle consists of myofibres that provide the contractile machinery for the tissue. Myofibres are post-mitotic, poly-nucleated cells arranged in longitudinal bundles that form the basis of skeletal muscle. Each myofibre contains bundles of myofibrils, which house the contractile filaments required for muscle contraction and movement. These filaments are further classified into either thin or thick filaments. Thick filaments comprised of myosin proteins that contain the ATPase used to convert chemical energy into mechanical energy by moving the myosin head. By using ATP as an energy source, these thick filaments move along thin filaments, which are largely composed of actin, troponin and tropomyosin. Together these

filaments make up sarcomeres, which compress during muscle contraction and elongate during muscle relaxation. While all skeletal muscle possesses the same contractile machinery, muscle fibres can be broadly categorized into Type I (Oxidative) or Type II (Glycolytic) fibres based upon their metabolic activity. Type I fibres contain a much greater amount of mitochondria, thus utilizing oxidative metabolism as a primary energy source of ATP, whereas Type II have far less mitochondria and rely on glycolysis.

In order to initiate muscle contraction, a stimulus is provided in the form of an action potential from an efferent neuron. A single neuron will innervate multiple fibres at once, which is referred to as a motor unit. The neuron interacts with the muscle at the neuromuscular junction, where acetylcholine is released from presynaptic terminal, causing depolarization of the muscle fibre. This action potential is propagated along the cell membrane until it reaches dihydropiridine receptors along the T-tubules. These T-tubules lie near the terminal cisternae of the sarcoplasmic reticulum, and signal widespread activation of ryanodine receptors in the sarcoplasmic reticulum. The sarcoplasmic reticulum, which is analogous to the endoplasmic reticulum, is a large intracellular membrane-bound organelle responsible for the storage, release, and uptake of Ca^{2+} ions in the cytosol of the muscle fibre. Upon activation of ryanodine receptors, the sarcoplasmic reticulum releases large amounts of Ca^{2+} into the cytosol. Ca^{2+} bind to troponin C on the thin filaments, which cause a conformational change and allow for the binding of myosin heavy chain ATPase, and proper function of the sliding filaments of the sarcomere.

1.2 Skeletal Muscle Disease

1.2.1 Sarcopenia

Skeletal muscle is susceptible to a variety of diseases including sarcopenia, cachexia and muscular dystrophy. Sarcopenia is the age-related muscle wasting that typically begins around the sixth decade of life. This decline in muscle mass is the product of a loss of both muscle fibre size and muscle fibre count (Lexell et al., 1988). Since sarcopenia involves muscle wasting, the role of satellite cells (or muscle stem cells, MuSCs; discussed at length in Chapter 1.5) to regenerate muscle throughout the progression of the disease is of interest. Although there is conflicting evidence, studies have shown that in certain muscles there is an age-dependent decline in satellite cell number, and that aged satellite cells tend to stray from the myogenic program and differentiate into fibrotic tissues (Brack et al., 2005, 2007). In mice, there is strong evidence that satellite cell function declines with age. A decline in Notch signalling in aged mice is associated with reduced regenerative potential in satellite cells (Conboy et al., 2003). Consequently, when aged satellite cells are transplanted into the muscle of young mice, their regenerative potential is restored (Conboy et al., 2005). This suggests that the decline in satellite cell function may be due in part to changes in the satellite cell environment, referred to as the niche, as discussed in Chapter 1.5.3.

1.2.2 Cancer Cachexia

Cachexia in cancer patients results in the gross loss of both skeletal muscle and adipose tissue mass. Up to 20% of cancer-related deaths are due to cachexia, underlying the importance of understanding the mechanisms of muscle wasting in order to develop improved therapeutics (Tan and Fearon, 2008). Treatment for cancer cachexia has been hindered by the difficulty to

identify driving forces that create the complex, pro-inflammatory, hyper-metabolic environment influencing the muscle (Fearon et al., 2012). By analyzing transcriptome and metabolomic data from human myotubes exposed to cachexic serum, Fukawa et al. (2016) demonstrated that pro-inflammatory cytokines triggers excessive fatty acid oxidation, resulting in elevated amounts of ROS. This elevation in ROS triggers p38 MAPK signalling in the muscle and primary myoblasts, which can be reversed with pharmacological inhibition of fatty acid oxidation (Fukawa et al., 2016). Interestingly, p38 MAPK signalling has been implicated in the loss of satellite cell self-renewal in aging mice, suggesting that a similar mechanism of impaired self-renewal is driving muscle atrophy in cancer cachexia (Bernet et al., 2014). Further, satellite cell differentiation is inhibited by maintained Pax7 expression via the increased NF-κB signalling present in cachexic serum (He et al., 2013). In parallel, these studies demonstrate that satellite cells in cachexic muscle activate, but are impaired in the differentiation steps required to regenerate muscle. Therefore, muscle wasting associated with cancer should be viewed as not only a disease of the muscle, but also a disease of the satellite stem cells within the muscle.

By understanding regulatory pathways controlling myogenesis, new therapeutic strategies can be developed to target these satellite cell deficiencies. Supporting this, Zhou et al. (2010) have shown that ActRIIB, a receptor for TGF-β family of ligands commonly present in cachexia, is required for muscle wasting in cachexic mice. Blocking ActRIIB with an antagonist resulted in a return to baseline or increase in muscle mass (Zhou et al., 2010). This restoration of muscle is due to an increase in satellite cell differentiation during cachexia, suggesting that inhibition of ActRIIB is sufficient to overcome the satellite cell differentiation impairment in cachexia. (Zhou et al., 2010). These studies suggest that the treatment of cancer cachexia is a muscle and muscle

stem cell disease associated with cancer, and thus treatments should continue to focus on overcoming impairments in satellite muscle stem cells.

1.2.3 Muscular Dystrophy

Muscular dystrophies are a family of genetic diseases known to elicit progressive weakness and muscle wasting throughout life. The most prevalent and widely studied is Duchenne muscular dystrophy (DMD), which affects 1 in 5000 male births and is caused by non-sense mutations in the *Dmd* gene (Romitti et al., 2015). *Dmd* is the largest gene in mammals and encodes for the dystrophin protein. Dystrophin is an integral component of the dystroglycan complex, which anchors the sarcolemma of the muscle fibre to the basal lamina. Without functional dystrophin protein, as in cases of DMD, muscle fibres begin to waste and are gradually replaced with adipose and connective tissue, resulting in the severe muscle weakness and frailty common in patients with DMD. Due to the persistent degeneration of muscle fibres, DMD can be viewed as an inflammatory disease. NF-κB signalling from macrophages is at least in part responsible for the obstructed muscle regeneration in mice with DMD (Acharyya et al., 2007). Despite the evidence, anti-inflammatory agents such as glucocorticoids show little, or no improvement in functional capacity (Manzur et al., 2008). Currently, there are no effective treatments or cures for patients with DMD.

When observing cross-sectional slices of tibialis anterior muscle from the Dmd^{mdx} mouse model of DMD, rare reverted dystrophin+ fibres can accumulate throughout a mouse's lifetime. These reverted fibers arise by skipping over the mutated exon, resulting in restoration of the original reading frame. The resulting dystrophin protein from these exon-skipped transcripts produce a truncated, yet functional, protein similar to those found in Beckers Muscular

Dystrophy – a much milder disease compared to DMD. Based on this principle, Lu et al. (2003) demonstrated that they could produce dystrophin-expressing fibres in *Dmd*^{mdx} mice Using 2-O-methylated phosphorothionated antisense oligoribonucleotides (2OMeAO) to facilitate exon skipping over the mutated exon-23. This study demonstrated that exon-skipping represents a viable strategy to produce a functional dystrophin protein for the treatment of DMD. However, because the mutation still persists in the genome, using 2OMeAO is limited by the duration in which they are administered.

Therefore, genetic correction of the mutated *Dmd* gene represents the only permanent cure for DMD. With precise gene-editing tools such as CRISPR/Cas9 available, the focus of recent work has been to excise the mutated *Dmd* exon in order to induce permanent exon-skipping. This has now been achieved in mice, by using adeno-associated virus (AAV) vectors to deliver CRISPR/Cas9 with sgRNAs targeting 5' and 3' of the affected exon (exon-23 in *Dmd*^{mdx} mice) (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). By creating double stranded breaks surrounding exon-23, the exon was permanently removed resulting in an in-frame dystrophin protein lacking the dispensable exon-23 coding region. The same strategy was used to restore dystrophin expression in the *Dmd*^{mdx4cv} mouse, which harbours a mutation in exon-53 (and contains fewer reverted fibres) with similar success (Bengtsson et al., 2017). However, satellite cells are not transduced efficiently by AAV vectors, and only one study demonstrated that host satellite cells were transduced, and at extremely low efficiency (Tabebordbar et al., 2016). While there are advantages to systemic delivery of CRISPR/Cas9 using AAV to restore dystrophin expression in muscle tissue, targeting the satellite cells is critical to establish a pool of healthy stem cells that respond to future bouts of regeneration. Additionally, intravenous administration of AAV poses risk for adverse immune responses, increasing the risk of the

procedure. Thus, more efficient ways to target satellite cells for gene editing, either by improving the efficiency of *in vivo* transduction, or by editing satellite cells *ex vivo* prior to engraftment is required.

1.3 Skeletal Muscle Development

Muscle can be divided into skeletal (Striated), smooth or cardiac muscle based on their location and function. During development, skeletal muscle originates from a region of the mesoderm that flanks the neural tube called the paraxial mesoderm. More specifically, a segmented region of the paraxial mesoderm further develops into epithelial buds called somites, that lie along the dorsal side of the developing embryo, laterally to the neural tube. The ventral portions of the somites differentiate into the sclerotome, which forms the mesenchyme, producing the bones and cartilage. The dorsal portion of the somites mature to form the dermomyotome and medially located myotome that gives rise to skeletal muscle. The spatial setting of cells in the dermomyotome determine which tissues they will eventually develop. The dorsal medial lip (DML) of the dermomyotome develop into the deep back muscles, while the ventral lateral lip (VLL) of the dermomyotome will develop into the myogenic progenitors making up the limb buds (Pownall et al., 2002). With the exception of the head and neck muscles, during development the somites produce all skeletal muscle in the body (Christ and Ordahl, 1995).

Multipotent progenitors that give rise to skeletal muscle within the early dermomyotome are marked not only by *Pax3*, but also by the *Pax3* paralogue *Pax7*. As discussed in Chapter 1.5.1, *Pax7* expression is eventually associated with the identification of adult muscle stem cells. However, in early embryogenesis, loss of *Pax7* does not effect muscle development, whereas

Pax3 mutants result in the loss of the hypaxial dermomyotome and failure to develop organized body muscle (Relaix et al., 2005). Further, double mutants of *Pax3* and *Pax7* produce normal muscle development until E10.5, followed by a severe impairment of differentiated muscle cells in later time points (Relaix et al., 2005). *Fgfr4* and *Sprouty1* are genetically downstream of *Pax3* and have and have been shown to regulated cell fate determination (Lagha et al., 2008). *Fgfr4* promotes myogenesis, while *Sprouty1* likely is a feedback inhibitor of *fgfr4*, which reduces precocious differentiation (Lagha et al., 2008). Since both downstream targets are driven, at least in part, by the expression of *Pax3*, these data demonstrate another control mechanism in which *Pax3* regulates between progenitor maintenance and myogenic differentiation.

Pax3 is genetically upstream of the four primary muscle-specific regulatory factors (MRFs): *Myf5, MyoD, Mrf4 (Myf6)*, and *Myogenin*, which are temporally regulated throughout muscle differentiation. Pax3 and Myf5 are required to activate the chief myogenic determining factor *MyoD*, as a double knockout of *Pax3* and *Myf5* result in a loss of *MyoD* and subsequent failure to develop trunk muscle (Tajbakhsh, et al., 1997). While the loss of trunk muscle observed in Myf5 null mice is partially rescued by MyoD overexpression, the double knock out of *Pax3* and *Myf5* cannot be. This underlines the role of *Pax3* in activating *MyoD*, and likely many other factors during myogenesis. Further, there is evidence that *Pax3* is also genetically upstream of *Myf5*, as it activates expression of *Dmrt2*, which has been shown to activate *Myf5* expression by binding to its upstream enhancer in epaxial muscle (Sato et al., 2010). In contrast, in the hypaxial dermomyotome, Pax3 directly activates *Myf5* by binding to a 145bp element upstream of *Myf5* (Bajard et al., 2006). In addition to *Pax3*, the Six family of proteins, specifically Six1 and Six4, also activate *Myf5* by binding to a conserved MEF3 site in the same upstream element as Pax3 (Giordani et al., 2007). Six proteins function by forming a complex

with the Eya family of proteins to activate transcription at the target site. As well as regulating *Myf5* with Pax3, *Six1/4* are also genetically upstream of *Pax3* itself, evidenced by the loss of Six1/4, or corresponding Eya proteins, result in a loss of *Pax3* expression (Grifone et al., 2005). Continued expression of Six1/4 in myocytes is required for the development of fast-type muscle fibres, while Blimp-1 expression in response to hedgehog signalling is required for the development of slow-type muscle fibres (Baxendale et al., 2004; Niro et al., 2010).

Further evidence that MRFs are activated in a hierarchal manner is demonstrated using the *Myf5* knockout mouse, which also reduces the expression of *Mrf4* due to cis-elements. These mice lose their *MyoD* expression and subsequent myogenic potential drastically. However, if *Myf5* is deleted without altering Mrf4 expression, MyoD is regained, suggesting that Mrf4 is sufficient to activate *MyoD* and drive myogenesis (Kassar-Duchossoy et al., 2004). This study also demonstrated that *Mrf4* not only drives the myogenic cell fate through activation of *MyoD*, but also independently, by the observation of developing skeletal muscle in $Myf5^{-/-}$; $MyoD^{-/-}$ mice, but not when *Mrf4* is absent. *Myogenin* is critical for the terminal differentiation of myoblasts, as evidenced by presence of un-differentiated myoblasts and severe muscle deficiency in *Myogenin* knockout mice (Hasty et al., 1993). When transcriptional control of *Myogenin* is under the promoter of a myogenic determining factor such as *Myf5*, *Myogenin* is unable to fully restore muscle formation (Wang and Jaenisch, 1997). This provides further evidence that myogenesis is a temporally control hierarchal process, in which *Myogenin* has a defined role as a terminal differentiation factor. Therefore, MRFs can be classified into myogenic determining factors, *MyoD, Myf5 and Mrf4* and terminal differentiation factors, *Mrf4* and *Myogenin*.

After *Myogenin* is expressed and cells have begun to differentiate, they fuse in a three-step process. First, they adhere to neighbouring myogenic cells, in a process regulated by a number of cell surface proteins. Some genes involved in the recognition of adhering partners include Jamb and Jamc, where mutations in these genes result in mononuclear myotubes (Powell and Wright, 2011). Jamb and Jamc recognize and bind one another to activate downstream signalling. Interestingly, both cells must express Jamb and Jamc in order to adhere properly, suggesting that cells require downstream signalling from both membrane bound proteins (Powell and Wright, 2011). The renal protein Nephrin has been implicated in the adhesion process, as C2C12 cells fail to fuse when Nephrin is absent (Sohn et al., 2009). Further, the extracellular matrix protein, Beta1-integrin, regulates adherence, as Beta1 knockout myoblasts fail to fuse in differentiation medium (Schwander et al., 2003). Loss of Beta1 results in the loss of membrane localization of other cell fusion proteins, such as CD9, suggesting that Beta1 is involved in a protein complex that regulates adherence.

Following recognition of fusing partners, cells have to be brought closer together so that their membranes can begin to fuse. This process is asymmetric, in that there is an attacking cell, known as a fusion-competent myoblast, which projects finger-like projections into the receiving cell, known as the founder cell. These finger-like projections are due to actin reorganization to create F-actin-enriched podosome-like structures (PLS) that invade the founder cell (Sens et al., 2010). In response to the mechanical stress from the PLS, the founder cell expresses Myosin II (MyoII)-mediated mechanosensory response that induces fusion pore formation (Kim et al., 2015). Following pore formation, the presence of phosphatidylserine (PS) plays a role in destabilizing the plasma membranes to allow for fusion (van den Eijnde et al., 2001). PS acts on the cell surface receptor BaI1, which is associated with phagocytosis of apoptotic cells (Park et

al., 2007). Interestingly, myoblasts in culture require the presence of apoptotic cells in order to secrete PS, while removing any dying cells or blocking BaI1 in culture resulted in greatly reduced fusion (Hochreiter-Hufford et al., 2013).

In 2013, Eric Olsen and colleagues (Millay et al., 2013) identified the gene now known as *Myomaker*, named for its significant role in myoblast fusion. Mice lacking in a functional *Myomaker* gene are not viable after birth, and are completely void of skeletal muscle. *In vitro*, overexpression of *Myomaker* in C2C12 cells results in massive myotubes with significantly more nuclei compared to controls, while knocking out *Myomaker* results in differentiated mononuclear myotubes, nearly void of polynucleated myotubes. Finally, ectopic expression of *Myomaker* in fibroblasts caused fusion into myotubes, demonstrating the driving power of *Myomaker* to induce cell fusion.

1.4 Somatic Stem Cells

Adult tissues with the capacity to regenerate do so by virtue of their somatic stem cells. Somatic stem cells are tissue-specific cells that give rise to the differentiated cells of the tissue within which they reside, while retaining their capacity to self-renew in order to cope with future bouts of regeneration. This phenomenon is first described in the context of blood, where James Till and Ernest McCulloch noted that red blood cells undergo quick turnover rates and lack a nucleus, and therefore must be sustained by a pool of proliferating cells (Till and McCulloch, 1961). By analyzing colonies formed in the spleen after intravascular injection of marrow-derived cells (Later known as hematopoietic stem cells), Till and McCulloch could observe that these cells are capable of proliferation and differentiation. Shortly after, they further characterized the blood system into three components: 1) The stem cell compartment that is

capable of proliferation or differentiation, 2) A more committed stage with limited capacity to proliferate and 3) the fully differentiated erythrocyte that serves its functional role, and has lost the capacity to proliferate (Till et al., 1964). This model not only describes the fundamental characteristics of a stem cell to have the capacity to differentiate and self-renew, but also demonstrates the heterogeneity and concept of differentially committed stem cells.

Much of the work by Till and McCulloch had built upon previous work by Yves Clermont and Charles Phillippe Leblond on rat spermatogonia. Clermont and Leblond coined the "Stem Cell Renewal Theory" to describe the process in which spermatogonia are replenished throughout an organism's lifetime. In their work, they describe spermatogonia as existing in type A, B, and intermediate type cells based on cell morphology (Clermont and Leblond, 1953). Further, they demonstrate that a small portion of mitotic type A cells are largely "Dormant", and when they divide, produce multiple intermediate type cells, and produce a new dormant type A cell (Clermont and Leblond, 1953). Stem Cell Renewal Theory laid the foundation for later work on stem cell quiescence, self-renewal and asymmetric divisions.

Tissue specific stem cells differ in their potency and capacity to regenerate after injury. For example, the blood represents the most robust model of regeneration in the body, as blood cells are restored at a rate of $\sim 10^7$ cells/second in adults (Ogawa, 1993). At the source of this regeneration lies the hematopoietic stem cell (HSC), which gives rise to all of the cell types within the blood. However, as Till and McCulloch postulated, it does this through committed progenitors that have defined lineages in which they can differentiate. HSCs give rise to a heterogeneous population of multipotent progenitors that have limited ability to self-renew (Morrison and Weissman, 1994). The first clear branch for blood cell commitment is when multipotent progenitors differentiate into common myeloid progenitors (CMPs) or common

lymphoid progenitors (CLPs). CLPs give rise to all lymphoid cells including B-Cells, T-Cells and NK-cells. CMPs make up all of the myeloid committed cells through two intermediates: 1) Megakaryocyte/erythrocyte progenitors (MEPs) and 2) granulocyte/macrophage progenitors (GMPs). As their names suggest, MEPs further differentiate into platelets and erythrocytes, and GMPs differentiate into granulocytes and macrophages. Interestingly, both CMPs and CLPs are capable of differentiating into a heterogeneous population of dendritic cells. All of the aforementioned progenitors can be isolated and characterized by their cell surface markers by flow cytometry (Seita and Weissman, 2010).

While HSCs represent one of the most robust models of regeneration, neural stem cells (NSCs) represent the opposite, as they rarely divide and are limited in their potency. NSCs reside in the ventricular zone in the brain in adult mice. In the developing embryo at E9-10, neuroepithelial cells begin a transition to form radial glial (RG) cells that develop into multipotent adult NSCs (Kriegstein and Alvarez-Buylla, 2009). RG cells divide in an apical-basal fashion, where Notch signalling in the apical domain prevents dividing neural progenitors from premature differentiation (Del-Bene et al., 2008). In the developed mammalian brain, B cells line the lateral ventricles and the sub-ventricular zone. These B cells are largely quiescent, but can divide and differentiate to proliferating C cells, which are transit amplifying or intermediate progenitor cells (Kriegstein and Alvarez-Buylla, 2009). These C cells can further differentiate into both neurons and oligodendrocytes within the sub-ventricular zone and migrate throughout the cortex.

The contrast between NSCs and HSCs demonstrate how tissue specific somatic stem cells differ based on the needs of their system. Like all somatic stem cells, they self-renew prior to differentiating into their functional roles in order to maintain a steady pool of progenitors for

future rounds of regeneration or injury. In addition to self-renewing, these cells demonstrate the range of potency found in adult stem cells, from HSCs that populate that populate the wide range of cell types in the blood, to NSCs that are limited to just a few cell types under normal conditions. While many other somatic stem cells are present in the body, this thesis will focus on the skeletal muscle tissue, and the muscle stem cells present within it.

1.5 Satellite Cells

In 1964, using electron microscopy, Alexandre Mauro identified a mono-nucleated cell within skeletal muscle, which he termed the "Satellite cell" for its position beneath the basal lamina surrounding a muscle fibre. Solely based on analysis of electron micrographs, he postulated "…satellite cells are merely dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of skeletal muscle fiber when the main multinucleated cell is damaged." (Mauro, 1961). Additionally, Mauro proposed that the inability for cardiac muscle to regenerate is related to the absence of satellite cells. Remarkably, experiments over the next 57 years have confirmed Mauro's predictions and the satellite cell is universally recognized as the adult somatic stem cell responsible for muscle regeneration (Relaix and Zammit, 2012).

Since myonuclei are post-mitotic, ^{3}H does not incorporate into the nucleus of mature fibres. Based on this, it was determined that satellite cells proliferate and differentiate into myofibres in response to injury, due to the pre-injury incorporation of ^{3}H only in satellite cells, and post-injury incorporation of ^{3}H into myonuclei (Snow, 1977). Minced muscle from mice treated with ^{3}H (^{3}H labelled satellite cells) engrafted into untreated mice also resulted in ^{3}H incorporation into myonuclei, demonstrating the myogenic potential of satellite cells, as well as

early efficacy for cell therapies (Snow, 1978). In addition to ^{3}H incorporation studies, *in vitro* experiments of cultured single muscle fibres demonstrate associated satellite cells ability to form myoblast colonies and differentiate into myotubes (Bischoff, 1975).

To be defined as a stem cell, satellite cells must demonstrate that they are able to differentiate into the cell types within the tissue they reside, as well as demonstrate the capacity to self-renew. While the previous studies demonstrated that satellite cells have the ability to differentiate into skeletal muscle, they failed to demonstrate that satellite cells self-renew in order to cope with future injury. Satellite cells robust capacity for self-renewal was demonstrated by the transplantation of a single myofibre containing a LacZ reporter into a mouse model of DMD. Remarkably, one myofibre (containing ~7 satellite cells) was able to produce over 100 new myofibres, each containing >1000 myonuclei, as well as new satellite cells in their native position beneath the basal lamina of these new fibres (Collins et al., 2005). Montarras et al. (2005) verified that isolated satellite cells are capable of contributing to new muscle fibers and self-renewing after intramuscular engraftment. By engrafting $10^4 – 10^5$ freshly isolated or satellite cells cultured for 3 days, they showed that satellite cells rapidly lose their regenerative potential in culture. Sacco et al. (2008) later demonstrated the robust potential of satellite cells, by engrafting single satellite cells in the tibialis anterior muscle of a mouse. These satellite cells were capable of contributing to new muscle fibres, as well as contributing to self-renewed satellite cells as evidenced by their re-isolation after 2 months. The low efficiency of engraftment (4%) was likely due to stochastic variation due to the difficulty of properly engrafting a single cell, but it also is likely the result of the heterogeneity of the engrafted satellite cells – evidenced by their variable expression of *MyoD*, *Pax3* and *Myf5* mRNA observed in this study. This paper

built upon the accumulating evidence that satellite cells represent a heterogeneous population of cells with varying degrees of regenerative potential.

1.5.1 Satellite Cell Heterogeneity

While satellite cells are heterogeneous for various transcription factors associated with myogenesis, virtually all satellite cells in adult muscle express Pax7. This was first proven in a seminal paper by Seale et al. (2000), which demonstrated that not only is Pax7 expressed in all satellite cells, but that *Pax7* null mutants lack satellite cells and have severe muscle deficits. *Pax7* was later confirmed as a discrete marker for satellite cells in a number of studies that utilize inducible Cre-recombinase under the control of the *Pax7* promoter to track the lineage of satellite cells into both muscle fibres and renewed satellite cells (Shea et al., 2010). Further, using diphtheria toxin expression driven by an inducible Pax7-Cre recombinase, 4 sequential papers published in *Development* in 2011 demonstrate that Pax7+ satellite cells are required for muscle regeneration (Lepper et al., 2011; McCarthy et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). Therefore, Pax7+ cells are required to regenerate new muscle, and the loss of muscle regeneration observed following the ablation of Pax7+ progenitors can be restored by engrafting healthy Pax7+ satellite cells into the injured muscle. In tandem, these 4 papers confirm that it is the satellite cell that is necessary for regeneration of adult skeletal muscle.

In some muscles, such as those in the diaphragm and trunk, the majority of satellite cells express Pax3 along with Pax7. In contrast to embryonic myogenesis, where Pax7 is rarely expressed and Pax3 drives myogenesis, Pax3 cannot compensate for loss of Pax7 in adult satellite cells (Relaix et al., 2006). This is unsurprising, as *Pax7* mutant mice were initially said to be devoid of satellite cells (Seale et al., 2000), however it was shown that young *Pax7* mutant

mice (D8) still possess a reduced, but quantifiable amount of satellite cells that do not require Pax7 expression (Oustanina et al., 2004). In this study, they showed that as the mice aged, satellite cell number diminished, regardless of if the cells expressed Pax3 or not. Therefore, while *Pax3* and *Pax7* seem to have similar function in the satellite cell, as evidenced by their ability to activate *MyoD* and induce the myogenic program, Pax3 is unable to compensate for loss of Pax7, likely due to anti-apoptotic effects of Pax7 expression (Oustanina et al., 2004; Relaix et al., 2006).

The transcription factor *Myf5* is responsible for the proliferation of activated satellite cells and has been used to exemplify the heterogeneity of satellite cell populations (Ustanina et al., 2007). Using a Myf5-Cre reporter mouse, it was shown that some populations of satellite cells never express *Myf5* (Kuang et al., 2007). Further, Pax7+Myf5- cells can divide asymmetrically to produce Myf5+ and Myf5- daughter cells (Kuang et al., 2007). The Myf5+ daughter cells underwent precocious differentiation after transplantation, while Myf5- cells preferentially self-renewed (Kuang et al., 2007). Therefore, this study indicated that the smaller population of Pax7+Myf5- satellite cells represent a less committed progenitor compared to Pax7+Myf5+ cells. Asymmetric division relative to Myf5 supports the notion that the satellite cell pool is maintained through asymmetric divisions resulting in self-renewing and differentiating progeny. Other theories along this line of thinking includes the Immortal Strand Hypothesis, which states that stem cells divide asymmetrically with the original DNA remaining in the self-renewing cell, and the new DNA segregating with the differentiating daughter cell (Rando, 2007). DNA segregation driving asymmetric division and cell fate has been contested in the satellite cell due to random DNA segregation in other somatic stem cell populations, such as the hematopoietic system (Kiel et al., 2007). However, there is mounting evidence that asymmetric segregation of

DNA is associated with asymmetric cell division and cell fate in the satellite cell, as evidenced by differences in co-segregation of DNA in $Pax7^{high}$ and $Pax7^{Low}$ populations of cells (Rocheteau et al., 2012; Shinin et al., 2006).

1.5.2 Satellite Cell Myogenesis

Under normal conditions, satellite cells reside in a dormant state beneath the basal lamina of their muscle fibre. In response to injury, satellite cells become activated and undergo a proliferatory phase prior to differentiation. It is unclear exactly how satellite cells sense muscle injury and activate, but many signalling pathways have been implicated in this process. One factor involved satellite cell activation is the stretching of the muscle fibre, which results in nitric oxide and hepatocyte growth factor expression leading to activation (Grand and Rudnicki, 2007; Wozniak and Anderson, 2007). Other extrinsic signaling pathways include activation of MAPK by FGF in response to injury, where MAPK induces satellite cell activation in quiescent cells (Jones et al., 2005).

Quiescent satellite cells are typically characterized by their expression of the transcription factors Pax3 and/or Pax7. Approximately 90% of quiescent Pax7+ satellite cells are actively expressing *Myf5* mRNA, as evidenced by β-gal expression in a *Myf5-nLacZ* mouse (Kuang et al., 2007). Therefore, after sensing cues to respond to injury, these Pax7+Myf5+ satellite cells are poised to enter the myogenic program and begin proliferating. Within 24hrs these cells begin expressing the myogenic transcription factor *MyoD*, which is the major driver of myogenic differentiation (Sabourin et al., 1999). Pax7+MyoD+ cells are thus said to be "activated" and proliferate without differentiation. Satellite cells from $MyoD^{-/-}$ mice continue to proliferate, with only a small number of cells progressing to express the terminal differentiation factor, *Myogenin*

(Yablonka-Reuveni et al., 1999). The eventual differentiation of muscle is likely due to the limited capacity of other MRFs, such as *Myf5* and *Mrf4* to initiate differentiation. This provides evidence that *MyoD* is critical for driving myogenesis to completion in adult muscle satellite cells. The importance of *MyoD* in the myogenic program is underlined by the observation that ectopic *MyoD* expression in fibroblasts drives the cells to a myogenic fate (Hollenberg et al., 1993). Interestingly, continued *MyoD* induction is not required, as once cells have begun to express *MyoD*, downstream transcription factors such as Myogenin are continually expressed.

While *MyoD* promotes differentiation, *Myf5* null satellite cells display reduced proliferation in culture, suggesting *Myf5* plays a role in maintaining proliferating populations of activated satellite cells (Gayraud-Morel et al., 2007). In these mice differentiation is not hindered, however a slight regeneration defect is observed, explained by the satellite cell proliferation deficit observed in culture (Ustanina et al., 2007). Therefore, it is likely that there is a "tug-o'-war" between *Myf5* and *MyoD*; where if Myf5 is more expressed, satellite cells will largely remain in an activated proliferatory phase, while if MyoD is more expressed, the cells will continue through the myogenic program, evidenced by their loss of Pax7 expression and activation of Myogenin (Rudnicki et al., 2008).

Expression of MyoD induces the expression of 678 genes after 48 hours – one of which is *Myogenin* (Cao et al., 2006). *Myogenin* is required for the terminal differentiation of muscle tissues. This requirement is evidenced by the perinatal lethality of double *Myogenin* mutants, where developing fetuses fail to develop mature muscle fibres (Hasty et al., 1993; Nabeshima et al., 1993). Interestingly, Myogenin relies on the histone acetylation activity of MyoD to drive gene expression in 69 out of the 78 genes that it targets (Cao et al., 2006). This provides a mechanism in which MyoD activates a vast set of genes, while Myogenin is recruited at later

time points to activate a subset of late-myogenic genes that induce terminal differentiation. Many of these genes include classic contractile machinery such as troponins and myosins, as well as genes required for cell fusion, as reviewed in Chapter 1.3.

While the majority of activated satellite cells will continue along normal progression of the myogenic program as described, some cells are required to revert to quiescence beneath the basal lamina of the muscle fibre for future injury. This process of self-renewal occurs when cells retain Pax7 expression and lose, or never express, MyoD, creating a small fraction (~4%) of Pax7+MyoD- cells (Zammit et al., 2004). As discussed in Chapter 1.5.1, this process can be accomplished via asymmetric division, in which one daughter cell adopts a myogenic fate and the other self-renews – a process that can be observed in Myf5+ and Myf5- cells, respectively (Kuang et al., 2007). However, in response to muscle injury, Wnt7a signaling can drive symmetric divisions to expand the population of Pax7+MyoD- satellite cells required (Grand et al., 2009). These expanded satellite cells can then undergo asymmetric or symmetric differentiation divisions to reconstitute the injured muscle.

As discussed in Chapter 1.2.1, satellite cells in aged mice have a reduced propensity to self-renew compared to young mice. However, culturing cells in the presence of a p38α/β MAPK inhibitor rescues this loss of self-renewal in aged cells (Bernet et al., 2014). Additionally, the activation of satellite cells occurs simultaneously with the expression of p38α/β MAPK, suggesting p38α/β MAPK pushes satellite cells out of quiescence to activate (Jones et al., 2005). p38α/β MAPK signaling pathway was identified as a satellite cell activator due to the fact that it is downstream of the FGF signaling receptors, FGFR1 and FGFR4. FGFR1 and FGFR4 are both expressed highly in activated satellite cells, suggesting that their activation stimulates p38α/β MAPK signaling to activate quiescent satellite cells (Sheehan and Allen, 1999). *Sprouty1*

expression is activated by FGFR4 signaling, where it also acts as a negative feedback regulator. In the developing embryo, overexpression of *Sprouty1* has been shown to inhibit myogenesis, likely due to FGFR4 inhibition, and thus p38α/β MAPK inhibition as well (Lagha et al., 2008). The importance of *Sprouty1* in satellite cells is highlighted by its expression in quiescent satellite cells, where it is lost in activated cells and then regained after cells have self-renewed (Shea et al., 2010). Moreover, deletion of *Sprouty1* resulted in a reduction of self-renewed satellite cells following injury (Shea et al., 2010). Therefore, FGF-p38α/β MAPK-Sprouty1 pathway exemplifies how one of the many extrinsic factors (FGF) influences satellite cell fate to self-renew or not.

Notch signaling represents an evolutionary conserved signaling pathway that is implicated in self-renewal in a variety of somatic stem cells including gut, mesenchymal, neuronal, hematopoietic, embryonic and pancreatic stem and progenitor cells (Liu et al., 2010). Like-wise, the interplay between Notch1 and Notch1 antagonist, *Numb*, has been shown to regulate satellite cell fate determination between proliferation and myogenic fates (Conboy and Rando, 2002). Constitutively overexpressing Notch1 results in an increase in self-renewal, evidenced by an increase in Pax7 and reduction in MyoD (Wen et al., 2012). Further, the authors showed that this was independent of simply delaying differentiation, as Pax7 was increased in *MyoD*-null mice as well. Lastly, overexpression of Notch1 *in vivo* resulted in a significant improvement in satellite cell self-renewal, but impaired muscle regeneration due to an impairment in the cell's ability to differentiate.

1.5.3 Satellite Cell Niche

The microenvironment in which cells natively reside within an organism is called the niche. In somatic stem cells, this area provides the grounds for complex interactions between the myofibre, extracellular matrix, blood vessels, growth factors, support and immune cells, and the stem cell itself. These niche components have been shown to regulate stem cell quiescence and activation in hematopoietic, intestinal, hair follicle, neural, spermatogonial and muscle stem cells (Rezza et al., 2014). The role of the niche in regulating satellite cell fate is unsurprising, evidenced by the simple fact that when satellite cells are taken from their niche, they immediately begin to activate their myogenic program (Machado et al., 2017).

Satellite cells reside between the sarcolemma and the basal lamina of its associated myofibre (Mauro, 1961). They maintain proper orientation within this niche by expressing surface M-cadherin on its basal interface and α7β1-integrins on its apical interface that interact with the myofibre and basal lamina, respectively (Brun et al., 2017). This orientation is important, as Kuang et al. (2007) demonstrate that satellite cells undergo lateral symmetrical divisions and apical-basal asymmetrical divisions, with respect to Myf5 expression. Apical-basal divisions result in a basal $Pax7^{+}Myf5^{-}$ and an apical $Pax7^{+}Myf5^{+}$ daughter cell, suggesting that the physical environment, or niche, is influencing the fate of dividing cells. More specifically, the $Pax7^{+}Myf5^{-}$ population is likely maintained by Notch-3 signalling from the myofibre, as basal cells have increased Notch-3 signalling, while apical cells express increased Delta-1 (Kuang et al., 2007). Additionally, it has been shown that Fibronectin induces Wnt7a directed proliferation of satellite cells following injury (Bentzinger et al., 2013). Fibronectin is expressed by satellite cells following injury, but is largely localized in the basal lamina of the ECM surrounding the apical side of the satellite cell. Together, Notch signalling from the myofibre

maintains quiescence, and fibronectin induced Wnt signalling from the basal lamina promotes proliferation – influencing apical-basal directed asymmetrical divisions.

Satellite cells reside in a "primed" stated, in which they are rapidly able to activate their cell cycle to respond to muscle injury (Crist et al., 2012). Under normal conditions, satellite cells are normally quiescent in G_0 phase, however Rodgers et al. (2014) coin a "G_{Alert}" cell phase, where satellite cells are quiescent, but respond more rapidly with greater myogenic potential after muscle injury. After injury to the contralateral tibialis anterior muscle, this G_{Alert} phase is induced by an increase in mTORC1 signalling due to an increase in systemic HGF (Rodgers et al., 2014). This study is an elegant example of how not only changes in the microenvironment, but also changes in circulating factors can play a role in cell state and cell fate choices during regeneration. Because of the niches role maintaining satellite cell homeostasis through self-renewal, efforts have been made to recapitulate the niche *in vitro* in order to create favourable culture conditions for satellite cells.

Satellite cell fate is finely controlled due to intrinsic and extrinsic factors. Extrinsic factors typically come in the form of signaling molecules from the environment, such as myokines from the muscle fibre or neighbouring satellite cells, but may arise from mechanical stimuli, such as forces during fusion or stress of muscle fibre damage. Most of the intrinsic factors discussed this far have been at the level of transcription – largely due to the expression of different transcription factors, such as *Pax7* or *Myogenin*. However, eukaryotic cells regulate gene expression by a variety of post-transcriptional mechanisms.

1.6 Translational Control

1.6.1 Alternative Splicing

The central dogma of pre-mRNA processing dictates that introns are spliced out, leaving only the coding regions within a transcribed mRNA. However, ~94% of genes in humans undergo alternative splicing or polyadenylation processes creating altered protein products or mRNA stability (Wang et al., 2008). Alternative splicing events can alter both the 5' and 3' UTR, both of which regulate protein translation, however splice sites in the 5' UTR are far more common (Modrek et al., 2001). As discussed in Chapter 1.6.4, 5' uORFs regulated selective translation of mRNAs through through P-eIF2α-mediated control. The 3' UTR of mRNAs are responsible for the majority of translational control, as this is where many proteins and microRNAs typically bind to affect mRNA stability or translation (As discussed in Chapter 1.6.2 and 1.6.3). Although the 3'UTR contains the poly-adenylated tail, RNA- and protein-binding sites that regulate mRNA stability and protein translation, the length of the 3' UTR does not necessarily directly control mRNA stability (Gupta et al., 2014). This emphasizes the complexity of regulation, as it is the mRNA binding properties and secondary structures that dictate mRNA stability, not simply the length of the 3'UTR.

1.6.2 mRNA Stability

mRNA degradation is controlled by four main processes; 1) ARE-mediated decay, 2) Non-ARE mediated decay, 3) Nonsense-mediated decay and 4) miRNA-mediated decay. ARE-mediated decay is regulated by AU repeats within the 3' UTR of mRNA. Shaw and Kamen (1986) were able to demonstrate this by inducing the degradation of the typically stable β-globin mRNA by inserting 51 AT nucleotide repeats in its 3' UTR. ARE-binding proteins recognize these AU repeats, and recruit deadenylases, and other mRNA degrading proteins to the mRNA (Carpenter et al., 2014). Non-ARE-mediated decay is regulated by pro- and anti-deadenylase proteins binding to the 3' UTR of mRNA that recognize other sequences such as UC-rich regions

(Carpenter et al., 2014). Nonsense-mediated decay of mRNA typically acts as a safeguard so that erroneous proteins are not translated when mRNAs contain premature termination codons. While nonsense-mediated decay safeguards against erroneous premature termination codons, it also regulates normal transcripts by fine tuning gene expression during development, immune response and stress response (Hug et al., 2016). Finally, miRNA, and other non-coding RNA, represent robust regulators of gene and protein expression. In addition to these four regulators of mRNA stability, other factors such as m^6A modifications regulate mRNA decay, translation, processing and folding patterns (Zhao et al., 2016).

1.6.3 microRNA

Fire et al., (1998) first discovered the influence of interfering RNAs after injecting dsRNA into *C. elegans* and observing a drastic reduction in protein expression. What the authors did not know at the time was that similar dsRNAs, known as pre-miRNAs, are transcribed from host DNA, capped, polyadenylated and then exported in to the cytoplasm. Interestingly, pre-miRNAs are typically transcribed from intronic regions of coding genes or intergenic regions, providing an explanation for evolutionary conservation of non-protein coding sequences in the genome (Ha and Kim, 2014). In the nucleus, *Dicer* RNase cuts these pre-miRNA hairpins into ~22nt sequences, leaving short dsRNAs. Once in the cytoplasm, Argonaute proteins are responsible for the separation of these two strands, and forms a complex in which one of the strands is used for anti-sense binding to native mRNAs in the cell. This protein-miRNA complex is referred to as a miRISC, and regulates protein expression largely by binding to the 3'UTR of mRNAs (Kaboli et al., 2015). Although miRNAs are 22nt long on average, the "seed" region, which is only ~ 6nt long near the 5' end, is largely responsible for target recognition (Wee et al., 2012). Because such a small motif is required for base-pairing, a single miRNA is capable of altering expression

of >100 genes (Bartel, 2009). Further, mRNA fate after miRISC binding is largely due to the complementarity of the rest of the sequence, where strong complementarity results in the degradation of the mRNA, while weaker binding results in translational inhibition (Bartel, 2009).

The importance of miRNA in development is underlined by the embryonic lethality of *Dicer* or *Dgcr8* knock-downs, as both are crucial for miRNA maturation (Bernstein et al., 2003; Wang et al., 2007). Some mRNAs with miRNAs bound to their 3'UTR are capable of de-repression in response to cellular stress following the recruitment of various RBPs. Bhattacharyya et al., (2006) provide evidence for this de-repression by demonstrating that *CAT-1* mRNA repression by miR-122 is reversed in response to stress, evidenced by CAT-1 mRNA exit from p-bodies and association with polysomes. This study underlines the dynamic role of miRNA in translational control, as changes in conditions within the cells allow for miRNA-bound mRNAs to be recruited away from p-bodies and into polysomes. Therefore, miRNAs not only control translation by degrading mRNAs, but also through inhibition of protein translation.

1.6.4 Regulation of mRNA translation

In addition to miRNA-regulated translational control, various proteins control the rate of protein synthesis at the translational level. While translation can be regulated at all three levels – initiation, elongation and termination – the most prevalent form of regulation is at the initiation step during the onset of translation. In eukaryotes, initiation is largely controlled by the aptly named eukaryotic initiation factors (eIFs).

Prior to initiation, eIF1, eIF1A, eIF3 and eIF5 form a scanning complex with the 40s ribosome, to create a 43s Pre-initiation complex (PIC) that will recognize start codons (Gebauer and Hentze, 2004). These eIFs help the ribosome scan the mRNA, and also play a role in

attaching the PIC to eIF4F at the m^7G cap. eIF4F binds to the m^7G cap of the mature mRNA, which allows for the 43S ribosome to dock to the mRNA and begin it's scanning function. The eIF4F complex is comprised of eIF4E, which bind to the m^7G cap, eIF4A, an RNA helicase, and eIF4G, a scaffolding protein. eIF4G also binds to the poly(A)-binding protein, which with the rest of eIF4F, circularize the mRNA. In particular, eIF4E represents a major regulator of cap-dependent protein translation. eIF4E expression is tightly controlled and acts as a protein-limiting step in initiation. Increasing eIF4E expression leads to a host of pathologies including autism-like phenotypes in the brain and a variety of malignant cancers (Gkogkas et al., 2012; Lazaris-Karatzas et al., 1990). Regulation of eIF4E is most well characterized through mTOR signalling, where phosphorylation of 4E-BP1 and S6K1 promote protein synthesis (Ma and Blenis, 2009).

In order to initiate translation, eIF2 is required for the recruitment of a Met-$tRNA_i$ referred to as the Ternary Complex (TC), to the PIC. eIF2 cycles between a GTP and GDP bound state, where GTP-eIF2 has a high affinity for Met-$tRNA_i$, and dephosphorylation facilitates its release (Erickson and Hannig, 1996). Once a scanning ribosome recognizes an AUG codon, GTP in the TC is hydrolyzed and Met-$tRNA_i$ is loaded into the P-site of the ribosome. Accompanying this is the loss of eIF1, eIF2 and eIF5, while the 60s subunit is added via eIF5B and translation is ready to begin (Hinnebusch, 2014). In order to reset, eIF2B exchanges eIF2-GDP, for GTP, enabling eIF2 to generate a new TC, and thus a primed PIC.

Since Met-$tRNA_i$ is critical for most initiation events to occur, the delivery of TCs by eIF2 represents another key regulatory bottleneck in protein translation. eIF2 is composed of eIF2α, eIF2β and eIF2γ subunits. Although eIF2γ is responsible for binding GTP, eIF2α is the regulatory subunit responsible for translational control within the eIF2 complex. When eIF2α is

phosphorylated at serine 51, it is no longer able to recycle GDP to GTP, and thus is considered in an inactive state where global protein synthesis is reduced. This phosphorylation event is facilitated by its four known kinases; HRI, GCN2, PKR and PERK, in response to iron deficiency, amino acid starvation, viral infection or ER stress, respectively (Chen et al., 1991; Dever et al., 1992; Harding et al., 1999; Kostura and Mathews, 1989). While a reduction in global protein synthesis is beneficial to help alleviate all forms of aforementioned kinase-associated stress, certain mRNA transcripts are selectively translated in response to P-eIF2α. When eIF2α is phosphorylated in yeast by its only kinase (GCN2), *GCN4* undergoes selective translation by virtue of its uORFs (Dever et al., 1992). This process is conserved in higher eukaryotes, where P-eIF2α results in the selective translation of many genes, including *Atf4* and *Chop* (Harding et al., 2000; Jousse et al., 2001). Further, uORFs regulate selective translation through P-eIF2α-dependent start codon skipping, resulting in translation of uORFs under normal conditions, and protein-coding regions during stress (Lu et al., 2004; Vattem and Wek, 2004). While these uORFs clearly have a regulatory function, they may have an alternative role in producing micro-peptides (<100AA long) translated from these relatively small regulatory uORF sites (Crappé et al., 2014). Just as miRNAs are regularly transcribed from the introns of protein coding genes, functional micro-peptides from uORFs may represent a novel role for previously trivial motifs.

1.6.5 Stress granules

Stress granules in eukaryotic cells are closely related to P-bodies, in that they are localized foci of untranslated mRNA within a cell. P-bodies are typically associated with the degradation of mRNAs, whereas stress granules are localized with many eIFs and other machinery required for the onset of translation (Buchan and Parker, 2009). Stress granules typically form under

conditions where translational initiation is blocked, such as conditions of high P-eIF2α (Kedersha et al., 1999). Interestingly, while P-eIF2α-dependent inhibition of initiation results in the formation of stress granules, inhibiting cap-recognition by blocking eIF4E resulted in substantially less stress granule formation, emphasizing that formation of stress granules is more complex than simply blocking translation initiation (Mokas et al., 2009). After the alleviation of stress, P-eIF2α is dephosphorylated, and mRNAs are capable of translation, either by direct recruitment into polysomes or by disassembly of mRNPs (Buchan and Parker, 2009). This represents how miRNA and translational machinery, together underline the dynamic nature of protein expression at translational level.

1.7 Translational control in satellite cells

Stem cells represent the most dynamic cells in an organism, as they are rapidly able to assume a variety of cell types or multiply in their stem form. Regulation of this process is highly coordinated, and it is thus unsurprising that miRNA provide an additional layer of control in these cells. As mentioned in Chapter 1.6.3, when Dicer expression is knocked-down in the developing embryo, mice do not progress past E7.5 (Bernstein et al., 2003). This embryonic lethal phenotype is due to the dysregulation of protein expression in ES cells, which typically rely upon miRNA to steer cell fate towards different lineages or self-renew.

Similar to early development in ES cells, skeletal muscle-specific genes are also regulated by miRNAs. The first evidence of miRNAs regulating myogenesis was the study of miR-1 and miR-133, which are transcribed in a single polycistronic pre-miRNA. During myogenesis, miR-1 and miR-133 promote opposite cell fates. miR-1 represses HDAC4, which induces myogenesis, whereas miR-133 targets SRF, delaying myogenesis and enhancing cell proliferation in *Xenopus*

laevis embryos and cultured myoblasts, respectively (Chen et al., 2006). There is also evidence that miR-133 targets Prdm16 in satellite cells, as antagonizing miR-133 leads to satellite cell differentiation in to brown adipose fat (Yin et al., 2013). In addition to miR-1, miR-206 is also required for differentiation of myoblasts in to myotubes (Kim et al., 2006). While mIR-206 represses a vast array of genes, the authors suggest that direct targeting of *Pola1*, a DNA polymerase downregulated during differentiation, is the clearest direct target of mIR-206. miR-27b targets the 3'UTR of *Pax3* in the developing embryo and in satellite cells, and has been shown to tightly regulate Pax3 (Crist et al., 2009). In satellite cells, miR-27b is upregulated just prior to differentiation, suggesting a translational control mechanism in which *Pax3* mRNA is rapidly degraded to allow for the onset of myogenesis (Crist et al., 2009). Further, miR-489 is expressed in quiescent satellite cells, but not activated cells (Cheung et al., 2012). miR-489 targets *Dek* mRNA, a known oncogene, and regulates self-renewal through *Dek*-dependent asymmetric division, where expression of *Dek* induces differentiation, but blocking *Dek* via mIR-489 promotes return to quiescence (Cheung et al., 2012).

The importance of translational control within satellite cells is highlighted by the expression of *Myf5* mRNA in the quiescent satellite cell, but little or no Myf5 protein detectable. This primed state is possible due to the sequestration of *Myf5* mRNA into mRNP granules via miR-31 binding in the 3'UTR of their mRNA (Crist et al., 2012). Upon satellite cell activation, these mRNP granules rapidly dissociate, allowing for the accumulation of Myf5 protein (Crist et al., 2012). mRNP granules share many features and proteins with stress granules, which accumulate non-translated mRNAs during cellular stress, and dissociate after alleviation of said stress, resulting in rapid translation of mRNAs (Protter and Parker, 2016).

1.7.1 Role of eIF2α in satellite cells

As mentioned in Chapter 1.6.5, eIF2α plays a significant role in responding to cellular stress, producing stress granules when phosphorylated and dissociates granules upon dephosphorylation. In support of Myf5 being held in mRNP granules in quiescent cells, it was posited that eIF2α may play a role in controlling this shift between quiescence and activation. Other stem cells, such as HSCs, tightly regulate protein synthesis, where synthesis is kept low at all times prior to differentiation (Singer at al., 2014). Therefore, Zismanov et al. (2016) investigated if protein synthesis is regulated through eIF2α in the satellite cell. Satellite cells on single EDL fibres all expressed high levels P-eIF2α, coinciding with their expression of Pax7 and not MyoD. Satellite cells rapidly activate the myogenic program in culture, as evidenced by their expression of MyoD and Myf5 between 6 and 24 hours after isolation. As discussed in Chapter 1.5.2, the majority of satellite cells will activate initially, with a population of cells self-renewing to regain their Pax7+MyoD- signature. Interestingly, while all cultured satellite cells along myofibres lose their P-eIF2α expression after 6 hours, cells that express Pax7 after 24 hours regain P-eIF2α, while cells that express MyoD do not. These data suggest that activated satellite cells rapidly lose P-eIF2α, but are re-phosphorylated after self-renewal. Similarly isolated satellite cells drastically lose their P-eIF2α signature after 3 days in culture compared to freshly isolated.

Using an elegant mouse model that inducibly abolishes the ability for eIF2α to be phosphorylated, and marks these cells with GFP, Zismanov et al. (2016) demonstrated that P-eIF2α is required to remain quiescent *in vivo*. After administering tamoxifen, satellite cells spontaneously began to express MyoD, signifying their departure from quiescence. After muscle injury with cardiotoxin, the authors showed that these P-eIF2α null satellite cells were capable of differentiating into new muscle fibres, as evidenced by the presence of GFP+ fibres. However,

the importance of P-eIF2α in satellite cell self-renewal is underlined by the scarcity of GFP+Pax7+ nuclei after injury.

As has been established, satellite cells rapidly lose their myogenic potential under normal culture conditions (Montarras et al., 2005). Therefore, the authors examined whether promoting P-eIF2α *ex vivo* would maintain satellite cell properties to differentiate and self-renew in culture. By blocking the dephopshorylation with the small compound Sal003, they demonstrated that a greater fraction of cells express Pax7, with a downregulation of MyoD and Myogenin. Further, after 4 days in culture, these cells could be engrafted into $Dmd^{mdx};Foxn1^{nu/nu}$ mice and contribute to new muscle fibres and renewed satellite cells. This study underlined the importance of P-eIF2α in preserving the 'stemness' of satellite cells and proved that maintaining the P-eIF2α signature *ex vivo* is necessary for ex vivo culture and expansion.

1.8 *Ex vivo* expansion of satellite cells

One of the greatest barriers for cell-based therapies using satellite cells is the inability to expand viable populations of cells *ex vivo*. The scarcity of satellite cells within skeletal muscle, coupled with their poor ability to migrate within the body, support the requirement for tools to expand satellite cells *ex vivo* (Schultz et al., 1985). Satellite cells expanded for 4 days in culture demonstrate a drastic reduction in their capacity to differentiate into new muscle, and self-renew to replenish the satellite cell pool (Montarras et al., 2005). Previous work in our lab, and others, have demonstrated that as early as 6 hours in culture, satellite cells begin to activate, characterized by their expression of MyoD (Zismanov et al., 2016).

Manipulating signaling pathways using genetic tools or small compounds represents the most effective way to promote self-renewal *ex vivo* in cultured satellite cells. However, it is

important that the cells not only expand, but retain their stemness during expansion so that they are capable of differentiating and self-renewing following engraftment. Early demonstration of *ex vivo* expansion studied individual cytokines such as EGF, FGF, SCF and IGF (Deasy et al., 2002). While these growth factors were capable of increasing cell number after 96 hours, the researchers failed to demonstrate that the cells retained their stem-like properties to differentiate and self-renew. Additionally, satellite cells cultured with four cytokines were capable of expanding nearly 40 days *ex vivo* with the ability to differentiate into new muscle following transplantation (Fu et al., 2015b). Despite robust expansion, the composition of these cells *ex vivo* is unclear, as is their capacity for self-renewal after engraftment.

In addition to the classical signaling pathways, the physical culture environment has been shown to regulate self-renewal in satellite cells. Satellite cells are found in their niche beneath the basal lamina of their associated muscle fibre. After isolating satellite cells, they fail to attach to plastic dishes during cell culture and must be plated on gelatin or collagen coated dishes. Thus it is unsurprising that satellite cell viability is related to the elastic modulus of the surface in which they are plated on (Gilbert et al., 2010). Satellite cells cultured on hydrogel-coated dishes with a similar elasticity to muscle retained their ability to differentiate and self-renew in culture, whereas matched controls did not (Gilbert et al., 2010). Similar results were shown in vivo, where absence of Collagen VI reduced the stiffness of host muscle fibres and impaired satellite cell self-renewal following injury (Urciuolo et al., 2013). Building upon this, engineering collagen-based synthetic muscle fibres for satellite cell culture was able to retain quiescence in cultured satellite cells, evidenced by their improved ability to differentiate and self-renew after 2.5 days in culture (Quarta et al., 2016). Therefore, in addition to the cell cycle signaling

pathways regulating the satellite cell fate, mimicking the physical niche represents an important aspect for the development of optimal long-term satellite cell culture conditions.

Small compounds are viable tools to enhance/inhibit molecular pathways regulating self-renewal, proliferation or differentiation in order to expand satellite cell populations. One example of this is the small compound PFI-2, which inhibits the protein methyltransferase Setd7. Under normal culture conditions, activated satellite cells accumulate β-Catenin in response to Wnt3a signaling, which in part drives the progression of the myogenic program (Brack et al., 2008). Judson et al. (2018) demonstrate that β-Catenin accumulation in the nucleus of satellite cells is regulated by Setd7, whereby Setd7 inhibition results in a delay in differentiation *in vitro*. Blocking Setd7 with the small compound PFI-2 allows for satellite cells to retain their ability to differentiate and self-renew in intramuscular engraftment assays following 7 days in culture (Judson et al., 2018). This study is analogous in many ways to our labs previous work on the role of eIF2α in the satellite cell (Zismanov et al., 2016). In both cases, the expansion of satellite cells *ex vivo* is facilitated by through pharmacological inhibition of a regulator of satellite cell exit from quiescence, whether that be β-Catenin accumulation in the nucleus or dephosphorylation of P-eIF2α.

Here, we build upon previous work done in our lab demonstrating the role of P-eIF2α in satellite cells (Zismanov et al., 2016). We observed that the small compound sal003 is capable of enabling *ex vivo* expansion of potent satellite cells for 4 days culture, as evidenced by differentiation and self-renewal in engraftment assays. Here, I aim to identify novel analogs of sal003 that allow for the *ex vivo* expansion of satellite cells with increased efficiency. In addition to expanding satellite cells for 4 days in culture, it is important to demonstrate that we can expand functional satellite cells over multiple passages, as this would be required for

downstream genetic or additional pharmacological modifications. As previously noted, *ex vivo* expansion of satellite cells is necessary for their use as a therapeutic, or research tool, in which expansion can facilitate genetic or pharmacological alterations for downstream applications. The ability to expand MuSCs that retain regenerative capacity *ex vivo* opens the door to genome editing, which will be important as a research tool and had potential therapeutic value. After demonstrating that satellite cells can be expanded *ex vivo*, one of the application in which we focus on is the use of CRISPR/Cas9 to create precise genetic modifications. To our knowledge, there have been no successful attempts to expand satellite cells from Dmd^{mdx} mice, likely owing to their accelerated activation and differentiation in culture (Yablonka-Reuveni and Anderson, 2006). Expanding satellite cells from Dmd^{mdx} mice is of importance, as expansion is required for the *ex vivo* genetic correction of this relatively common genetic disease of the muscle and satellite cell.

1.8.1 CRISPR/Cas9

CRISPR/Cas9 is a tool derived from the adaptive anti-viral immune system of bacteria, engineered for the use of precise genetic manipulations in eukaryotic cells (Ran et al., 2013). While genetic editing tools, such as zinc-finger nucleases and TALENs have been used for years, CRISPR/Cas9 represents a breakthrough advancement due to its specificity, ease of design and low cost. The Cas9 nuclease is guided by a customizable 20nt RNA guide sequence followed by a PAM sequence. Following a double-stranded break in DNA 3nt upstream of the PAM site, DNA is repaired through NHEJ or HDR (Cong et al., 2013). NHEJ is useful for creating genetic knockouts, as it relies upon error-prone repair of the double stranded break, which typically introduces small indels at the repair site, resulting in frameshift mutations. HDR represents a precise mode of repair, relying on a template DNA to facilitate accurate repair, introduce

insertions or deletions at a desired loci. Although HDR provides the means to easily insert transgenes and perform precise mutagenesis, depending on the cell type, HDR is occurs at very low frequencies (0.5%-20%) and thus increasing the efficiency of HDR is an emphasis of study (Chu et al., 2015; Maruyama et al., 2015; Yu et al., 2015). CRISPR/Cas9 can be used as an efficient tool for genetic screening in human cell lines, as new sgRNAs can quickly be designed to create genome-wide knockout screens (Shalem et al., 2014). Further, the therapeutic potential of CRISPR/Cas9 has been demonstrated to repair various genetic diseases in ES cells and mice, such as cystic fibrosis (Schwank et al., 2013) and muscular dystrophy (Chapter 1.2.3). As discussed in Chapter 1.2.3, exon excision of the mutated locus in muscular dystrophy has been successful *in vivo*. By following the same strategy, we aim to leverage our ability to expand muscle satellite cells to facilitate the correction of DMD *ex vivo*.

1.9 Hypothesis

Satellite cells are expanded *ex vivo* with 10μM sal003, a relatively high concentration that precludes its use in vivo. I hypothesize that structure activity relations can be used to identify novel analogs of sal003 with increased activity at lower concentrations. An improved compound may facilitate expansion of satellite cells isolated from both wild-type and *Dmd*^{mdx} mice, which can be used to facilitate genome corrections using CRISPR/Cas9 tools. My specific objectives are:

1.10 Objectives

1) Identify a novel analogue of sal003 through our medium-throughput compound screening pipeline.
2) Demonstrate that this novel small compound is capable of maintaining satellite cell 'stemness' *ex vivo* during 4 day culture.
3) Demonstrate that satellite cells cultured in the presence of the small compound can be cultured beyond 4 days, and have the potential to be passaged multiple times.
4) Demonstrate that passaging satellite cells allows for *ex vivo* genetic manipulations with CRISPR/Cas9 in primary satellite cells.

Chapter II:

Ex vivo expansion and genome editing of skeletal muscle stem cells with a novel inhibitor of eIF2α dephosphorylation

Graham Lean, Matt Halloran, Jean-Phillip Lumb and Colin Crist

An article in preparation as a Short Report for submission to eLife.

2.1 Abstract

Regeneration of adult tissues requires the activity of rare, mitotically quiescent somatic stem cells. There is large interest in harvesting the potential of somatic stem cells in regenerative medicine based applications. However, partly owing to their scarcity, these cells are difficult to study, manipulate or use in cell based therapies. Using skeletal muscle regeneration as a paradigm to study somatic stem cells, we have previously shown that muscle stem cells (MuSCs) require tightly regulated protein synthesis through the phosphorylation of eIF2α. Sal003, an analog of salubrinal that blocks eIF2α dephosphorylation, promotes *ex vivo* expansion of MuSCs retaining regenerative capacity after engraftment into the Dmd^{mdx} mouse model of Duchenne muscular dystrophy. However, micromolar concentrations of sal003 (10μM) are required to expand MuSCs *ex vivo*, which potentially limits the effectiveness of this compound *ex vivo* and *in vivo*. Here, we have synthesized and screened chemical analogs of sal003 to identify a novel compound promoting the *ex vivo* expansion of MuSCs. The novel compound expands wild-type and *mdx* MuSCs more efficiently than sal003, enables passaging of MuSCs with regenerative capacity, and facilitates genome modification. Our novel compound can be a component of standard MuSC culture conditions to enable their further study, genetic manipulation and cell based therapies.

2.2 Introduction

Adult tissues with the capacity to regenerate do so by virtue of their somatic stem cells. The regeneration of skeletal muscle is facilitated by normally quiescent muscle stem cells (MuSCs), or “satellite cells”, originally named for their satellite position beneath the basal lamina of the myofibre (Mauro, 1961). Quiescent MuSCs normally express PAX7 and, in a subset of muscle, PAX3. In response to injury, MuSCs activate the cell cycle and the expression

of members of the myogenic regulatory family of transcription factors (MYF5, MYOD). MuSCs proliferate, differentiate to repair the injured muscle, and self-renew to re-establish the MuSC pool.

Although the isolation of MuSCs from skeletal muscle can be routinely performed, the isolation conditions alone dramatically influence gene expression (Machado et al., 2017) and furthermore, ex vivo culture of MuSCs results in their entry into the myogenic program and loss of MuSC regenerative capacity. Efforts to understand molecular and biophysical cues that regulate quiescence are useful to mitigate the loss of MuSC powers during ex vivo culture (Gilbert et al., 2010, Quarta et al., 2016, Zismanov et al., 2016, Judson et al., 2018). For example, there is rich evidence that translational control mechanisms play a role in the determination of MuSC fate (Cheung et al., 2012; Crist et al., 2012; Zismanov et al., 2016). We have shown that the phosphorylation of eIF2α is a translational control mechanism regulating MuSC quiescence and self-renewal, while MuSCs that lose their P-eIF2α signature activate protein synthesis, the myogenic program (Pax7+, MyoD+) and differentiate (Pax7-, MyoD+ and/or Myogenin+) (Zismanov et al., 2016). Furthermore, pharmacological inhibition of the eIF2α phosphatase Gadd34/PP1c permits the ex vivo expansion of MuSCs that retain regenerative capacity, as illustrated by engraftment into the *Dmd*^{mdx} mouse model of Duchenne muscular dystrophy (Zismanov et al., 2016).

2.3 Results

2.3.1 Identification of novel sal003 analogs maintaining P-eIF2α and promoting MuSC expansion ex vivo

To identify novel analogues of sal003, we utilized structure-activity relations to synthesize and screen novel sal003 analogs. To initially screen novel compounds, we tracked *ex vivo* expansion in the presence of 10μM compounds by measuring bioluminescence from MuSCs isolated from *Tg(Cag-luc,-GFP)* mice (Figure 1A). In parallel, we directly tested the effect of novel compounds on P-eIF2α levels in C2C12 myogenic cells (Figure 1B). We classified novel compounds into those that a) did not increase bioluminescence or P-eIF2α levels, b) increased bioluminescence without increased P-eIF2α, c) increased bioluminescence and P-eIF2α levels and d) reduced bioluminescence and increased P-eIF2α levels (Figure 1C). Thapsigargin (a toxic compound isolated from the Mediterranean plant *Thapsia garganica*) is used a positive control, as it greatly elevates levels of P-eIF2α, but does so by inducing endoplasmic reticulum stress, not through inhibition of P-eIF2α dephosphorylation (Thastrup et al., 1990). One compound, C9, which has an o-hydroxyl substituted for the p-chloro on the sal003 N-phenyl group, caused a rapid increase in bioluminescence, without increasing P-eIF2α levels. A second compound, C10, which has a 3,5-bis(trifluoromethyl) substituted for the p-chloro on the sal003 N-phenyl group, decreased bioluminescence but increased P-eIF2α levels greater than sal003 (Figure 1A-C).

We focused our attention on C10, as it was the only molecule to increase P-eIF2α to a greater extent than sal003 at 10μM. In our initial bioluminescence screen, we posited that the higher P-eIF2α levels would account for an overall decrease in bioluminescence by promoting

lower initial rates of proliferation followed by robust expansion, as we have previously shown for sal003 (Zismanov et al., 2016). We titrated C10 from 10μM to 100nM, to determine that 5μM C10 potently expands MuSCs (Figure 1D) at P-eIF2α levels more similar to 10μM sal003 (Figure 1E). In contrast, 5μM sal003 does not expand MuSCs (Figure 1D).

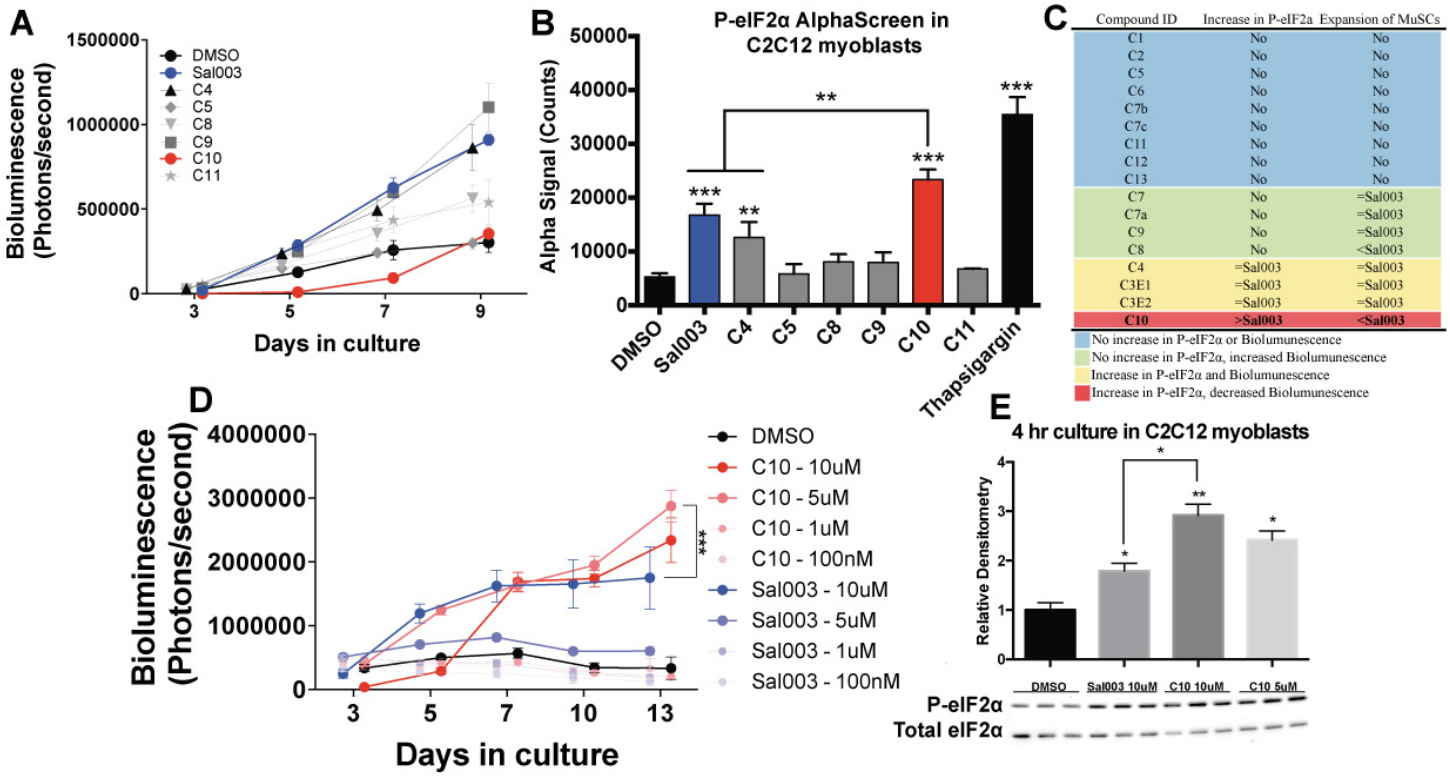

Figure 1. Identification of C10 as a candidate novel small compound inhibitor of eIF2α dephosphorylation. (A) Bioluminescence from MuSCs isolated from *Tg(Cag-luc,-GFP)* adult mice and cultured in the presence of 10μM or DMSO (control). (B) P-eIF2α levels of C2C12 myoblasts after 4 hour culture with 10μM candidate compounds or DMSO (control) using AlphaScreen SureFire eIF2α (Ser-51) assay. (C) Classification of novel compounds based on bioluminescence and P-eIF2a. (D) Bioluminescence from MuSCs isolated from *Tg(Cag-luc,-GFP)* adult mice cultured in the presence of C10, Sal003, at the range of indicated concentration or in DMSO (control). (D) Western blotting against P-eIF2α from cell lysates of C2C12 cells cultured for 4 hours in C10, Sal003 or DMSO (control). All values indicate mean (n≥3) ± SEM.

2.3.2 Inhibition of eIF2α dephosphorylation by the novel sal003 analog C10 promotes MuSC self-renewal during ex vivo culture

We next examined the ability of 5μM C10 to expand a population of MuSCs that maintain PAX7 expression and reduce MYOD/MYOGENIN expression after four days in culture. MuSCs isolated from adult *Pax3*$^{GFP/+}$ mice were immunolabelled with antibodies against PAX7 and MYOD, or PAX7 and MYOGENIN, after 4 days in culture. Culture in the presence of 5μM C10 for four days resulted in a 2-fold increase in the numbers of PAX7(+), MYOD(-) cells that have not entered the myogenic program and a 1.5-fold decrease in numbers of differentiating PAX7(-), MYOD(+) cells (Figure 2A,B). We also observe a greater than 2-fold increase in PAX7(+) progenitor cells and a 2-fold decrease in MYOGENIN(+) differentiating cells (Figure 2C,D). Immunoblotting with antibodies against PAX7 and MYOGENIN revealed a 3-fold increase in PAX7 and a 4-fold decrease in MYOGENIN levels in *Pax3*$^{GFP/+}$ MuSCs after 4 days in culture with 5μM C10 (Figure 2E,F). Similarly, RT-qPCR revealed a decrease in *MyoD* and *Myogenin* mRNA. In contrast to PAX7 protein levels, there was no significant change in *Pax7* mRNA (Figure 2G), similar to our observations with 10μM sal003 (Zismanov et al., 2016).

2.3.3 C10 expands MuSCs isolated from *Dmd*^{mdx} mice

The progression of Duchenne muscular dystrophy is preceded by a progressive reduction in the MuSC pool (Sacco et al., 2010), in part through the loss of DYSTROPHIN-dependent asymmetric MuSC divisions (Dumont et al., 2015). We asked whether our novel compound C10 can overcome some of the defects of *Dmd*^{mdx} MuSC expansion. Similarly to *Pax3*$^{GFP/+}$ MuSCs, *Pax3*$^{GFP/+}$*; Dmd*^{mdx} MuSCs cultured in 5μM C10 had a greater than 2-fold increase in PAX7(+) cells and a 2-fold decrease in MYOGENIN(+) cells (Figure 2 – Supplement 1A,B). While there

was not a significant difference in PAX7(+),MYOD(+) that have activated the myogenic program, or in differentiating PAX7(-),MYOD(+) cells, we observed a greater than 2-fold increase in Pax7(+), MYOD(-) *Dmd*^{mdx4cv} MuSCs that have not activated the myogenic program, when cultured with 5uM C10 (Figure 2 – Supplement 1C,D).

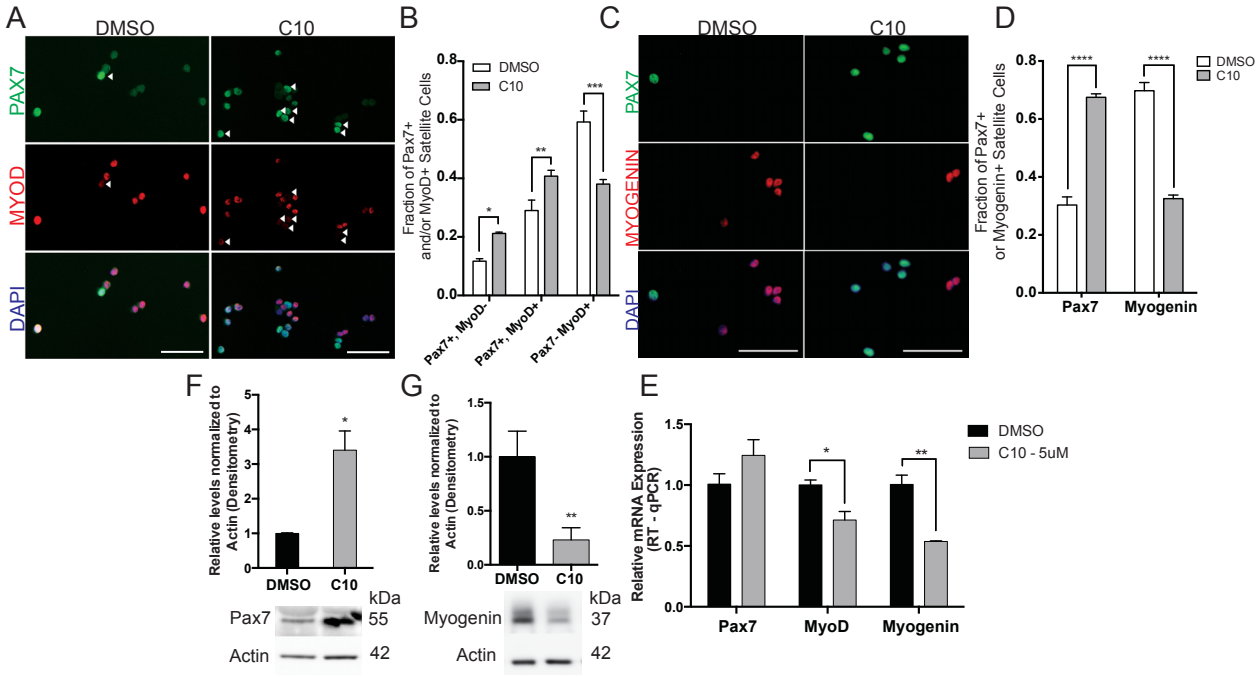

Figure 2. MuSCs cultured in C10 have delayed differentiation and improved self-renewal *ex vivo*. (A) Immunofluorescence with antibodies against Pax7 (Green) and MyoD (Red) on MuSCs isolated from Pax3$^{GFP/+}$ mice after 4 day culture in 5μM C10 or DMSO (control). Quantification of results are to the right of representative images. (B) Immunofluorescence with antibodies against Pax7 (Green) and Myogenin (Red) on MuSCs isolated from Pax3$^{GFP/+}$ mice after 4 day culture in C10 or DMSO (control). Quantification of results are to the right of representative images. (C) Relative mRNA levels of Pax7, MyoD and Myogenin by RT-qPCR after 4 day culture of MuSCs in C10 or DMSO (control). mRNA levels are normalized to *actb* and are relative to DMSO. (D,E) Western blotting with antibodies against Pax7, myogenin and actin of MuSC lysates after 4 day culture in C10 or DMSO (control). Relative densitometry is normalized to actin and presented relative to DMSO (control). Scale bars represent 50μm. All values indicate mean (n≥3) ± SEM.

2.3.4 C10 maintains the regenerative capacity of cultured MuSCs

MuSCs rapidly lose their regenerative potential under normal culture conditions (Montarras et al., 2005). To confirm that MuSCs expanded *ex vivo* in the presence of C10 retain their stem cell properties to self-renew and regenerate muscle, we engrafted cultured MuSCs into

the *tibialis anterior* muscle of the mouse model of Duchenne muscular dystrophy (*Dmd*^{mdx}). Satellite cells were isolated from *Pax3*$^{GFP/+}$ mice and cultured for 4 days in 5μM C10 or DMSO (control). 10 000 cultured cells were engrafted into 18 Gy irradiated hindlimbs of *Dmd*$^{mdx/mdx}$; *Foxn1*$^{nu/nu}$ immunodeficient mice. 21 days after engraftment, we examined whether engrafted donor cells retained their capacity for self-renewal (PAX7(+), GFP(+)) and differentiation (DYSTROPHIN+ myofibres). Engraftment of 10 000 C10-treated MuSCs led to significantly more DYSTROPHIN(+) myofibres (Figure 3A,B) and more PAX7(+), GFP(+) cells of donor origin (Figure 3C,D), compared to DMSO.

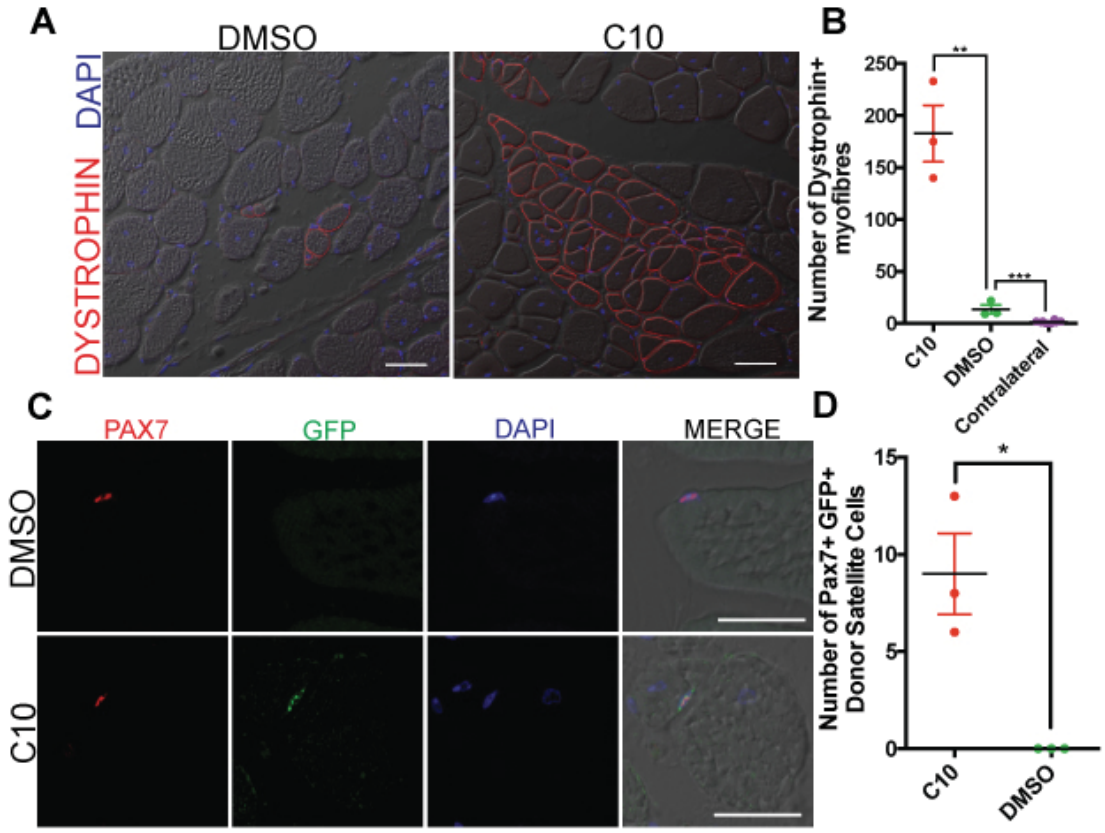

Figure 3. MuSCs maintain their capacity to differentiate and self-renew after 4-day culture in C10. (A) Dystrophin (Red) Immunolabelling of tibialis anterior muscle 21 days after engraftment of 10 000 MuSCs cultured with DMSO (control) or 5μM C10. Scale Bars represents 50μm. (B) Scatter plot of the number of dystrophin(+) muscle fibres per immunolabelled cross-section from (A). (C) Immunolabeling with antibodies against Pax7 (Red) and GFP (Green) on transverse sections of tibialis anterior muscle following intramuscular engraftment of MuSCs cultured in DMSO (control) or C10 5μM. Scale bars represent 25μm. (D) Scatter plot of Pax7+GFP+ donor derived cells per cross-section from (C). All values indicate mean (n≥3) ± SEM.

2.3.5 C10 enables serial passaging and genome modification of MuSCs

Since MuSCs cultured under normal conditions have limited potential to expand after passaging, we next asked the extent to which 5μM C10 can enable serial passaging of MuSCs. Culture of 7500 cells isolated from wild-type and *Dmd*^{mdx} mice leads to total counts of greater than 6 million (wild-type, 4 passages) and 7 million (mdx, 5 passages) cells, compared to a total of greater than 200 000 (wild-type, 4 passages) and 400 000 (mdx, 3 passages) cells passaged and cultured under normal conditions (Figure 4A). When MuSCs are passaged in the presence of 5μM C10, they also retain the ability to differentiate into large, multinucleated myotubes, in contrast to culture under normal conditions that result in small, thin myotubes populated with fewer myonuclei (Figure 4B,C). Furthermore, large myotubes cultured in the presence of C10 are populated with numerous PAX7(+) nuclei (Figure 4 B,D), suggesting that these mature myogenic cultures in the presence of C10 have spontaneously organized to recreate an *ex vivo* niche for perturbing PAX7(+) satellite-like cells. Even after 2 passages, both wild-type and *Dmd*^{mdx} MuSCs cultured in the presence of 5μM C10 increased numbers of PAX7(+) cells and decreased numbers of MYOGENIN(+) (Figure 4E-H). These data explain cell passaging and expansion (Figure 4A), as MuSCs cultured with C10 are largely PAX7(+) and are likely to continue to proliferate through later passages, while controls (DMSO) are largely MYOGENIN (+) and likely exit the cell cycle rendering them unable to expand further.

Finally, we demonstrate the utility of cultured MuSCs in the presence of 5μM C10 to facilitate genome modification of MuSCs. We isolated MuSCs from *Dmd*^{mdx} mice and transduced them with lentiviral vectors containing Cas9 and sgRNAs targeting intron 51 and intron 53 of *Dmd,* as well as an mCherry reporter. These CRISPR/Cas9 constructs will create a double stranded break, excising the mutated exon-53 in *Dmd*^{mdx4cv} mice and restore functional

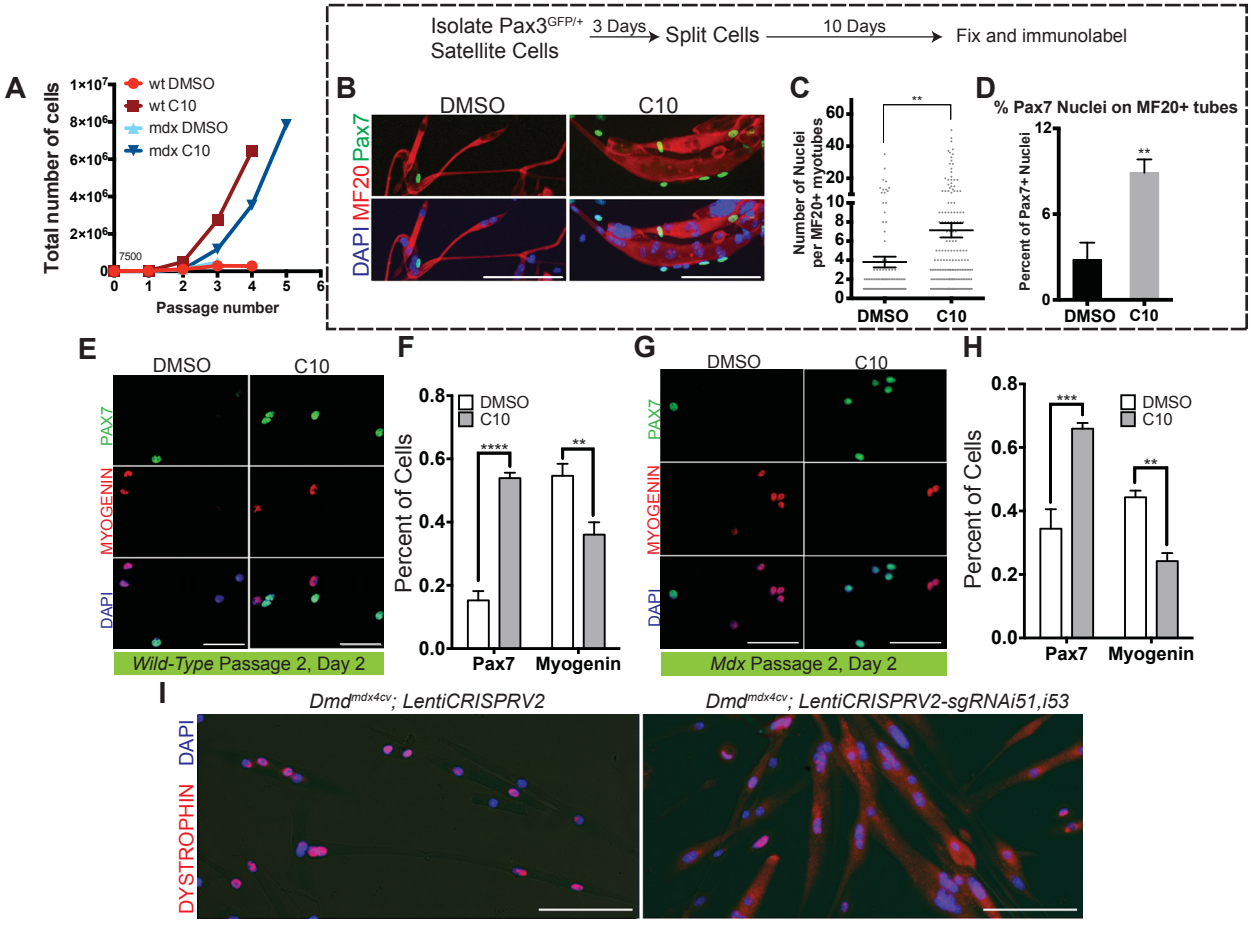

Figure 4. MuSCs cultured in C10 allow for *ex vivo* genetic editing with CRISPR/Cas9. (A) Expansion of MuSCs derived from wild-type and *mdx* over 4 and 5 passages, respectfully. MuSCs are plated at an initial density of 7500 cells/plate, passaged at a density of 2000 after 3 days, and then passaged every 2 days at a density of 2000 cells/plate for subsequent passages. (B) Immulabelling for Pax7 (Green) MF20 (Red) of MuSCs cultured for 10 days following passage 1. Scale Bars represents 100μm. (C) Quantification of the number of DAPI+ nuclei per MF20+ myotubes from (B). n≥100 myotubes. (D) Quantification of Pax7+ nuclei associated with MF20+ myotubes from (B). (E) Immunolabelling for Pax7 (Green) and Myogenin (Red) of wild-type MuSCs after passage 2, cultured with DMSO (control) or 5uM C10. (F) Quantification Pax7+ or Myogenin+ MuSCs cultured in DMSO (control) or C10 from (E). (G) Immunolabelling for Pax7 (Green) and Myogenin (Red) of *mdx* MuSCs after passage 2, cultured with DMSO (control) or 5uM C10. (H) Quantification Pax7+ or Myogenin+ MuSCs cultured in DMSO (control) or C10 from (G). (I) Immunolabelling of Dystrophin (Red) of transduced MuSCs with lentivirus containing Cas9 and sgRNAs targeting i51 and i53 or without sgRNAs (control). Scale bars represent 100μm. Scale bars represent 50μm unless indicated otherwise. All values indicate mean (n≥3) ± SEM.

DYSTROPHIN protein as previously shown *in vivo* (Bengtsson et al., 2017). Transduced MuSCs were cultured for 2 passages (5 days) and then mCherry(+) cells were sorted into single cell clones at a density of one cell per well in 96 well plate formats. Colonies that emerged from

these single cell clones were grown for a further 6 days in the presence of C10, then four days in differentiation media to promote differentiation. While no colonies were dystrophin positive in un-transduced controls, 44.5% ± 6.7% of colonies transduced with lentiviral constructs targeting sgRNAs to *Dmd* i51 and i53 differentiated into DYSTROPHIN(+) myotubes *ex vivo* (Figure 4I).

Here we have reported a novel compound that expands wild-type and Dmd^{mdx} MuSCs *ex vivo*, enabling their engraftment *in vivo*, as well as passaging and genome modification *ex vivo*. Further optimization of sal003/C10 analogs may facilitate the development of cell-based therapies for muscle disease, or potentially overcome defects in MuSC expansion *in vivo*, which would be expected to delay the progression of DMD.

2.4 Materials and Methods

2.4.1 *Mice*

Pax3$^{GFP/+}$ mice were a gift from the Buckingham lab (Pasteur Institute, France). These mice contain an eGFP cassette replacing the first exon of Pax3 (Relaix et al., 2005). FVB-Tg(CAG-Luc,-GFP) L2G85Chco/J mice (Cao et al., 2004) referred to as *Tg(CAG-Luc,-GFP)* mice, were purchased from Jackson Laboratories. *Dmd*$^{mdx4cv/mdx4cv}$ referred to as *dmd*^{mdx} mice harbouring a non-sense point mutation in exon-53 of the *dmd* gene were purchased from Jackson Laboratories (Chapman et al., 1989; Shin et al., 2011). In order to isolate MuSCs by FACS from *Dmd*^{mdx} mice, these mice were crossed with aforementioned *Pax3*$^{GFP/+}$ mice to produce *Dmd*$^{mdx4cv/mdx4cv}$; *Pax3GFP*$^{+/-}$ mice. *Dmd*^{mdx} mice were also crossed with *Foxn1*$^{nu/nu}$ (Purchased from Jackson Laboratories) to produce *Dmd*$^{mdx4cv/mdx4cv}$; *Foxn1*$^{nu/nu}$ mice – an immunocompromised mouse model of Duchenne muscular dystrophy to act as recipients for transplantation assays.

2.4.2 *MuSC isolation and culture*

Satellite cells were isolated from the abdominals and diaphragm muscle of 5- to 8-week old *Pax3*$^{GFP/+}$, *Pax3*$^{GFP/+}$; *Dmd*^{mdx}, and *Pax3*$^{GFP/+}$; *Dmd*$^{mdx/y}$ mice as previously described (Monterras et al., 2005) using a FACSAriaIII cell sorter. Satellite cells were also isolated from the abdominals and diaphragm muscle of 5- to 8-week old *Tg(Cag-Luc,-GFP)* mice via the Miltenyi MACS Satellite Cell Isolation Kit, following by positive selection with Miltenyi Anti-Integrin α7 beads. Isolated MuSCs were cultured in 39% DMEM, 39% F12, 20% FBS, 2% UltroserG and when indicated, 0.1% DMSO (Control) and 5uM C10, 10uM sal003 or 100nM thapsigargin. C2C12 and Hek293T cells were cultured in DMEM with 10% FBS.

2.4.3 *Luciferase expansion assay*

400 MuSCs from *Tg(Cag-Luc,-GFP)* mice were seeded on a 96-well gelatin-coated plate in MuSC media with 10uM of candidate compounds, or DMSO (control). Bioluminescence (photons/second) was measured at regular intervals by replacing 10% of media with MuSC media supplemented with 1.5mg/ml D-Luciferin (Gold Biotechnology) and recorded using an IVIS Spectrum (Perkin Elmer)**.**

2.4.4 *Immunodetection*

Immunofluorescence labelling of cultured satellite cells and transverse sections of TA muscle was performed as previously described (Crist et al., 2009, Zismanov et al., 2016). For immunoblotting, cell lysates were prepared as previously described (Crist et al., 2009). ImageJ was used to determine the densitometry from immunoblots. Antibodies are described in Table S2

2.4.5 *RNA analysis*

RNA was isolated from cells in culture using TRIzol reagent (Life Technologies) and treated with DNase (Roche). RNA was reverse transcribed using the Superscript III reverse transcriptase (Life Technologies) using oligoDT primers. Primer sequences are described in Table S1.

2.4.6 *MuSC engraftments*

Immunocompromised 5- to 7-week-old *Foxn1*$^{nu/nu}$*; Dmd*^{mdx} mice were used as recipient mice for engraftment assays. Mice were exposed to 18Gy of irradiation to their right tibialis anterior muscle 24 hours prior to receiving the engraftment. 10,000 cells are suspended in 10ul PBS, loaded into a 10ul Hamilton syringe and introduced in a single injection longitudinally

throughout the tibialis anterior muscle. Mice were euthanized 21 days after the engraftment and the tibialis anterior muscle was isolated for immunodetection.

2.6.7 *Passaging MuSCs*

MuSCs were isolated by FACS as previously described and plated on 35mm gelatin-coated dishes at a density of 7,500 cells/plate. After 3 days in culture, cells were washed with PBS once and trypsinized with 0.05% trypsin at 37°C for 1 minute. Cells were re-suspended in 1ml 10% FBS, F12 media and centrifuged at 600g for 10 minutes at 4°C. Pellets were re-suspended in 100ul MuSC media, counted with a haemocytometer and re-plated at 2,000 cells/plate for wild-type and 1,000 cells/plate for Dmd^{mdx}. Cells were passaged every 2 days after this, following the same protocol.

2.6.8 *Lentiviral transduction of MuSCs for CRISPR/Cas9*

MuSCs from $Pax3^{GFP/+};Dmd^{mdx}$ mice were isolated by FACS as described earlier. After isolation, MuSCs were plated at 7,500 cells/plate in MuSC media and inoculated with LentiCRISPRv2-mCherry with sgRNAs targeting intron 51 and intron 53 or without sgRNA (Control) 1 day after plating. 24 hours later, satellite cells were washed and passaged as described earlier, at 1,000 cells/plate. 2 days after re-plating, single-cells were sorted based on mCherry expression into 96 wells by FACS and cultured with MuSC media for 6 days and differentiation media containing 10% horse serum instead of FBS for the next 4 days.

2.6.9 *Statistical analysis*

Graphical analysis is presented as mean ± SEM. At least three independent replicates of each experiment were performed. Unless otherwise indicated, significance was calculated using unpaired Student's t tests with two-tailed p values: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

2.5 Supplementary Materials

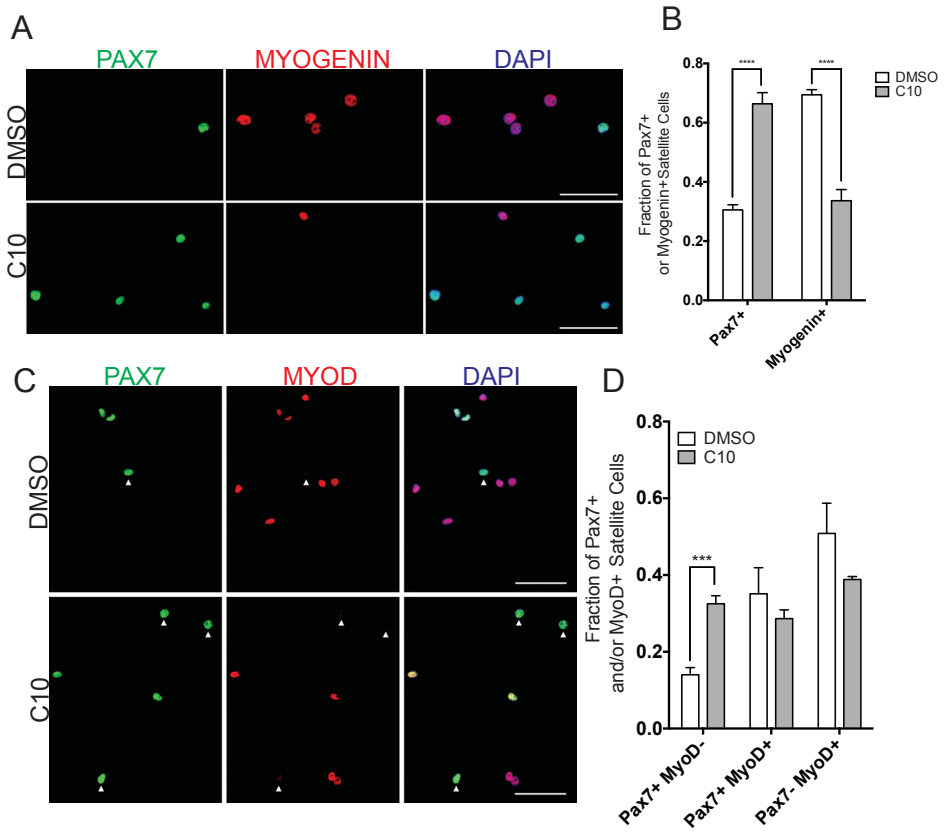

Figure 2 – Supplement 1. MuSCs isolated from *mdx* mice cultured in C10 have delayed differentiation and improved self-renewal *ex vivo*. (A) Immunofluorescence with antibodies against Pax7 (Green) and MyoD (Red) on MuSCs isolated from $Pax3^{GFP/+}$ mice after 4 day culture in 5μM C10 or DMSO (control). (B) Quantification of results from (A). (C) Immunofluorescence with antibodies against Pax7 (Green) and Myogenin (Red) on MuSCs isolated from $Pax3^{GFP/+}$ mice after 4 day culture in C10 or DMSO (control). (D) Quantification of results from (C). Scale bars represents 50μm. All values indicate mean (n≥3) ± SEM.

Table S1. List of primers

Type	*Gene*	*Fwd Primer*	*Rev Primer*
qPCR	Pax7	CTCAGTGAGTTCGATTAGCCG	AGACGGTTCCCTTTGTCGC
qPCR	MyoD	CCCCGGCGGCAGAATGGCTACG	GGTCTGGGTTCCCTGTTCTGTGT
qPCR	Myogenin	CAACCAGGAGGAGCGCGATCTCCG	AGGCGCTGTGGGAGTTGCATTCACT
qPCR	Actin	AAACATCCCCCAAAGTTCTAC	AAACATCCCCCAAAGTTCTAC
sgRNA	Dmd-i51	GATACTAGGGTGGCAAATAG	
sgRNA	Dmd-i53	GTGTTCTTAAAAGAATGGTG	

Table S2. List of antibodies

Antibody	*Source*	*Identifiers*	*Dilution factor*
Pax7	DHSB (Monoclonal mouse)		1/100
MyoD	Abcam (Polyclonal rabbit)	Ab203383	1/200
Myh1e	DHSB (MF20, Monoclonal mouse)		1/100
Myogenin	Abcam (EPR4789, Monoclonal rabbit)	Ab124800	1/100
Dystrophin	Pierce (Polyclonal rabbit)	PA137587	1/3000
GFP	Life Technologies (Polyclonal rabbit)	A-11122	1/3000
β-Actin	Sigma (AC-15, monoclonal mouse)	A5441	1/5000
P-eIF2α	Abcam (E90, monoclonal rabbit)	Ab32157	1/1000
eIF2α	Cell Signalling (L57A5, Monoclonal mouse)	2103	1/1000
Alexa Fluor 488, anti-mouse	Life Technologies (Polyclonal donkey)	A-21202	1/500
Alexa Fluor 488, anti-rabbit	Life Technologies (Polyclonal donkey)	A-21206	1/500
Alexa Fluor 594, anti-mouse	Life Technologies (Polyclonal donkey)	A-21203	1/500
Alexa Fluor 594, anti-rabbit	Life Technologies (Polyclonal donkey)	A-21207	1/500

Chapter III: Discussion

3.1 Novel analogs of sal003 promote *ex vivo* expansion in P-eIF2α-dependent and independent mechanisms

Through structure-activity relations, we developed 17 analogues of sal003 and screened them for their ability to expand satellite cells *ex vivo*. These compounds were developed based on the sal003 backbone, where small alterations to sal003 changed the functional properties of the molecule. Through this process we identified a number of interesting compounds – one being C9, which expanded satellite cells comparably to sal003 at 10μM, but did not increase P-eIF2α in the AlphaScreen (Figure 1B). This is a compelling finding, as it is well documented that sal003 increases P-eIF2α through inhibition of the eIF2α phosphatase Gadd34/PP1c (Boyce et al., 2005), and that maintenance of P-eIF2α is required for self-renewal (Zismanov et al., 2016). It has been shown that Gadd34 drives the specificity of PP1c catalytic activity, and sal003 inhibits the formation of this complex, thereby inhibiting eIF2α dephosphorylation (Boyce et al., 2005). Therefore, it would be intriguing to ask whether the small change in sal003 to generate C9 changes a putative interaction with PP1c to one of the many different protein phosphatase 1 regulatory subunits that are distinct from Gadd34. In order to test this, pull-down experiments of PP1c in cells cultured with C9 or DMSO controls, may reveal regulatory PP1c cofactors that are unable to bind during culture with C9. Candidate targets are likely involved in controlling satellite cell proliferation, as evidenced by the bioluminescence expansion assay of satellite cells cultured with 10μM C9 (Figure 1A). Since high levels of P-eIF2α are required for the *ex vivo* expansion of satellite cells that maintain the capacity to differentiate and self-renew, it would be interesting to test if the satellite cells cultured with C9 are capable of self-renewal and maintain

regenerative capacity, independent of P-eIF2α. Alternatively, C9 may promote the proliferation of committed myoblasts that have lost regenerative capacity.

Alternatively, Gadd34/PP1c has many other targets, including TGF-β Type I receptors, which have shown to inhibit both proliferation and differentiation of satellite cells (Shi et al., 2004; Allen and Boxhorn, 1989). Thereby, compounds that expand satellite cells *ex vivo*, but do not alter P-eIF2α levels, may still regulate phosphorylation events by interfering with the interaction between Gadd34/PP1c and alternative targets other than eIF2α. Therefore, alternative targets of Gadd34/PP1c may represent an additional mechanism by which analogues, and sal003 itself, allow for *ex vivo* expansion of satellite cells. We previously showed that the effect of sal003 is lost in satellite cells isolated from mice that are unable to phosphorylate eIF2α (Zismanov et al., 2016). However, since maintenance of P-eIF2α is necessary for *ex vivo* expansion, perhaps alternative targets of Gadd34/PP1c that promote cell proliferation were unable to overcome this initial deficit. Therefore, identifying proteins that interact with Gadd34/PP1c may represent novel targets for sal003 and its analogues that promote satellite cell proliferation.

We selected to study C10 as a novel analogue of sal003, due to its potent ability to increase P-eIF2α (Figure 1B), but initially expanded satellite cells poorly at 10μM (Figure 1A). However, titrating C10 from 10μM to 5μM allowed for continued expansion of satellite cells for 13 days (Figure 1D). It is unclear whether the delay in expansion of satellite cells in 10μM C10 was due to cells undergoing apoptosis, or if the satellite cells were maintained in quiescence. Although high levels of P-eIF2α selectively translate *Atf4* to alleviate stress, unresolvable stress leads to accumulation of Atf4 and Chop protein, eventually triggering apoptosis (Szegezdi et al., 2006). In order to resolve this, TUNEL assay and EdU incorporation can be used to mark apoptotic and

proliferating cells, respectfully, during culture with 10μM C10. If satellite cells cultured with 10μM C10 are apoptotic, culture with 5μM C10 and 10μM sal003 may represent a "Sweet Spot" of eIF2α phosphorylation, in which satellite cells may be expanded *ex vivo*. Therefore, compounds with increased potency to phosphorylate eIF2α may have a ceiling effect with regards to maintenance of satellite cell regenerative potential *ex vivo*, and may only function similarly to sal003 or C10, but at lower concentrations. In other words, it may not be feasible to elevate the percentage of Pax7(+)MyoD(-) population of cells greater than 40%, as increasing the concentration of inhibitors may simply result in apoptosis.

3.2 Proposed mechanism of C10: Do selectively translated mRNAs regulate satellite cell self-renewal?

Comparing the transcriptomes between freshly isolate satellite cells, to activated satellite cells in culture for 3 days, and satellite cells isolated from *Dmd*^{mdx} mice, has revealed key regulatory genes in maintaining the stemness signature of the satellite cell (Pallafacchina et al., 2010). However, as reviewed in Chapter 1.7, satellite cell fate is tightly controlled by post-transcriptional processes. Previous work in our lab has demonstrated that P-eIF2α is necessary for satellite cell quiescence, and that blocking the dephosphorylation of eIF2α promotes functional satellite cell expansion (Zismanov et al., 2016). While it is possible that simply reducing protein synthesis leads to the improved regenerative capacity observed when satellite cells are cultured in the presence of sal003 or C10, regulating global protein synthesis is likely only in part responsible. Alternatively, as discussed in Chapter 1.6.4, certain mRNAs are selectively translated under conditions of high P-eIF2α, and it is likely that mRNAs for regulators of satellite cell stem cell properties are selectively translated in a similar manner.

As discussed previously, canonical stress response genes such as *Atf4* and *Chop* are selectively translated because of uORFs in the 5'UTR of their mRNAs during conditions of high P-eIF2α (Harding et al., 2000; Vattem and Wek, 2004). There is some evidence to suggest *Atf4* may be responsible for maintenance of quiescence and self-renewal in satellite cells expressing P-eIF2α. *Atf4* function seems to be context-dependent, as in cancer, high P-eIF2α results in Atf4 expression that is necessary for proliferation, however overexpression of Atf4 in the mammary epithelium results in inhibited proliferation and differentiation (Bagheri-Yarmand et al., 2013; Ye et al., 2010). Aft4 has been shown to to form a heterdimer with C/EBPβ to activate transcription of various genes (Fu et al., 2015a). Further, C/EBPβ is involved in maintaining Pax7 expression in satellite cells, and is rapidly degraded upon differentiation (Tominaga et al., 2008). Therefore, experiments where Atf4 is over-expressed in satellite cells independent of P-eIF2α could reveal a novel role for Atf4 in maintaining Pax7 expression and thus delaying differentiation.

Alternatively, uORFs in the 5'UTR of mRNAs are prevalent in 49% of human and 44% of mouse genes, and have been widely shown to have regulatory function (Calvo et al., 2009). The regulatory properties of uORFs controlling protein translation are widely conserved across human, mouse and zebrafish, underlining their importance in protein expression (Chew et al., 2016). While the presence of uORFs in the 5'UTR of mRNAs do not necessitate that they are selectively translated, the location and size of uORFs dictate changes in translation due to P-eIF2α (Vattem and Wek, 2004). Because of their high prevalence in the genome, it is likely that mRNAs are selectively translated in similar mechanisms to *Atf4* and *Chop* during conditions of elevated P-eIF2α in order to maintain the stemness of muscle satellite cells. Experiments such as ribosome profiling to identify selectively translated mRNAs require ~10^6 cells (Ingolia et al.,

2012), limiting their feasibility in satellite cells, as typical satellite cell isolations yield ~4 x 10^4 cells/mouse. *Ex vivo* expansion of satellite cells with C10 can overcome this hurdle, as we demonstrate that we can expand cells ~1000-fold over 4 passages (Figure 4A). Therefore, expansion of satellite cells with C10 may facilitate previously impractical experiments to identify novel regulators of satellite cell stemness that are selectively translated during conditions of high P-eIF2α.

RT-qPCR and Western blotting revealed that Myogenin mRNA and protein was decreased when satellite cells were cultured with C10, but Pax7 protein increased with no significant change in mRNA (Figure 2). This disconnect between Pax7 mRNA and protein expression raises an intriguing possibility that *Pax7* mRNA is selectively translated when eIF2α is phosphorylated. *Pax7* lacks uORFs in its 5'UTR, so it is unlikely that it is selectively translated in a mechanism similar to *Atf4* or *Chop* (Vattem and Wek, 2004). Alternatively, *Pax7* has 5 in-frame start codons within the first 440bp of its mature mRNA, suggesting a potential mechanism for P-eIF2α-dependent translation of *Pax7*, as translation initiation at alternative AUG codons has been shown during conditions of stress (Sasaki et al., 2003). To identify if *Pax7* is selectively translated at alternative initiation sites, luciferase reporters cloned downstream of each start codon can reveal which initiation sites are most utilized during normal conditions and conditions of high P-eIF2α via culture with 5μM C10. Further, Western blotting could reveal if alternative Pax7 isoforms are translated during conditions of high P-eIF2α based on the size of the transcribed protein.

Additionally, control of Pax7 protein expression has been shown through other mechanisms, such as miRNA regulation via miR-206 and miR-486-dependent downregulation of Pax7 during differentiation (Dey et al., 2011). Although P-eIF2α does not have a direct

interaction with miRNAs, perhaps unknown selectively translated mRNAs discussed earlier control Pax7 protein expression indirectly through regulation of the micro-transcriptome. For example, Myostatin negatively regulates mIR-486 expression, thereby indirectly maintaining Pax7 protein expression in quiescent cells (Hitachi et al., 2014). Interestingly, *Myostatin* has an uORF in its 5'UTR, as well as 3 alternative start codons, marking it as a potential candidate for selective translation during conditions of elevated P-eIF2α.

3.3 *Ex vivo* expansion of *dmd*^{mdx} satellite cells with C10 implies role for inhibitors of eIF2α dephosphorylation for *in vivo* use

Similar to satellite cells derived from wild-type mice, we demonstrated that we were able to expand satellite cells derived from *Dmd*^{mdx4cv} mice for five passages: from 7,500 cells to ~8x10^6 cells. Previous work suggested that DMD is not simply a disease of the muscle, but also a disease of the satellite cells (Chapter 1.2.3). An explanation of this theory can be discerned by the fact that muscle is in a constant state of regeneration in children with DMD, which results in the exhaustion of satellite cells that lose their regenerative potential. Evidence of this was provided by the observation that satellite cells isolated from children with DMD had much shorter telomeres than age-matched controls, suggesting shortened telomere length from continuous cell division results in depletion of the satellite cell pool (Decary et al., 2000). This would explain why mouse models of DMD show a very mild phenotype compared to humans, likely due to their maintenance of long telomeres. Consistently, when *mdx* mice lose their telomerase activity, a severe muscle wasting phenotype is accentuated (Sacco et al., 2010). Further, dystrophin expression in the satellite cell itself is required for asymmetric division through PAR polarity (Dumont et al., 2015). *Ex vivo* expansion of functional *mdx* satellite cells has yet to be shown, likely because many satellite cells isolated from *mdx* mice are already

activated, and that self-renewal is likely inhibited because of the absence of functional dystrophin protein.

Because *mdx* satellite cells are efficiently expanded *ex vivo* with the small compound C10, systemic or intramuscular delivery of C10 may promote *in vivo* expansion of satellite cells as well. Interestingly, satellite cells isolated from *mdx* mice were ~40% Pax7(+)MyoD(-), compared to just over 20% in wild-type mice, while in both cases DMSO treated controls were ~10% Pax7(+)MyoD(-) (Figure 2). The robust effect of C10 on *mdx* satellite cells suggests that *in vivo* delivery of inhibitors of eIF2α dephosphorylation to *mdx* mice may produce greater regeneration compared to wild-type mouse models of injury. The regenerating muscle in *mdx* mice has a reduced percentage of quiescent satellite cells compared to healthy mice, due to its constant need to regenerate muscle, and state of chronic inflammation (Pannérec et al., 2012; Perandini et al., 2018). Therefore, it is likely that the satellite cell pool is not completely depleted because of clonal selection of more "resilient" satellite cells that are able to self-renew or remain quiescent in such a harsh environment. Perhaps this population of satellite cells is more sensitive to pharmacological intervention by virtue that they need less inhibition of eIF2α dephosphorylation to be coerced into a self-renewing Pax7(+)MyoD(-) state. Single-cell mRNA sequencing of satellite cells isolated from *mdx* or wild-type mice may reveal the heterogeneity of satellite cell populations through clustering analysis. Following delivery of C10 to *mdx* mice *in vivo*, satellite cell population and properties can be assessed by observing cross sectional slices of tibialis anterior muscle immunolabelled with antibodies against Pax7 and MyoD. Therefore, we could quantify the number of satellite cells present, as well as the percentage of activated satellite cells. Further, satellite cells along single isolated EDL muscle fibres can be observed to

determine if *in vivo* expansion with C10 reverses the failure of *mdx* satellite cells to divide asymmetrically (Dumont et al., 2015).

While future experiments will focus on the *in vivo* delivery of C10, systemic delivery of small compounds with a K_d <1μM are ideal as a therapeutic (Hajduk and Greer, 2007). C10 is effective at 5μM, therefore more novel inhibitors of eIF2α dephosphorylation should continue to be identified in future studies. The identification of novel inhibitors of eIF2α dephosphorylation can be pursued using three distinct strategies. First, we can use a structure activity relations approach by analyzing the structures of salubrinal, sal003 and C10 to create more potent analogs of salubrinal. Next, utilizing the crystal structure of the Gadd34:PP1 holoenzyme, novel compounds can be effectively designed to disrupt their interaction with each other or with eIF2α (Choy et al., 2015). Lastly, a shotgun, high throughput assay can be used to identify compounds that may inhibit eIF2α dephosphorylation. By cloning the *Atf4* 5'UTR upstream of a luciferase reporter, Sidrauski et al. (2013) identified the compound ISRIB, which reverses the effect of eIF2α phosphorylation. Therefore, this screen can be used to identify compounds that promote selective translation of luciferase in a P-eIF2α-dependent mechanism.

3.4 Applications of *ex vivo* expanded satellite cells cultured with C10

By expanding viable satellite cells, we developed culture conditions that enable the use of CRISPR/Cas9 to make genetic modifications in satellite cells *ex vivo*. In order to restore Dystrophin expression in Dmd^{mdx4cv} mice, we transduced satellite cells with lentiviruses coding for mCherry, Cas9 and sgRNAs targeting intron 51 and 53. These sgRNAs create double stranded breaks flanking the mutated exon 53 in Dmd^{mdx4cv} mice, restoring the original reading frame and thus a functional dystrophin protein (As discussed in Chapter 1.2.3). By expanding

mCherry(+) or mCherry (-) satellite cells, and then allowing them to differentiate, we were able to observe mCherry(+),Dystrophin(+), but not mCherry(-),Dystrophin(+) myotubes. While this CRISPR/Cas9 strategy to restore Dystrophin expression has been implemented *in vivo* via systemic delivery of AAV, it has yet to be demonstrated in *ex vivo* expanded satellite cells (Bengtsson et al., 2017; Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). By transducing satellite cells *ex vivo* prior to engraftment, many of the adverse effects, such as the host-immune response, are mitigated compared to systemic delivery of virus. Additionally, by correcting satellite cells *ex vivo*, the self-renewed cells after engraftment will be capable of contributing to new healthy muscle following future injury. This is an important distinction, as systematic delivery of AAV have been shown to target myofibres efficiently, but failed to efficiently transduce resident, quiescent satellite cells. Our future experiments will focus on engrafting DMD-corrected satellite cells into an *mdx* mouse model, thus demonstrating that *ex vivo* correction of *Dmd*, followed by engraftment of autologous satellite cells is possible.

The use of satellite cells for cell-based therapies is primarily hindered by: 1) the scarcity of satellite cells in muscle, 2) the inability to deliver satellite cells systemically, and 3) satellite cells migrate poorly from the site of engraftment. While the *ex vivo* expansion of satellite cells with C10 overcomes the low number of cells, other strategies must be developed to overcome the other hurdles before satellite cells are used to treat muscle disease.

Genetic alterations and subsequent expansion of satellite cells can be used as a valuable research tool. Since satellite cells rapidly lose their regenerative potential, culture with C10 allows for researchers to create precise genetic modifications and expand functional satellite cells *ex vivo*. CRISPR/Cas9 genome-wide screens have been used to effectively identify genes involved in cancer-drug resistances in human cell lines and can be transitioned to satellite cells

(Shalem et al., 2014). For example, CRISPR/Cas9 screens combined with our luciferase expansion assay (Figure 1A) allow for the screening of genes involved in the proliferation of satellite cells. Further, we have shown that satellite cells cultured in C10 are capable of differentiating and fusing after expansion, allowing for the screening of genes that play a role in differentiation and fusion. Other experimental outcomes, such as mRNA levels using RT-qPCR, can be used to assess the composition of these expanded satellite cells in a high-throughput pipeline. Further, in addition to transduction of lentiviruses containing CRISPR/Cas9, overexpression vectors can be used in similar screens to identify the role of a particular gene in the context of the satellite cell.

Studying the properties of satellite cells cultured in 5uM C10 may reveal novel regulatory pathways that promote self-renewal. While we have demonstrated that P-eIF2α drives the *ex vivo* expansion of satellite cells with regenerative capacity (Zismanov et al., 2016), it likely does so by maintaining an expression profile of genes that promote self-renewal and proliferation in culture, while delaying differentiation. As discussed in Chapter 3.2, inhibition of eIF2α dephosphorylation may promote selective translation of certain mRNAs that play a role in maintaining the stemness of satellite cells. Further, comparing the transcriptome of satellite cells cultured in 5uM C10 to previously published RNAseq data from quiescent, *Dmd*^{mdx} and cultured satellite cells, will likely reveal regulatory pathways that promote self-renewal, activation and differentiation (Pallafacchina et al., 2010). Therefore, the culture of satellite cells with C10 may facilitate future research by acting as a tool to expand satellite cells *ex vivo*. The study of satellite cells cultured with C10 *ex vivo* may also directly reveal novel regulators of satellite cell fate.

Chapter IV: Conclusion and Future Directions

Here we identified the sal003 analog, C10, as a novel inhibitor of eIF2α dephosphorylation. We demonstrate that C10 is a more potent inhibitor of eIF2α dephosphorylation than sal003, and is capable of increasing levels of P-eIF2α at lower concentrations. Satellite cells cultured in the presence of 5μM C10 were able to be expanded *ex vivo*, while retaining their regenerative potential. *Ex vivo* expansion of satellite cells is critical for the future study and therapeutic potential of satellite cells. We demonstrated the application of *ex vivo* expanded satellite cells by using CRISPR/Cas9 to produce functional dystrophin protein in Dmd^{mdx4cv} derived satellite cells. As discussed in Chapter III, future research will be threefold: 1) Identify novel analogs of salubrinal with increased potency, 2) Test the effectiveness of *in vivo* delivery of C10 to expand satellite cells in mouse models of DMD, and 3) Leverage the ability of C10 to expand viable satellite cells *ex vivo* for the use as a research tool.

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