Ex Vivo Expansion of Skeletal Muscle Stem Cells by Selective Targeting of the eIF2α Pathway.

Master's Thesis

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AUTHOR CONTRIBUTIONS:

A significant number of the results presented in this thesis were published as part of an extended manuscript, below are listed all the contributions of the authors. Colin Crist conceived and designed the study. Antonis E. Koromilas provided guidance throughout. Victor Chichkov performed experiments and collected data for all figures presented in this thesis. Victoria Zismanov, Veronica Colangelo, Solene Jamet, and Colin Crist performed experiments and collected data for all other figures presented in *Zismanov et al,Cell Stem Cell. 2016 Jan 7;18(1):79-90* . Shuo Wang provided expertise for sucrose gradient and polysome fractionation. Alasdair Syme irradiated mouse hindlimbs. Colin Crist analyzed data and wrote the manuscript for *Zismanov et al, Cell Stem Cell. 2016 Jan 7;18(1):79-90.*

Abstract:

Disorders of skeletal muscle characterized by degeneration of muscle make up the family of muscular dystrophies, aging associated sarcopenia and cancer cachexia. Development of stem cell based therapies for the muscular dystrophies aim to replace the pool of skeletal muscle stem cells required for muscle regeneration. For long-term stem cell based therapy to be effective, transplanted stem cells must not only contribute to the regeneration of healthy muscle, but must also 'self-renew' and be present throughout the lifetime of the individual. The importance of self-renewal is illustrated by the total failure of engrafted myoblasts, which lost the stem-cell capacity to self-renew, to provide any benefit to the muscle of patients with Duchene muscular dystrophy in early clinical trials performed in the 1990s. These failures indicate that strategies are necessary to optimize the self-renewal capacity of skeletal muscle stem cells both during *ex vivo* expansion and after their engraftment. Analysis by our group showed that satellite cell self-renewal is regulated by phosphorylation of eIF2α, as its genetic ablation inhibits self-renewal *in vivo*. In this manuscript, we provide additional evidence to support this theory. We show that pharmacological inhibition of eIF2α dephosphorylation promotes skeletal muscle stem cell self-renewal and expansion, and improves their capacity to engraft.

Abrégé :

Les maladies des muscles squelettiques caractérisés par une dégénérescence des muscles constituent la famille des dystrophies musculaires, la dégénérescence associée au vieillissement (sarcopénie) et la cachexie cancéreuse. Le développement de thérapies à base de cellules souches pour les dystrophies musculaires a pour but de augmenter la quantité de cellules souches musculaires squelettiques nécessaires pour la régénération musculaire. Pour que la thérapie de cellules souches à long terme soit efficace, les cellules souches transplantées ne doivent pas seulement contribuer à la régénération du muscle, mais doivent aussi «se renouveler» et être présents pendant toute la durée de vie de l'individu. L'importance de l'auto-renouvellement est illustré par l'échec total de myoblastes greffées, qui ont perdu leur capacités de cellules souches : soit d'auto-renouvellement, de fournir toute prestation au muscle des patients atteints de dystrophie musculaire de Duchenne dans les premiers essais cliniques effectués dans les années 1990. Ces échecs indiquent que les stratégies sont nécessaires pour optimiser la capacité d'auto-renouvellement des cellules souches du muscle squelettique à la fois pendant l'expansion *ex vivo* et après leur greffe. L'analyse effectuée par notre groupe a montré que l'auto-renouvellement de cellules satellites est régulée par la phosphorylation de eIF2α, presque son ablation génétique inhibe l'auto-renouvellement *in vivo*. Dans ce manuscrit, nous fournissons des preuves supplémentaires à l'appui de cette théorie. Nous montrons que l'inhibition pharmacologique de la déphosphorylation de eIF2α favorise l'auto-renouvellement et l'expansion des cellules souches ainsi que l'amélioration de leur capacité à greffer.

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List of Abbreviations:

2'OMe-2'O- methylphosphorothioate

ADARs - Adenosine Deaminases Acting on RNA

ADP- Adenosine Diphosphate

AGO – Argonaute

ARE -AU Rich Element

ASC -Adult Somatic Stem Cells

ATF4 –activating Transcription Factor 4

ATP – Adenosine Triphosphate

CDK Cyclin Dependent Kinase

CHOP - CCAAT/Enhancer-Binding Protein Homologous Protein

CPSF -Cleavage and Polyadenylation Specificity Factor-

DGC-The Dystrophin-Glycoprotein Complex

DMD- Duchene's Muscular Dystrophy

E- Embryonic Day

eIF –Eukaryotic Initiation Factor

ELAV –Embryonic Lethal, Abnormal Vision

ER- Endoplasmic Reticulum

FSHD-Facioscapulohumeral Muscular Dystrophy

GFP –Green Fluorescent Protein

GSCs-Germ line Stem Cells

HNRNP - heterogeneous nuclear ribonucleoproteins

HSC-Hematopoietic Stem Cells

HSR-Heat Shock Response

ISC- Intestinal Stem Cells

kDa- kilodalton

LGMD -Limb-Girdle Muscular Dystrophies

microRNA – miR or miRNA

Mrf4 – Myogenic Regulatory Factor 4

mRNP – messenger Ribonucleoprotein

Myf5 – Myogenic Factor 5

MyoD –Myogenic Differentiation 1

MyoG – Myogenin

NMD-Nonsense Mediated mRNA Decay

Pax3 – Paired Box 3

Pax7 –Paired box 7

PERK - PKR-Like Endoplasmic Reticulum Kinase

PMOs-phosphoroduamidate morpholino oligomers

PP1-Protein Phosphatase 1

RISC-RNA induced Silencing Complex

ROS- Reactive oxygen species

Ser51- Serine at position 51

SERCA-Sarcoplasmic Reticulum Calcium ATPase

SOCS3 –Suppressor of cytokine signalling

SR – Sarcoplasmic Reticulum

SRSF - Ser/Arg Rich splicing factors

TAC-transiently amplifying progenitor cell population

TnT –TroponinT

uORF-Upstream Open Reading Frame

1. Introduction:

1.1. Skeletal Muscle – Function and Structure:

Striated skeletal muscle is a structurally complex tissue largely composed of functionally specialized post-mitotic cells termed myofibers[1-4]. Each myofiber is surrounded by a basement membrane enriched with extracellular matrix proteins, commonly known as the endomysium[1-4]. Bundles of myofibers are connected by perimysium, and form a structure termed fascicle[1-4]. Multiple fascicles are encapsulated within a layer of epimisium to form a functional muscle unit. The three different layers of connective tissue provide a biomechanical scaffold for extensive blood vessel formation and innervation, ensuring adequate nutrient supply and coordinated signalling from the CNS[1-4] (Figure 1). Each muscle belly is connected to its relevant bone structure by a tendon, which is composed of a specialized connective tissue enriched with Collagen Type-I and adopted to withstand tension[1-4]. Broadly, muscle fibres are divided into two categories: Fast twitch muscle- “Type II”, is responsible for producing short and rapid contraction, suitable for voluntary movement; Slow twitch muscle – “Type I” is oriented towards slow and prolonged contractions, commonly necessary in maintenance of posture[1-4]. Despite differences in functional specialization, all myofibers contain several evolutionary conserved, structural components that permit their contractile function. They are primarily:

The Sarcomere:

The sarcomere is the central unit of contraction, is microscopically defined by the repetitive formation of I- and A-bands. The I-band mostly contains "thin" filaments principally

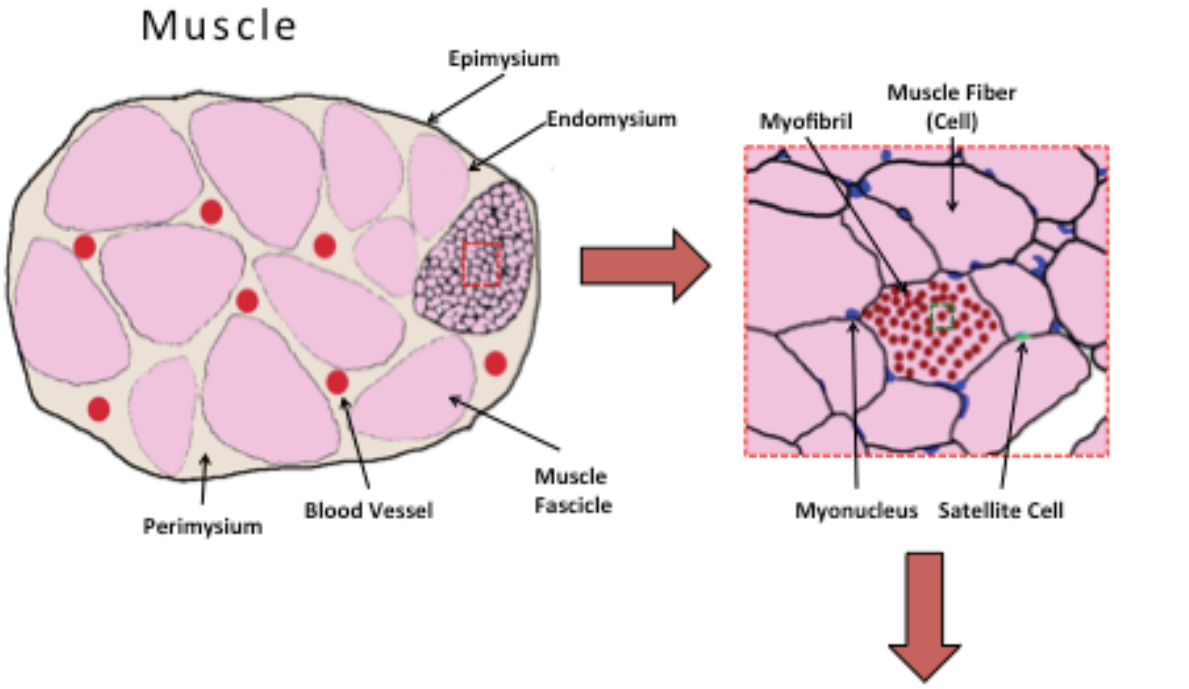

Figure 1.1 Schematic representation of muscular structure units. From left to right, increased magnifications of a muscle cross-section. Muscle is composed of bundles of specialized muscle cell, termed muscle fibres, which retain a hierarchy of increasingly thinner fibres grouped within larger fibre groups. Connective tissue composes the Epi-,Peri- and Endomysium and permits vascularization (depicted) and innervation (not depicted). Satellite cells (marked green) reside on top of myofibers and under the basement membrane (endomysium). Myofibril structure is presented longitudinally to facilitate depiction of sarcomere structure.

made of G-actin, which protrude towards the center of the sarcomere and overlap with the A-band[2, 4]. It is bisected by a structure termed the Z-Disk, which serves to anchor the sarcomere to the cytoskeleton as well as the plasma membrane[4]. A lighter band, termed the

H-zone, bisects the A-band, itself bisected by a middle M-line. The M-line serves as an anchoring point for “thick” Myosin chains, which protrude from the M-line towards the Z-disk and overlap the thin filaments throughout the A-band[2, 4]. Extensive analysis of this structure showed that muscle contraction is mediated through an ATP dependent bind-and-release mechanism, called the “sliding-filament model”. Briefly, inactive myosin maintains a stable steady state conformation by binding of ATP to its head domain. Upon initiation of a contractile event, the ATP is hydrolyzed to ADP, which physically re-orients myosin and energizes the protein. In parallel, Ca^{2+} mediated signalling induces conformational re-orientation of the thin filament and exposes an actin binding site for myosin. Upon binding the filament, ADP is hydrolyzed and myosin regains its original conformation, thus pulling the thin filament and increasing the overlap between the thick and thin filaments[2, 4]. Thus, sarcomeres are shortened and force is transmitted longitudinally along the muscle fibre, and perpendicularly through interaction with the Z-disks. Relaxation is mediated by de-novo binding of ATP to myosin, which permits thick filaments to detach from actin, and “slide back” to their original position. Repetitive sarcomere units within the myofiber form a myofibril and several myofibrils are found within each myofiber[2, 4].

The Sarcoplasmic Reticulum:

The second major component that ensures proper coordinated muscle contraction is the sarcoplasmic reticulum (SR), which is a specialized extension of the endoplasmic reticulum dedicated to control and storage of intracellular Ca^{2+} within muscle fibres[5]. Again, multiple proteins within the SR are orchestrating Ca^{2+} release upon electrophysiological stimulation. Ryanodine and dihydropiridyine receptors are responsible for depolarization induced and calcium induced Ca^{2+} release from the ER, respectively[5]. After depolarization, Sarcoplasmic Reticulum Calcium ATPase pumps (SERCAs) are responsible for uptake of cytosolic Ca^{2+} back to the SR, which is than sequestered by Ca^{2+} binding elements, such as calsequestrin. Different isoforms, protein abundance and signalling differences are responsible for a broad range of contractile specialization found in different fibre types[2, 5].

The Dystrophin-Glycoprotein Complex (DGC):

It has been noted early on that coordinated muscle contraction poses a significant burden on the structural composition of the myofiber. In order to efficiently transmit force, maintain integrity and preserve flexibility, a complex system of cytoskeletal and extracellular components was evolved[6, 7]. Before considering the DGC, it is important to note that numerous other components of the sarcomere, such as various Z- and M- band associated proteins play a significant role in stabilization and coordination of contractile activity. For example, Titin is a protein that plays a key role in stabilization and anchoring of contractile machinery to the Z-disk as well as the M-band[2]. Several isoforms of the protein have been characterized, and their different biophysical properties are thought to play a role in defining muscle fibre characteristics. Nevertheless, the DGC is another major component of the muscle fibres cytoskeleton, localized to the Z-Disk and forms a special structure known as costamere at the cell membrane[2, 7]. The DGC facilitates Z-disk anchoring to the plasma membrane, as well as to elements of the extracellular matrix surrounding the fibre. The DGC is composed of multiple intracellular and trans-membrane components. The principle components embedded in the cell membrane are dystroglycan, sarcoglycan and sarcospan. The first is a laminin receptor composed of one α and

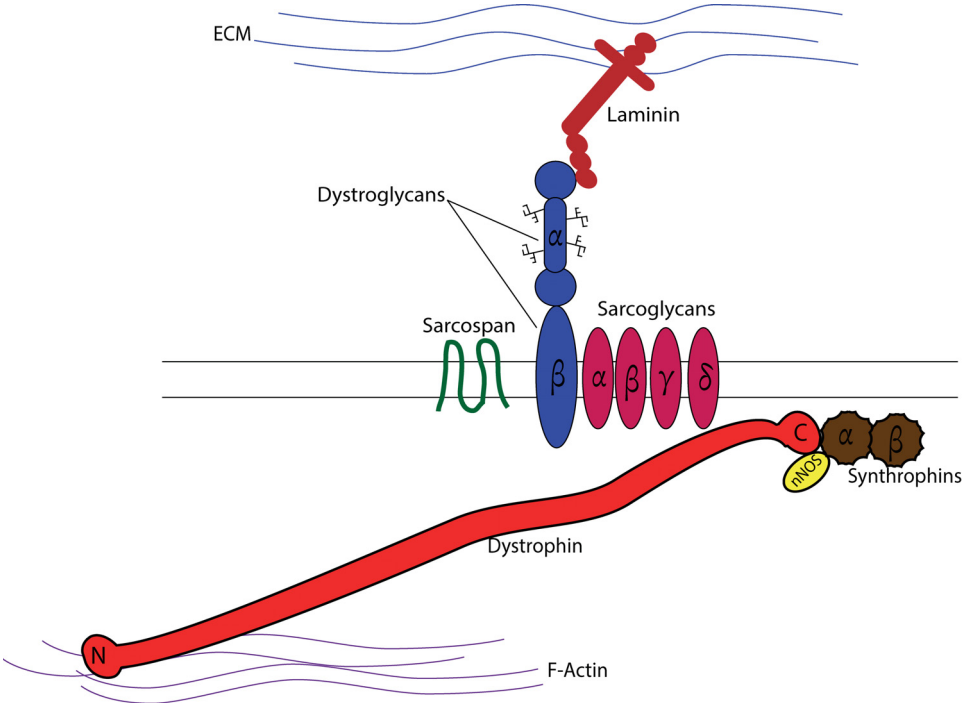

Figure 1.2 Schematic representation of the Dystrophin-Glycoprotein Complex: Multiple components of the Dystrophin-Glycoprotein Complex interact together to promote structural and mechanical stability during muscle contraction and rest.

one β subunits that together bind extracellular matrix in a broad range of tissues; the second is a multiunit subcomplex believed to interact and stabilize other members of the DGC; and the last is a four-domain trans-membrane protein that is co-localized to the DGC but is non-essential for DGC functionality[2, 7]. On the cytoplasmic side, the principal component of the complex is dystrophin, a large 427 kDa Rod-like protein. Its N-terminal domain contains a γ-actin binding site that permits binding of the cytoskeleton, while its C-terminus domain forms a complex with caveolin, nNOS, dystrobrevins and syntrophin to bind the plasma membrane[2, 4, 6-8]. The elongated Rod-domain comprises 24 spectrin-like repeats, which are 100 amino acids long, triple helix structures with four hinge sites interspersed between them. The presence of hinges provides the protein with flexibility while the spectrin domains are also believed to interact with a variety of cytoskeletal elements. The effect of mutations in several of the above mentioned components are further detailed in section 1.3 (Myopathies) [2, 4, 6-8].

While broadly described, all three components discussed above ensure successful and coordinated activation of skeletal muscle contraction, thus guaranteeing a rapid functional response. While great inter-species variation in muscle types, fiber compositions and general anatomy occurred throughout evolution; the core structure of contractile muscle fibers remained highly conserved.

1.2. Muscle Development:

Myofibers are long, contractile and multi-nucleated syncytia, which arise from fusion of dermomyotome derived myoblastas throughout embryogenesis[1-3]. Embryonic development of skeletal muscle is a precisely orchestrated and highly regulated process. In early development, paraxial mesoderm is sequentially segmented into epithelial like structures known as somites. While the ventral segment of the somites undergoes epithelial-to-mesenchymal transition to generate the sclerotome, later giving rise to bone and cartilage, the dorsal part termed dermomyotome, remains epithelial and upregulates high levels of Pax3 by embryonic day (E) 8.5. These progenitors go on to delaminate and form the underlying myotome, the first skeletal muscle structure of the embryo. Progressively, Pax3 progenitors in the dermomyotome upregulate the myogenic determination factors Myf5 and MyoD, followed

by Myogenin and Mrf4[4, 9, 10]. Later on, Pax3 and Pax7 expressing dermomyotome cells delaminate to further expand the myotome up to E12[4, 9-11]. By E14.5, myotome derived progenitors fuse to form multinucleated syncytia, which are then cleaved and reorganized to give rise to tissue masses that will underlay trunk musculature. In a closely related process, Pax3+ dermomyotome cells delaminate from the hypaxial dermomyotome and migrate to the emerging limb bud to give rise to limb muscle. As this process continues, Pax3 is gradually downregulated and Pax7 expression dominates. Throughout this process, certain muscle progenitors go on to occupy the adult skeletal muscle position to become adult satellite cells, which closely recapitulate their embryonic counterparts later in life[4, 9-11]. The transcriptional cascades that dictate how single progenitors multiply and fuse to form muscle were largely understood from observation of adult myogenesis, and are therefore listed in section 1.7 below. Initial muscle development gives rise to primary myofibers with slow twitch properties, with later establishment of neuromuscular junctions and excitation-contraction coupling that leads to secondary, termed "foetal", myogenesis by E17[4]. In this process, a pool of ever expanding progenitors forms secondary, smaller myofibers with exclusive fast twitch properties. Finally, further refining of muscle fibre functionality occurs in the post natal stage, where some fast twitch fibres are gradually converted to slow-twitch, and vice versa. Development of head and neck skeletal muscle follows a distinct transcriptional cascade, where the transcription factor pitx2 plays a dominant role in coordinating genetic events that lead to MRF upregulation and progenitor generation[4]

1.3. Myopathies:

Skeletal muscle provides the underlying physiological foundation to our daily activities. Peripheral muscles in our limbs permit complex manipulation of objects, which underlies the basis of most human activity and technological development. Hence, loss of muscle functionality greatly impacts an individual's quality of life. In addition to locomotion, skeletal muscle in the trunk also supports involuntary activity, such as respiration and posture. *Respiratory* muscle dysfunction is the common underlying cause of death in patients suffering from skeletal muscle disorders[1, 3, 6]. Skeletal muscle disease collectively referred to as myopathies, comprise a variable group of underlying causes. Many environmental factors can

lead to decline in muscle functionality, such as life style choices that reflect in an individual's diet and physical activity regimes, as well as substance consumption[1]. Muscle function loss can also occur in the context of a broader disorder such as autoimmune or infectious diseases[1]. Importantly, inherited genetic factors can produce congenital myopathies that manifest in childhood and exacerbate towards adolescence, often ending in premature death. Here we will discuss the most prevalent genetic disorders that impact quality of life at an early age, as well currently available and prospective treatments.

1.3.1. Dystrophinopathies*:*

Pathophysiology of Dystrophinopathies:

The most common genetic myopathy is Duchene's Muscular Dystrophy, which affects 1 in 3500 male patients due to the *DMD* gene's localization to a 2.4 Mb region of chromosome Xp.21 [6]. The *DMD* locus spans 79 exons and its enormous size makes it prone to various mutations, but the majority of de-novo changes were found to be deletions that are confined to two mutation prone hot spots, at the 5' end and the center of the gene[12]. The severity of the DMD phenotype is caused by the occurrence of out-of frame, nonsense mutations that completely disrupt the reading frame or introduce early termination codons to the translational reading frame of the protein[8, 12]. In instances where translation of such transcripts is possible, it produces truncated proteins that lack many, if not all, functional domains of dystrophin and result in loss of function[13, 14]. Loss of dystrophin leads to complete disruption of the DGC, structural destabilization of the muscle fibre cytoskeleton, and suboptimal force distribution throughout contractions[1, 6]. Thus, frequent muscle contraction damages the muscle fibre and induces fibre necrosis, immune infiltration and activation of a regenerative response. The lack of stable DGC complexes In DMD patients ensures that this event occurs repeatedly throughout their lifetime, creating a vicious cycle of muscle degeneration followed by regeneration[1, 6, 15]. Eventually, tissue remodelling induces replacement of muscle tissue by fibrotic scar and fat tissues, and loss of contractile function.

In frame *DMD* mutations induce internal truncations, but maintain a reading frame and lead to generation of a viable transcript that can be translated to protein with partial or almost complete function. The disorder presents a broader spectrum of symptoms, and is medically

recognized as Becker's Muscular Dystrophy[6].

Diagnosis and Treatment of Dystrophinopathies:

DMD patients are typically identified in early life, between the ages of 3 to 5. Primary symptoms include ambulatory abnormalities such as delayed walking; frequent falls; difficulty navigating obstacles; toe curling while walking; waddling gait and progressive difficulty in limb movement[8, 12]. Patients also demonstrate pseudohypertrophied calves, which are enlarged due to excessive deposition of fat and scar tissue, and posture abnormalities that include protruded belly and slanted back shoulders[1]. Another common symptom that can assist diagnosis is detection of elevated levels of serum creatine kinase, which is released from muscle tissue upon cell death[6]. Clinical diagnosis today can be confirmed through genetic analysis and identification of the precise mutation present in each patient, which helps distinguish de-novo from inherited alleles, which benefits both patient and family[6, 16]. Muscle biopsies are also subjected to analysis by molecular and immunohistological methods, which permits quantitation of Dystrophin expression and assessment of disease progress[6, 17]. Importantly, dystrophin positive fibres, termed revertant fibres, are commonly detected in DMD patients and shown to increase with age[6, 12].

Currently, no significant treatment of Duchene's Muscular Dystrophy is available. Improvement in screening and diagnosis method allow early identification of the disease, which permits proactive management of symptoms[18]. Administration of corticosteroids is the most common treatment, which reduces inflammation in diseased tissues and slows down muscle wasting and loss of ambulation by 1 to 3 years. However, corticosteroids exert a systemic effect on the body, and frequent administration is associated with negative side effects such as weight gain and reduced bone density.[6, 18] Physical therapy and, in later years, assisted ventilation are used to improve quality of life and extend patients' lifespans[19]. Alternative approaches to slow or halt physiological decline of dystrophic muscle are actively sought for, with some reviewed below.

1.3.2. Limb-Girdle Muscular Dystrophies:

Pathophysiology of LGMDs:

[1] https://www.mda.org/disease/duchenne-muscular-dystrophy/signs-and-symptoms

Limb-Girdle Muscular Dystrophies (LGMD) are a highly heterogeneous group of disorders classified together based on the phenotypic feature of progressive weakness and wasting of specific proximal and distal muscle groups[17]. Many subtypes of the disease also present some form of cardiomyopathy and other symptoms that may overlap with other disorders due to the underlying mutation[6, 17]. Traditionally, LGMD are classified into two classes: LGMD 1 refers to disorders with autosomal dominant inheritance, while LGMD 2 refers to autosomal recessive transmission. In recent years, Next Generation Sequencing techniques identified over 30 subtypes of LGMD, with further identification of various rare variants within the underlying genes[6, 17]. The genes identified are either direct members of the DGC (sarcoglycans and dystroglycan [LGMD2C-F, P]) or involved in their processing (Fukutin-related proteins and Protein-O-Mannosyl trasnferase [LGMD2I and K]). Other genes are involved in sarcomere function (Myotilin, Calpain3, Titin and Telethonin [LGMD1A, LGMD2A, G, J]). Finally, genes involved in membrane repair and integrity, such as dysferlin, caveolin 3 and desmin were also identified (LGMD2B, LGMD1C and LGMD1E)[6, 17]. The increasing number of identified genes, as well as the molecular heterogeneity of the pathways involved, has pushed for reconsideration of nomenclature conventions for these disorders, replacing phenotype based nomenclature by a protein based system (ex. Dystroglycanopathies, Calpainopathies, etc.). Most of the causative mutations contribute to fibre structure destabilization, ultimately leading to cell death, inflammation and regeneration. While not as severe as DMD, loss of function still occurs in LGMD patients to variable extents[18].

Diagnosis and Treatment of LGMDs:

The autosomal nature of LGMD disorders usually presents in families with previous history of the disease, which made them amenable to traditional linkage mapping for gene identification. Today, the continuous decrease in cost of next generation sequencing techniques, and specifically exome sequencing, made patient specific diagnosis and cataloguing feasible. Immunodetection techniques are still used to confirm the pathological progress of the specific disease, as well as to analyze any additional effects in specific mutations[17]. Due to the genetic nature and broad spectrum of the disorders, no long-term solutions are available.

Similar to DMD and BMD patients, LGMD patients receive supportive care and symptom management[6].

1.3.3. Facioscapulohumeral Muscular Dystrophy

Pathophysiology of FSHD:

Facioscapulohumeral Muscular Dystrophy (FSHD) is a common disorder that affects between 1:8000 and 1:20000 people[20]. It is inherited in an autosomal dominant manner and with variable degrees of penetrance in different patients and even family members (down to 70% in some families). Clinical symptoms include progressive weakness of specific muscle groups of the face, torso, and limbs, often starting in the head and moving down the body. Distribution and order of muscle weakening is also variable, with some 15% of patients not presenting facial muscle weakness at all[6, 20]. Mild symptoms have been observed in older relatives of symptomatic patients, which first indicated a potential mechanism of genetic anticipation in the disease. The molecular pathogenesis of the disease has been recently identified, and shown to be promoted by aberrant expression of the transcription factor DUX4. DUX4 is normally dormant in somatic tissue due to its specific location in a highly methylated subtelomeric region of chromosome 4q35[6, 20]. In normal conditions, DUX4 coding sequences are located within 3.3kb tandem macrosatellite repeats, termed D4Z4, which number between 11 and 100 in asymptomatic carriers. When the number of macrosatellite repeats is reduced to less than 10, as found in FSHD patients, chromatin remodelling occurs, and extensive decrease in methylation allows access of transcription machinery to the DUX4 promoter. In addition, a pLAM1 Polyadenylation signal must be present downstream of the transcribed DUX4 locus, to ensure mRNA stability. The above features are found in 95% of FSHD patients, while a minority of less than 5% demonstrates hypomethylation despite high numbers of D4Z4 repeats[6, 20]. In these patients, other genes such as structural maintenance of chromosomal hinge 1 (SMCHD1) and DNA methyltransferase 3B (DNMT3B) are lost in a digenic manner that reduces methylation. Thus, the inverse correlation between D4Z4 repeat length and methylation status was confirmed, with patients that have 3-4 repeats demonstrating the highest rates of penetrance >85%). Overexpression of DUX4 *in vitro* showed high levels of toxicity in muscle

where increased rates of apoptosis and oxidative stress were observed along with inhibited myogenesis[6, 20]. Histology and gene expression profiling of FSHD muscles show severe and progressively increasing immune cell infiltration and activation of innate, adoptive immune response pathways[6, 20]. Thus, it is believed that DUX4 gene products as well as DUX4 target genes actively induce immune responses and perturbed cell function when expressed in adult somatic context. However, the direct cause of muscle fibre degeneration is yet to be definitively elucidated.

Diagnosis and Treatment of FSHD:

FSHD is diagnosed clinically and often confirmed with standard genetic testing methods such as restriction digests and southern blot to determine the length of D4Z4 truncation. Additional testing is required in cases of non-truncated FSHD. No cure or significant treatment is available now, and symptoms are mostly managed through physical and occupational therapy, while severe complications require surgical interventions such as scapular fixation.

1.3.4. Muscle Ageing:

As reviewed above, genetic as well as acquired factors can lead to major skeletal muscle pathology resulting in loss of function and impact on our quality of life. Nevertheless, it is important to note that in most individuals, muscle function loss occurs naturally with ageing and is termed “sarcopenia”. Age dependent loss of function has been well documented in multiple tissue types of a variety of organisms, especially in post-mitotic tissue such as muscle and brain[1]. Phenotypically, extensive loss of type II fibre number and innervation has been documented in aged muscle[1, 21]. Fibre loss has been documented as early as at the age of 25, and total mass reduction is estimated to be at 40% by the age of 80 in both men and women. Metabolic activity in aged muscle fibres is skewed towards increased catabolism, as evidenced by increased expression of lysosomal factors, and reduced protein synthesis. Elevated levels of pro-inflammatory cytokines such as TNF-a are believed to be playing a role in stimulation of reactive oxygen species (ROS) production in mitochondria, further contributing to oxidative stress in the muscle fibre[1, 21].

1.4. Prospective Strategies for Treatment of Muscular Dystrophies:

Advances in our understanding of muscle function and disease have opened the avenue to multiple new approaches for treatment of these debilitating conditions. Improvements in gene delivery methods and stem cell biology have made gene therapy and cell therapy based approaches feasible at the clinical levels. However, previous trials with either approach highlight the challenge of applying such strategies in the context of skeletal muscle. Each method and its challenges will be further elaborated below.

1.4.1. Gene Therapy:

Endogenous Repair by Exon Skipping and Anti-Sense Oligonucleotides:

These strategies are primarily directed at patients of Duchene muscular dystrophy, although if successful, they could be applied to other diseases in a context specific manner. The rational behind this strategy is derived from the observation that BMD patients do not exhibit severe disease phenotypes compared to DMD patients[12]. This it attributed to the observation that BMD mutations involve internal truncations that shorten dystrophin, but preserve large parts of the N and C terminal segments of the protein and thus maintains function, unlike DMD mutations where large segments are lost, including all C terminal domains. Therefore, strategies were developed to allow skipping of the premature stop codon and generation of an internally truncated protein isoforms found in BMD patients. To achieve this, antisense oligonucleotide technologies have been deployed. Among several developed chemistries, two are currently in clinical trials: 2'O- methylphosphorothioate (2'OMe) and phosphoroduamidate morpholino oligomers (PMOs)[8, 18, 22]. In both cases, the oligomers are specifically designed to target the exon 51 region of *DMD* pre-mRNA, blocking it from the splicing machinery and resulting in its removal from the final mRNA, which allows restoration of the reading frame and enables mRNA translation. Clinical testing of this strategy has been conducted in the last few years, with controversial results. Treatment with both types of nucleotides proved to be non-hazardous in both healthy and affected subjects, however efficacy could not be definitely demonstrated. 2'OMe treatment, clinically referred to as Drisapersen, has failed to achieve its primary endpoint in a placebo-controlled, phase 2B trial test[22]. Patients treated with a continuous dose of 6mg/kg of Disapersen did not show any improvement in walking distance in

the clinical 6-minute walking test (6MWT). However, re-evaluation of the treatment in younger patients is currently under way and may show better efficacy in delaying muscle deterioration. PMO testing, commercially known as Eteplirsen, showed more encouraging results. Phase 2B trials with a cohort of 12 patients, who were older than the Disapersen cohort, with 1:1:1 treatment of placebo, 30mg/kg/week and 50mg/kg/week showed significant increase in dystrophin levels in muscle of treated patients by 24 weeks of treatment. 6MWT results were not significant by 24 weeks, at which point the trial became open-label and placebo patients began receiving treatment. Over 168 weeks, a significant difference in 6MWT results was observed between the fully treated and the late treated patients[22]. In addition, no deterioration of respiratory capacity was noted in treated patient, and most patients remained ambulatory[22]. These results contrast sharply with the normal course of DMD, where most patients require assisted ventilation by their second decade of life. While these results are encouraging, several challenges remain. First, the designed PMOs only target exon 51, which would help only an estimated 14% of DMD patients[12]. Thus, it would require additional generation and testing of PMO constructs that would aim to help a larger cohort of patients. Second, the patient cohort that participated in the above mentioned study might be considered too small (n=10) for regulatory approval[22]. Further evaluation is expected.

Read-Through Strategies for Nonsense Mutation Suppression:

This strategy aims to tackle in-frame nonsense mutations that introduce a premature stop codon to the DMD and other genes. This is achieved by administration of a small molecule that induces a conformational change in the target mRNA, which in turn permits the translating ribosome to bypass the premature stop codon and replace it with a non-specific amino acid. Importantly, this process is selective for premature codons, as normal stop codons are not affected[12]. Gentamicin and Ataluren are two small molecules tested for this purpose[23]. Gentamicin was the first to show efficacy in an mdx mouse models (animal model of DMD), with significant increase in dystrophin production after treatment. However, Gentamicin proved to be unsafe in humans, as high dosage and no oral bioavailability precluded it from human testing. This was mitigated by Ataluren, which is a safe, orally bioavailable molecule that

acts at lower doses.[24] Its complete mechanism of action is undemonstrated, but it has been shown to bind the 60S subunit of the ribosome and induce mRNA conformational change throughout elongation[24]. In 2014, this molecule was approved for treatment of DMD patients with nonsense mutations in Germany. Issues of regulatory requirements and cost currently hinder its approval in the rest of the EU and USA.

Gene Replacement Strategies:

Gene replacement therapy does not aim to modify existing elements of the patients' genome, or correct expression of the mutated gene. Instead, it aims to deliver an additional, wild type copy of the missing gene in order to restore function in the tissue. This strategy is particularly pertinent to the variety of LGMD disorders, where pathologies arise in smaller genes that can be readily packed in to existing delivery vectors for either transient or permanent introduction into patients' cells.

It must be emphasized that the size of certain genes, such as dystrophin, made them unsuitable for gene therapy, as even their cDNA (11 kb for *DMD*) is too large to fit in a standard delivery vector[6, 8, 12, 18]. Nevertheless, studies from BMD patients showed that partial function could be restored with highly truncated proteins. Thus, mini- and micro- dystrophin (mDYS and µDYS, respectively) constructs were developed and delivered with adeno-associated viral vectors (AAVs). Clinical testing showed that optimization of AAVs was required, as injected muscles showed strong immune reaction to AAVs capsid antigens[19]. In addition, certain patients developed antigenicity to mDYS or µDYS themselves, as was shown by identification of mDYS specific T cells and eventual suppression of mDYS expression in muscle[19]. While further optimization of AAV vectors is required in order to reduce immunogenicity, this application for larger genes like *DMD* is probably not suitable. However, delivery of smaller genes such as α-sarcoglycan, gene editing tools such as CRSPR-Cas9 elements, or gene silencing through RNAi is more appealing for other conditions. For example, FSHD can be targeted by anti-DUX4 RNAi. A proof-of-concept study conducted by Wallace et al. has shown that FSHD phenotype can be successfully supressed *in vivo* by AAV mediated introduction of DUX4 RNAi[25]. While DUX4, expression was equally achieved by AAV transduction in this model; the study stands as a first

proof of concept and paves a direct road towards concrete FSHD treatments.

Non-viral Vectors such as Plasmids, Human Artificial Chromosomes (HACs) and transposons are also under investigation for gene therapy purposes. The advantage of these vectors is their quasi-unlimited storage capacity, with whole genes and regulatory sequences being cloned in easily[18]. However, direct delivery into muscle cells is highly inefficient, as it requires electroporation that can result in cell death and tissue damage, and is difficult to achieve *in vivo.* The use of these vectors is therefore more appropriate for *ex vivo* gene correction and combined with one of the various cell therapy techniques described in the following sections.

1.4.2. Stem Cell Therapy Strategies for Treatment of Muscular Dystrophies:

Advances in cell biology which led to the identification of adult stem cells in almost all tissues of the body, including muscle, have made the perspective of stem cell transplant for therapeutic purposes an attractive strategy[18]. Identification of resident muscle stem cells, as well as other cell populations that exhibit myogenic potential led researchers to adopt the following two approaches in clinical experiments involving stem cell transplantation: *ex vivo* expansion and genetic modification of autologous stem cells, and transplantation of donor derived allogeneic stem cells. The strengths and weakness of each approach are detailed below.

Transplantation of Satellite Cells and Satellite Cell-Derived Myoblasts:

The early discovery of skeletal muscle satellite cells and their rapid characterization as the resident myogenic stem cells in adult skeletal muscle has driven researchers to investigate their potential use in treatment of Duchene's Muscular Dystrophy. Initial positive results of myoblast grafts in the mdx *(*mouse dystrophy *X)* mouse model have quickly paved the way for researchers to initiate clinical testing of *ex vivo* expanded myoblasts[26]. These cells were derived either from allogeneic (i.e. donor derived) satellite cells, or from autologous cells that underwent subsequent genetic correction to restore dystrophin expression. Beginning in the early 1990's, several trials attempted to restore dystrophin expression in DMD patients by injecting up to 100 million myoblasts into single or several muscles[16]. The results of these trials showed no adverse effects, but also any improvement over control or placebo condition.

Further experimentation demonstrated that successful restoration of function was hindered by several key issues: First, intramuscularly injected myoblasts showed poor migratory capacity, essentially localizing to the site of injection without deeper tissue penetration and dispersion, while systemic administration resulted in rapid clearance of cells[12, 16, 26]. In addition, satellite cells and myoblasts were shown to be incapable of traversing the endothelial cell wall. This indicated a requirement for a large number of repeat injections for complete coverage of a single muscle. Second, transplanted myoblasts showed high levels of immunogenicity and were quickly rejected in non-immunosuppressed patients[19]. Subsequent re-evaluation of the immune response in animal models showed high numbers of infiltrating CD4+ and CD8+ T cells in myoblast grafts, as well as myoblast reactive antibodies in the animals' sera. Thus, immunosuppression is required in order to maintain partial survival of engrafted cells. The third and final issue observed was the massive cell death of transplanted myoblasts within the first 72 hours post injection. This was partially attributed to an immediate innate immune response, specifically neutrophil mediated cell clearance, as well as ischemic necrosis of transplanted cells[19]. Subsequent experimentation in the field has led to improvement of some of the above challenges, including identification of Tacrolimus as an optimal immunosuppressant and development of novel cell injection tools. However, a final challenge remained in the inability to preserve the satellite cell pool throughout *ex vivo* expansion, leading to progenitor depletion and absence of self-renewal upon engraftment.

Other Cell Populations for Therapeutic Applications in Muscle:

As part of the effort to overcome the challenges of myoblast transplantation, several groups have decided to examine other cell populations that were reported to have myogenic potential *in vivo*. Among these, CD133+ cells and Mesangioblasts were recognized as potentially therapeutic. Initial analysis of CD133+ cells isolated from the circulation showed that upon systemic administration into mdx mice, they were able to contribute to dystrophic muscle regeneration[6, 16, 18, 26]. Subsequently, CD133+ cells were isolated from muscle biopsies and were shown to be partially associated with muscle vasculature *in vivo*. Subsequent demonstration of their differentiation in animal models after lentivirus mediated genetic correction led to initiation of Phase I safety study in humans. While this study showed no

adverse effects of CD133+ cell administration, no subsequent attempts to test genetically modified CD133+ cells in the context of muscular dystrophy were reported in humans[6, 16, 18, 26].

Mesangioblasts are blood vessel associated progenitor cells that can be isolated from the vasculature of muscle biopsies. Lineage tracing experiments showed that mesangioblasts posses the capacity to contribute to regenerating muscle *in vivo*, as well as travel in the vasculature and cross the endothelial cell wall. These qualities made mesangioblasts an attractive candidate cell type for cell-based correction of genetic muscular disease. Currently, a Phase I safety study was completed in five DMD patients, showing that intravascular administration of mesangioblasts does not pose any significant safety risk to patients. Further studies in this field are currently expected[27].

Ultimately, more effort is required to properly characterize mechanisms that regulate myogenic stem cell activity and survival in order to improve their engraftment capacity and long-term self-renewal post treatment. Equally important is the role of the immune response in stem cell transplantation, and how it affects long-term cell survival in the host.

1.5. Homeostatic Muscle Repair Mechanisms:

In adult vertebrates, postnatal muscle growth is mainly driven by growth and enlargement of existing myofibers, as no significant postnatal increase in myofibers has been reported past three weeks in mice[28]. Muscle injury is variably defined by several morphological and functional criteria, such as decrease in force production and physical disruption of myofibrils. In addition, sarcolemma disruption is a common indicator of focal fibre damage. In vertebrates, skeletal muscle possesses high load-bearing capacity, routinely exceeding 300 kPa of force generation at high frequency[29]. However, acute injury resulting in loss of membrane integrity can occur when loading capacity is exceeded, or when external insults occur. As such, when damage is inflicted, adult muscle must quickly achieve tissue homeostasis, followed by restoration of complete structure and function.

Lifetime muscle usage can lead to occasional eccentric contractions, which cause mild damage to accumulate, and are mostly characterized by membrane disruption. In such cases, muscle fibres have been shown to initiate self-repair through vesicle accumulation and membrane fusion[29, 30]. Vesicle mediated membrane repair was shown to be partially mediated by a cytosolic and sarcolemma protein named dysferlin. *Dysferlin* mutations have been linked to muscle pathologies in humans, namely limb girdle muscular dystrophy 2B, and Myoshi's myopathy[6, 29, 30]. Dysferlin knockout in mice also correlates with progressive dystrophy throughout the lifetime of the animal. Dysferlin function in membrane resealing appears to be positively regulated by Ca^{2+} levels, since membrane repair is delayed in knockout fibres when homeostatic Ca^{2+} is present, but not when Ca^{2+} is depleted. Nevertheless, dysferlin mutants present high variability in muscle phenotype, and some appear to lack significant loss of function[30]. This evidence shows that physiological membrane disruption is a minor cause of loss of skeletal muscle function.

However, when significant damage is inflicted due to freeze, burn or toxin-induced injuries, skeletal muscle shows remarkable regenerative response resulting in complete restoration of muscle architecture and function to pre-injury levels in young individuals[3, 29]. This rapid and efficient repair is mediated by resident skeletal muscle stem cells, referred to as satellite cells due to their physical confinement between the muscle fibre sarcolemma and the surrounding basement membrane[31]. To appreciate the role of the satellite cells as the bona fide stem cell of adult skeletal muscle, one must first examine the broad definition of somatic stem cells and their biology in other tissues.

1.6. Adult Somatic Stem Cells (ASC):

The most basic definition states that stem cells must possess two essential characteristics. First, a stem cell must be able to activate a differentiation program that will allow it to undergo proliferation and differentiation into a downstream somatic tissue cell type. Second, a stem cell must also be able to self-renew and maintain itself for future tissue generation[32, 33]. The sections below detail general aspects of ASC biology, as well as specific

examples in hematopoietic and intestinal stem cells, which would allow their comparison to adult satellite cells.

1.6.1. Cell Potency:

The differentiation capacity of cells is known as their potency, follows a hierarchical organization defined by daughter cells' ability to differentiate down a distinct and gradually restricted cell lineage[32]. At the top of the hierarchy are the totipotent cells, capable of generating all embryonic and extra-embryonic tissue. Currently, this definition only applies to the cells of the early pre-implantation blastocyst, as no totipotent stem cells have been identified *in vivo* or *in vitro*[32]. Next, pluripotent cells are capable of generating cell types belonging to all three embryonic tissue lineages, the ectoderm, mesoderm, and endoderm. Embryonic stem cells, derived form the inner cell mass, and more recently derived induced pluripotent stem cells (iPSCs) belong in this category and are beyond the scope of this discussion[32]. Multipotent stem cells are further restricted to generation of multiple cell types within a restricted tissue lineage. A clearly defined example of this is the hematopoietic stem cell that resides within the bone marrow and whose responsibility lays in generation of all erythroid and myeloid cell lineages[34]. Lastly, unipotent progenitor cells are restricted to generation of only one cell type with restricted functionality. Nevertheless, unipotency does not indicate lack of stemness, as can be seen in skeletal muscle tissue. Resident skeletal muscle stem cells generate a pool of differentiated myocytes to repair damaged tissue, and subsequently self-renew.

Somatic stem cells were identified in most major types of tissue derived from all three germ layers and were shown to be responsible for all tissue homeostasis in the adult organism [32, 33]. Tissues with physiologically high turnover rates, such as the blood, skin and gut, require constant replenishment of a variety of cell types to maintain functionality. This is maintained by resident stem cells that divide to generate a transiently amplifying progenitor cell population (TAC) that will later terminally differentiate and provide the necessary numbers of functional tissue cells[32, 33]. In parallel, a self-renewed stem cell will be generated and returned to a dormant, quiescent state or maintained as an active progenitor [35].

1.6.2. Stem Cell Quiescence:

Cellular quiescence (G_0) is defined as a long-term, yet reversible, exit from the cell cycle[36]. Quiescent has been extensively studied in *in vitro* culture systems of both yeast and mammalian cell lines, and recently *in vivo*[36, 37]. Single cell organisms were shown to enter the quiescent state upon induction of adverse environmental conditions, such as amino acid starvation, mitogen deprivation and contact inhibition. Historically, radioactive DNA labeling techniques allowed determination of proliferation rates by measurement of label dilution throughout cell division over time. It was noted that while most tissue cells were dividing in most tissues, select subpopulations of cells retained labels over time. Development of new chemical labelling techniques, such as bromodeoxyuridene (BrdU) and 5-Ethynyl-2´-deoxyuridine (EdU) incorporation, as well as genetic labels such as inducible H2B- GFP, greatly enhanced control and sensitivity of detection, leading to detection of variably quiescent populations in most tissues[36, 37]. Of note, these techniques helped illustrate that quiescence is not exclusive to ASCs, but also applies to certain terminally differentiated cells, such as hepatocytes. In addition, it was later determined that certain ASCs do not retain label at all during homeostasis, but could incorporate it when external stimulus, usually by a form of injury, was provided[33].

1.6.3. The Stem Cell Niche:

Efforts to identify isolate and characterize ASCs has led to the discovery of specialized tissue specific microenvironment, termed niche, that helps regulate stem cell fate decisions[32]. While the molecular composition and anatomical position vary greatly between tissues, a common theme prevails: a mix of secreted signalling molecules and cell adhesion elements within the niche provide stem cells with extrinsic cues that combine with intrinsic regulatory pathways to maintain a signalling balance that retains their stem cell phenotype[31, 32, 35, 37]. Predominantly, both canonical and non-canonical Wnt signalling, as well as BMP were shown to influence ASC behaviour.

Evidence from studies of Germ line Stem Cells (GSCs) in *Drosophila* has demonstrated that the adult GSC niche possesses an asymmetric, physically limited architecture that promotes GSC differentiation through asymmetric cell division. In this model, a niche resident stem cell giver rise to two daughter cells: the daughter cell that retains contact with the niche microenvironment remains exposed to the molecular cues that promote stem cell phenotype, and therefore self-renews; the second daughter cell is physically displaced outside the niche, loses relevant extrinsic signalling, and begins a process of differentiation. Thus, the niche hypothesis stipulates that self-renewal signalling is both finite and confined to a narrow physical space[31, 32, 35, 37]. Hence, removal of stem cells from their niche leads to loss of self-renewal capacity [35, 38]. Observation of quiescent and cycling populations in multiple stem cell systems led researchers to postulate that the adult stem cell niche contains multiple "zones" that support maintenance of different effector cells[35, 39]. Thus, the niche contains a segregated population of "primed", activated stem cells that maintain homeostatic replenishment of tissue cells, and a population of quiescent stem cells that sporadically cycles to replenish the primed population. The zone model favourably rationalizes stem cell activity and self-renewal in high turnover tissues. In tissues that are predominantly post-mitotic like brain, heart and skeletal muscle, resident stem cells exist in a prolonged quiescent state and activated only in exceptional circumstances to regenerate heavily damaged tissue. Skeletal muscle dominates this category by retaining its capacity to achieve complete tissue restoration far into adulthood[38, 40].

1.6.4. Hematopoietic Stem Cells:

Research into hematopoiesis accelerated during the second half of the 20th century, when the devastating effects of ionizing radiation on human health be came evident. As bone marrow was rapidly identified as a highly radiosensitive organ, researchers began to actively seek methods to reconstitute hematopoiesis in lethally irradiated subjects[34]. Soon after, it was realized from experimentation in rodents that allogeneic transplantation of donor bone marrow cells into lethally irradiated hosts could completely rescue hematopoiesis, generating all erythroid and myeloid lineage cells with donor genotype[34, 41]. This finding pointed out the

presence of one or several types of bone marrow residing hematopoietic progenitors. Subsequent experiments, specifically spleen colony forming assays, demonstrated several key features of hematopoietic stem cells, which later became applicable to other adult stem cells as well: First, all cells within a single spleen colony (or “nodule”) were derived from a single progenitor cell, and thus were considered to be genetic clones of that cell. Second, each colony was a heterogeneous mixture of differentiated progeny of the original progenitor, with erythroid and myeloid cells found within many colonies. Third, certain cells within individual spleen colonies possessed the capacity to repopulate the hematopoietic compartment of a secondary irradiated host, as well as generate multiple spleen colonies. This observation provided evidence of hematopoietic stem cell self-renewal capacity, where a subset of progeny cells retained progenitor status for future use. In addition, it was found that these progenitors were normally insensitive to chemotherapeutic drugs that targeted cycling cells, which showed that they were quiescent in homeostatic conditions[34].

Embryonic development of Hematopoietic Stem Cells (HSCs) is complex multistep process that occurs in different anatomic locations throughout the developing embryo[32, 33]. Briefly, a primary population of hematopoietic progenitors migrates to the yolk sac at E7.0, and give rise to primitive erythrocytes that express embryonic forms of haemoglobin[41]. These progenitors support embryonic hematopoiesis throughout development, and were later shown to migrate to the embryonic liver and persist shortly in postnatal life. However, yolk sac progenitors are incapable of reconstituting the adult hematopoietic compartment, and are therefore restricted to the embryo proper. Proper HSCs populations that can reconstitute hematopoiesis in the adult arise much later, around E11.0 and from a different origin, known as the aorta-gonadmesonephros region[41]. Studies have shown that these cells undergo further conditioning in the foetal liver and postnatal bone marrow, which allows them to properly populate the niche and establish long term stem cell phenotypes.

Adult HSCs are commonly isolated based on their low retention of viability dies (Rhodamine or Hoechst), lack of lineage (lin) surface markers, and the presence of several other surface markers, such as CD34, CD38 and CD150 on their surface[34, 35]. Recent technological advances allow further separation of cell types based on the relative expression of each surface

marker. In the hematopoietic system, slow cycling label retaining cells were shown to divide every 145 days on average during physiological homeostasis, but rapidly and actively repopulate the myeloid and lymphoid hematopoietic compartments in a clonal serial transplantation model of lethally irradiated hosts[34, 35]. These observations led to the hypothesis that a more active, cycling yet self-renewing progenitor must exist to ensure homeostatic balance. New evidence demonstrates that HSCs can be retain lineage biases, and fall within three main categories: Myeloid-HSC, Lymphoid-HSCs and balanced (equally differentiating). Furthermore, long-term HSCs, which completely repopulate the hematopoietic compartment over the animals' lifetime, can be distinguished from short-term HSCs, which repopulate the compartment but show limited self-renewal[34].

The localization of various types of HSC within the bone marrow correlates with their differentiation capacity. It has been observed that quiescent HSCs localize to the endosteal region of the bone marrow, while active HSCs can be located in the central region of the bone marrow. Osteoblastic cells present in the endosteum were shown to secrete BMP, Osteopontin and sFRP1, all implicated in inhibition of HSC differentiation. The central region is devoid of osteoblasts, but enriched in CAR and endothelial cells thought to provide stimulatory factors such as Wnt, FGF and SDF1 signalling molecules[34, 35].

1.6.5. Intestinal Stem Cells*:*

The small intestine's efficient regenerative capacity has been the subject of many studies in recent decades. The impact of its role as a nutrient absorbing surface, while simultaneously act as an efficient barrier against noxious external agents, leads to an estimated cell death figure of 10^{11} cells per day[38]. Effective cell replacement throughout the different sections of the small intestine is mediated by several groups of crypt-based stem cells.

The embryonic origin of intestinal stem cells (ISC) closely follows the embryonic development of the gastrointestinal system. As single layer of pseudostratified epithelium, which later gives rise to the small intestine, is detectable at E9.5 in the primitive gut of mouse[38]. However, formation of intestinal villi rises from extensive tissue remodelling that

begins at E14.5 and continues into the postnatal stages. Acquisition of regenerative capacity parallels formation of intestinal crypts, which begins at E16.5 and lasts until postnatal day 7. As intestinal crypts continue to expand throughout the first few weeks of life, individual progenitors become dominant, and give rise to a clonal population of stem cells that resides within each crypt[38].

In the adult intestine, resident stem cells and their downstream transiently amplifying (TAC) progeny remain localized within the intestinal crypts throughout adult life. As TACs differentiate into enterocytes, enteroendocrine and goblet cells, they migrate towards the intestinal villus and renew the gut epithelium on a weekly basis[38]. Intriguingly, several types of cell were identified and confirmed by lineage tracing methods as possessing stem like characteristics in the gut. Primarily a population of crypt based columnar (CBC) cells, which express the surface marker Lgr5, is continuously dividing to replenish the gut epithelium during homeostatic conditions and is able to form gut-like organoids *in vitro*[35, 38]. In addition, a rare (2-4 cells) and label retaining population located at the 4+ positions in the crypt was shown to reconstitute crypt and villus architecture, even upon genetic removal of Lgr5 expressing cells.

Given the presence of at least two functionally distinct stem cell types, several niche factors are required for their maintenance. It is believed that increased Wnt signalling and BMP inhibition is mediated by submucosal cells under intestinal CBC cells, while BMP production near the 4+ cells maintains their quiescence[38].

1.6.6. Genetic Regulators of Stem Cells:

Important advances in molecular biology and cell culture produced powerful tools for fluorescent tagging of multiple ASC based on intracellular markers. In parallel, flow cytometery technology has improved to allow detection of increasing number of markers. Together, these technologies were used to isolate large numbers of ASCs from multiple tissue types, which provided researchers with material to carry out high-throughput analysis of ASC transcriptomes, epigenomes and, more recently, translatome and proteome[33].

Tissue turnover speed indicates that the quiescent state of ASC may vary for individual cell types. Nevertheless, comparative analysis of transcriptome data of several ASC types shows a common pattern of down regulation of cell cycle effectors genes such as cyclins A2, B1 and E1; Mitochondrial biogenesis genes such as CYCS and others involved in chromatin structuring (SMARCA2) and RNA processing (Dicer1)[32, 33, 36]. Negative regulators of the cell cycle, primarily the p53 and Retinoblastoma (Rb) family proteins were shown to play a key role in G_0 maintenance. P53 ablation in Neural ASC induced over proliferation and tumour formation, while ablation of Rb in muscle ASCs induced proliferation without differentiation. Similarly, HSC depletion occurred when Rb was genetically removed. Other inhibition of cell cycle activity is mediated by a group of CDK inhibitors such as P27 and p57, which regulate cell cycle entry by inhibiting translocation of Cyclin D1 to the nucleus. Double knockout of these genes impairs HSC quiescence[34, 36]. Notch signalling, which plays a key role in regulating cell fate decisions in differentiating progenitors cells during development and regeneration, was also implicated in regulation of ASC quiescence. As previously mentioned, RNA profiling techniques showed active expression of distinct miRNA groups within various ASCs. miRNA activity was shown to act at multiple levels, by either attenuating signalling pathways, or promoting post-transcriptional control of gene expression in HSC and MuSC, respectively[36].

1.7. Satellite Cells:

1.7.1. History of Discovery:

The process of muscle regeneration has been documented as an object of scientific interrogation since, at least, the 19th century. Gabriel Gustav Valentine and Theodore Schwan were early researchers who observed muscle formation during embryogenesis, and proposed the hypothesis that “primitive muscle bundle[s are a secondary structure formed by fusion of individually aligned mononucleated progenitors]”[42]. Subsequently, about 120 years had to pass before formal documented proof of this hypothesis was presented. *In vivo* injury and transplantation experiments showed the demonstrated the remarkable regenerative potential of skeletal muscle in multiple species, but could not shed light on the exact mechanism of

action[1, 3]. Progress was achieved with the development of tritiated thymidine labeling techniques, which allowed radioactive labeling of mitotic cells as well as their lineage tracing *in vivo*. A series of experiments with this technique showed that upon muscle injury and pulsing, only mononucleated cells retained the label at early stages of regeneration[1, 3, 42]. Radioactivity was later detected in differentiated myotubes, but not if pulsing occurred several days after injury. This demonstrated that a population of mononucleated progenitors fused to generate muscle in the adult. Development of tissue and cell culture methodologies further confirmed the notion of progenitors cells. Observation of chick muscle explants showed that tissue outgrowth was mediated by proliferating myoblasts, which then fused to form multinucleated myotubes[1, 3, 42]. Radioactive pulsing of muscle culture monolayers showed exclusively that myotubes from labeled myoblasts contained radiolabeled nuclei. Later on establishment of immortal myogenic cell lines from adult muscle, primarily the C2C12 line, allowed extensive analysis of *in vitro* myogenesis[1, 3, 42].

While the role of single cell progenitors that appear during injury to fuse and repair muscle was well accepted, their source was a topic of debate. Several theories were proposed, including dedifferentiation and encapsulation of myonuclei of dead fibres, or recruitment and differentiation of interstitial cells to the task[42]. However, two papers published in 1961 proposed a novel hypothesis to explain the origin of progenitors in muscle. During observations of electron micrograph data, both Bernard Katz and Alexander Mauro pointed out the presence of small nuclei, wedged between the sarcolemma and the basal lamina of myofibers, which they termed “Satellite Cells”[43, 44]. Mauro even speculated that these cells are ‘merely dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of skeletal muscle fibre when the main multinucleated cell is damaged’.[43] Modern advances in molecular biology and genetics led to further investigation and confirmation of satellite cells as bona fide muscle stem cells.

1.7.2. Developmental Origin:

The developmental origin of satellite cells in adult muscle has been extensively investigated. For example, *Gros et al.* used several tools, including GFP electroporation and quail-chick grafting to demonstrate embryonic myogenesis in a spatial and temporal manner[9]. In addition to elegantly documenting the process of embryonic myogenesis by tracking GFP+ (positive) cell migration; they also show that late development and post hatch chick chimeras grafted with quail myotome segments exhibit Pax7+, QCPN+ (quail cell marker) cells in the satellite cell position within the graft[9]. Relaix et al confirmed the notion that satellite cells arise from somitic dermomyotome progenitors that express Pax3 and Pax7[11]. In their study, they use a series of GFP and nLacZ reporter mouse lines to track and document the role of Pax7 and Pax3 positive cells within the dermomyotome. They show that Pax3+/Pax7+ cells are indispensible muscle progenitors in later embryonic stages, as their ablation blocks myogenic progress[11]s. Crucially, they also show that a portion of Pax3+/Pax7+ cells become embedded under the basement membrane of embryonic muscle fibres after secondary myogenesis has occurred at E18.5[4, 11]. Large numbers of satellite cells persist during the perinatal stage and contribute to myonuclear content of existing myofibers. After postnatal day 21 (P21), these cells downregulate active myogenesis, stop proliferating and enter a state of quiescence[45].

1.7.3. Molecular Signature:

Initial analysis of satellite cells was conducted according to Mauro's method, transmission electron microscopy[43]. Analysis of electron micrographs of satellite cells from various species showed several common morphological characteristics: Poor organelle content, high nucleo-cytoplasmic ration, generally small nucleus and abundant presence of heterochromatin. These observations indicate low metabolic activity at all levels[42, 46, 47].

At the molecular level, satellite cells are canonically identified by the robust expression of Pax3 and Pax7 paired domain transcription factor proteins[48]. Pax7 is expressed in adult satellite cells all throughout the body, while Pax3 remains co-expressed in a restricted manner in trunk and diaphragm muscles[48, 49]. Other markers of satellite cells in muscle are mostly

cell surface proteins, including: cluster of differentiation 34 (CD34); Caviolin-1; M-Cadherin; Neural Cell Adhesion Molecule (NCAM, CD56); Calcitonin Receptor (Calcr); Integrin-α7 and others[31, 47, 50]. It is important to note that unlike Pax7 and/or Pax3, these markers are not satellite cell exclusive in skeletal muscle. In addition to these, it has been extensively shown that satellite cells transcribe the Myf5 locus and, more recently, the MyoD locus[31, 47, 50-54]. While post-transcriptional regulation of these mRNAs ensures repression of protein generation, the active locus of Myf5 has been extensively used to mark satellite cells with lineage tracing tools[55].

Pax7 gene is essential for postnatal satellite cell maintenance and proliferation, since its ablation leads to gradual depletion of resident stem cells in uninjured muscle, and completely abolishes tissue regeneration during injury[56]. In addition, Pax7 positive satellite cells were shown to be the primary source of muscle progenitors in adult skeletal muscle, as their elimination with a diphtheria toxin transgene abolished regenerative capacity[57].

1.7.4. Population Heterogeneity:

Satellite cells were considered a homogenous population of primitive cells based on initial observations by electron microscopy. Since its validation as a positive marker of satellite cells in adult muscle, Pax7 has become the canonical marker of this muscle stem cell population[49]. It is commonly accepted that all quiescent satellite cells express Pax7 in all adult muscle, but recent evidence indicates that they are in fact differences in gene expression, differentiation capacity and self-renewal ability[40]. Hence, it has become increasingly accepted that there may be heterogeneity in the satellite cell population. For example, it has been shown that satellite cells in specific muscle groups retain Pax3 expression, primarily in trunk and diaphragm. Interrogation of this phenotype showed no specific correlation with muscle fibre type, innervation or embryonic origin[48]. Furthermore, Pax7+/Pax3- cells can be detected in generally positive muscles like the diaphragm as well. Grafting experiments of Pax3GFP/+ cells shows that they retain GFP expression, indicating a cell-intrinsic property[11, 48]. In addition, Pax3 was demonstrated to not be able to compensate for Pax7 knockout in adult muscle,

demonstrating non-redundant functionality, while selective knockout of Pax3 did not affect regenerative potential[58].

In addition, it has been widely demonstrated that virtually all quiescent satellite cells transcribe Myf5 at variable levels, as been repeatedly shown by Myf5-nLacZ and Myf5-Cre knock-in alleles[55]. Post transcriptional repression of Myf5 mRNA translation is prevented by miR-31 mediated post-transcriptional repression however, there is also evidence that small population of adult satellite cells that has never transcribed Myf5 posses enhanced self-renewal ability and may be responsible for maintenance of the satellite cells pool[59, 60]. Satellite cells have been also reported to vary in their differentiation potential, as engraftment of cells originating from different muscles (such as TA, Soleus and EDL) has shown variable de-novo muscle generation and self-renewal capacity. A similar degree of difference was reported for label retaining cells isolated from within the same muscle belly. Overall, evidence indicates that there may be a hierarchy of differentiation potential within the satellite cell group, specifically a minority of true self-renewing stem cells and a majority of increasingly restricted progenitors[40, 50, 61].

1.7.5. Adult Muscle Regeneration:

Acute muscle injury typically induces physical disruption of tissue morphology and leads to degeneration. In muscle, injured fibres undergo necrosis, in the process of which the sarcolemma becomes increasingly permeable and leakage of intracellular contents occurs. At the same time, the muscle membrane becomes permeable to low molecular weight dyes, such as Evan's blue, which allows tracking of muscle damage *in vivo*[3, 29, 31]. In parallel, necrosis disrupts the fibre's Ca^{2+} balance, leading to release of Ca^{2+} from the SR and activation of calcium based proteolytic cascades[29]. Following that, activation of complement cascades induces an immune response at the site of injury, leading to several waves of immune cell infiltration. First, phagocytic cells, primarily neutrophils, are recruited followed by at least two distinct subsets of macrophages[4, 29]. This response clears tissues debris and potentiates muscle progenitors to further activate and regenerate tissue.

1.7.6. Satellite Cell Activation:

Upon injury, satellite cells were shown to re-enter the cell cycle and activate the myogenic program. This process involves many of the genetic factors that orchestrate embryonic myogenesis, and show similar deployment hierarchy in adult regeneration. Activation and differentiation is marked by sequential expression of myogenic regulatory factor proteins, of which there are four key members: Myf5, MyoD, Mrf4 and Myogenin[4, 46, 50]. This family of basic helix-loop-helix (bHLH) transcriptions factors directs a sequence of activation, proliferation and differentiation steps that convert most satellite cells into transiently amplifying adult myoblasts. The majority of myoblasts then undergo terminal differentiation into myocytes, which then exit the cell cycle to fuse and form multinucleated myotubes in culture and *in vivo*[60, 62].

The first step of satellite cell activation begins with rapid translation of the myogenic transcription factors Myf5 in virtually all satellite cells near the site of injury[60, 62]. This rapid response is enabled by the constitutive presence of Myf5 mRNA transcripts in the quiescent satellite cell[53, 55]. Emerging evidence shows that Myf5 mRNA is subject to post-transcriptional regulation in the quiescent satellite cell, which represses protein expression yet primes the cell for myogenic commitment[60]. Myf5 helps initiate the myogenic program in satellite cells, yet its eventual downregulation is required to allow cell commitment to the myogenic fate[63]. Within a period of 6 hours after injury, upregulation of MyoD is detected within the majority of Myf5+ cells, and then all satellite cells after 24 hours[55]. Conditional knockout models of these genes showed potential redundancy in their function, as MyoD was shown to compensate for Myf5 in a Myf5 knockout model, while Myf5 could not compensate in the absence of MyoD[64]. MyoD was identified as the first MRF, and subsequent analysis of its function showed numerous regulatory functions both in muscle progenitors as well as differentiated fibres. MyoD has been shown to by to regulatory elements and drive expression of downstream MRFs (Myogenin), Muscle structure proteins (Myosin light and heavy chain), Metabolism (Creatine Kinase) and cell cycle regulators ($p21^{waf/cip1}$)[65]. Regulation of MyoD has been shown to occur on multiple levels. For example, Pax7 was shown to directly upregulate inhibitor of DNA proteins (Ids), which bind E box factors that normally heterodimerize with

MRFs and promote their function[46, 65, 66]. P38 MAPK activity was shown to regulate MyoD activity in a dose and time dependent manner. More recently, it has been shown that MyoD mRNA is actively expressed in quiescent satellite cells, but at the same time targeted for degradation by an active mRNA decay pathway mediated by p38[54]. During the proliferation phase, Pax7 expression is gradually lost and the majority of cells upregulate Myogenin and Mrf4, thus committing to differentiation[13]. After several rounds of proliferation, several Pax7 positive, MyoD positive satellite cells were shown to downregulate MyoD and re-enter quiescence[67].

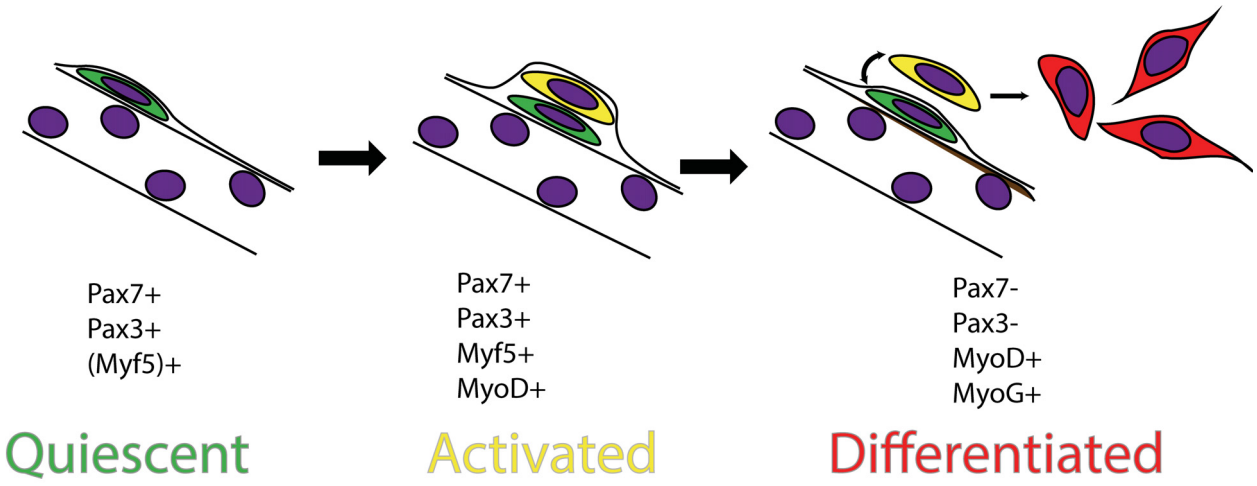

Figure 1.3 Schematic representation on satellite cell activation. Quiescent satellite cells express high levels of Pax7 and Pax3 in trunk muscle. Myf5 transcripts are also present, but their translation is actively supressed through miR-31 mediated silencing. Activated satellite cells rapidly upregulated Myf5 and MyoD, and enter the myogenic program. Finally, satellite cells upregulate Myogenin and MRF4 to exit the cell cycle and terminally differentiate, or downregulate MRFs and maintain Pax7 to self-renew.

1.7.7. Mechanisms that Regulate Satellite Cell Quiescence and Self-Renewal:

At later stages of adult myogenesis, most of the satellite cell progeny population commits to differentiation, stops cycling and fuses to form new muscle fibres or repair existing damaged ones. However, a subset of cells exits the cell cycle, returns to the satellite cell position under the basement membrane to self-renew, and replenishes the stem cell pool. As detailed above, the mechanisms that direct the satellite cell towards differentiation have been relatively well characterized and extensively studied. However, our understanding of cell fate choice mechanism(s) that direct satellite cells to self-renew only began to emerge recently. It is

important to note that the distinction between regulatory mechanisms of the quiescent state and of active self-renewal during myogenesis is often used interchangeably in the literature. However, it falls to logic that if the ability to maintain quiescence is compromised, self-renewal cannot occur.

Many of the pathways that were determined to be important for hematopoietic, neural and epithelial ASC quiescence and self-renewal are also implicated in the muscle satellite cell. For example, satellite cells inhibit cell cycle entry by expressing high levels of CDK inhibitors 1B and 1C; Rb tumour suppressor family members; RGS 2/5 and Spry1, a negative modulator of the ERK1/2 mediated IGF signalling cascade[36, 47]. At the same time, many positive regulators of the cell cycle are downregulated. Epigenetic mechanisms are also believed to be involved in controlling self-renewal, as it has been recently demonstrated that histone methyltransferases Prmt5 and Prmt7 play an active role in the process[68, 69]. Both Prmt5 and PRMT7 variably supress p21 expression in satellite cells via distinct mechanisms that when perturbed ablate stem cell self-renewal. In addition, new evidence shows that Suv4-20H1 mediated facultative heterochromatin maintenance helps repress transcriptional activity at the MyoD locus, but further analysis is required to understand the mechanism[70].

Niche signalling factors also play a crucial role in directing satellite cell self-renewal. For example, Ca^{2+} signalling is a key modulator of quiescence since satellite cells express high levels of calcitonin receptor which, when knocked-out, results in loss of self-renewal and apoptosis of satellite cells after injury[71]. Muscle fibres also express Delta, the canonical ligand of Notch pathway signalling, which was shown to play various, context-dependent roles in homeostatic quiescence and activation[47]. Blood vasculature is another source of signalling, as satellite cells are often found in proximity to blood vessels in muscle. Angiopoietin-1, which is expressed at high levels from vascular smooth muscle cells and fibroblasts, binds Tie2 receptors on proliferating satellite cells and directs them to enter quiescence through and ERK1/2 tyrosine kinase signalling cascade. Satellite cells then continue expressing high levels of Tie2 throughout their quiescent state[72]. Differentiation promoting ligands like HGF, IGF and FGF are released from microenvironment elements during injury, but are also present during homeostatic conditions[3]. Negative regulation of their signalling is mediated by intracellular factors in

satellite cells. For example, IGF signalling is mediated through the ERK1/2 tyrosine kinase pathway, and is inhibited in quiescent satellite cells by high levels of Sprouty1 expression[47]. Notch signalling in the adult satellite cells supports quiescence, as it was shown that genetic deletion of Notch intracellular Domain (NCID) breaks satellite cells quiescence and leads to premature differentiation without entry into the cell cycle, while overexpression expands satellite cells levels at the expanse of differentiation[73]. In addition, it has been shown that Notch signalling is required for hypoxia mediated satellite cell self-renewal[61].

Regulation of satellite cell fate decisions throughout regeneration was also shown to be important for self-renewal. It is currently understood that multiple niche and cell extrinsic factors paly a role in dictating satellite cell fate through symmetric and asymmetric cell division[46]. For example, *in vitro* observation of satellite cell divisions in single cell culture as well as on intact myofibers shows asymmetric segregations of myogenic fate determinants such as Myf5, MyoD and Myogenin after cell division[61, 67, 74, 75]. Intriguingly, non-random segregation of DNA strands has also been proposed as a mechanism, where it was shown through EdU pulse tracking that newly synthesized strands are inherited by committed daughter cells together with MyoG[75]. On the other hand, "Old" DNA was segregated into a $Pax7^{Hi}$ daughter cell that self-renewed. The mechanism that dictates determinant segregation is poorly understood, but recent evidence shows that Partioning-defective proteins (PAR) are involved in determination of cell polarity. It has been shown that PAR3-PKCλ complexes co-localize with P38α/β during activation, which induces asymmetric MyoD expression and differentiation in the daughter cell[74, 76]. Similarly, it has been recently proposed that activated satellite cells express detectable levels of Dystrophin, which interacts with PARB1 to direct PAR3 localization and organize the mitotic spindle for symmetric or asymmetric division[15].

Satellite cells remain quiescent over long periods of time in the absence of injury. Like other non-cycling cell populations, they employ mechanisms that reduce damaging effects of environmental stressors. Quiescent satellite cells express high levels of multidrug resistance channel pump genes, oxidative stress resistance genes, and members of the Cytochrome P450 and other endotoxin modifying genes[52]. Quiescent satellite cells also actively regulate their

metabolism through autophagy and its regulation by the mTOR pathway[77]. High levels of Ulk1 expression induce basal autophagy in satellite cells. mTOR activity was shown to down regulate Ulk1 and cause exit from quiescence. The mTOR pathway is highly complex and involves multiple regulators that sense and respond to metabolic status. Systemic signals during injury were shown to activate the mTORC1 pathway in a global manner and push satellite cells, as well as other ASC to increase their metabolic activity and be come 'Alert', resulting in higher propensity to exit the quiescent state[78]. In addition to its role in maintaining basal satellite cell metabolism and preventing senescence, autophagy was also implicated in satellite cell activation, where this pathway is believed to provide the necessary energy during transition from glycolytic metabolism to oxidative phosphorylation[79].

Indeed, current evidence shows that satellite cells, like other ASCs, actively regulate their quiescent state by repressing cycling and actively regulate their cellular processes. Evidence to an additional layer of regulation has recently emerged. Post-transcriptional regulation of gene expression, mediated through microRNAs, was demonstrated when the miRNA-processing gene *Dicer* was selectively ablated from quiescent satellite cells, leading to spontaneous activation[80]. Subsequent analysis of miRNA showed downregulation of multiple miRs in activated satellite cells[80]. Specific analysis of miR- 489 showed that it functions to repress translation of the oncogene *Dek*, which when expressed is asymmetrically localized to myogenically committed daughter cells. Interestingly, miR-489 is transcribed from the intron 4 region of *Calcr* and is rapidly downregulated with it[71]. Similarly, another group has identified miR-195 and miR -497 as repressors of cell cycle genes cdc25a and cdc25b, as well as ccnd2. Repression of these genes allows satellite cells to transition and maintain quiescence during juvenile muscle growth[81]. Subsequently, miRNA involvement was demonstrated for regulation of satellite cell activation (miR-133a/b), proliferation (miR-29) and differentiation (miR-1, miR-206, miR486)[82].

Finally, our group provided evidence that post-transcriptional regulation holds satellite cells in a differentiation poised state during quiescence. Previous analysis showed that Myf5 transcripts contain a miR-31 consensus sequence in their 3'- UTR[60]. As mentioned above,

Myf5 reporter alleles showed activity at that locus in virtually all quiescent satellite cells, yet satellite cells remained inactive. Our group showed that translation of Myf5 transcripts is repressed by miR-31 mediated sequestration into mRNP granules. These granules contained many components traditionally found in stress induced granules and P-bodies, but did not contain HuR, a characteristic marker of the both types of granules[60, 83]. Myf5 transcripts co-localized with mRNA and other factors that mediate translational repression of transcripts, such as Argonaut, Tia 1 and phosphorylated FMRP. Inhibition of miR -31 activity showed accumulation of Myf5 protein, activation and impaired self-renewal *in vitro* and *in vivo*, while the opposite was observed when mir-31 was transfected into satellite cells *in vitro*. To better understand this process, we review several commonly studied aspects of post-transcriptional gene regulation.

1.8. Post-Transcriptional Regulation of Gene Expression in Eukaryotic Cells:

Eukaryotic cells differ from their prokaryotic predecessors in their levels of structural and functional complexity. Throughout evolution, the acquisition of specialized roles within the context of multicellular organisms has necessitated development of greater control of the molecular products that were encoded in cells' genomes. While control of gene expression at the transcriptional levels are well characterized in both eukaryotes and prokaryotes, the decoupling of transcription and translation in eukaryotes, which occur in the nucleus and cytoplasmic compartments respectively, has added multiple post-transcriptional control tools to the cells repertoire[84]. Reviewed below are several common as well as novel mechanisms of post-transcriptional control.

1.8.1. Alternative Splicing:

Alternative splicing is a well-defined mechanism of gene regulation in eukaryotic cells[84]. Briefly, it utilizes the fact that all protein-coding sequences in the genome are segmented into sections termed exons. Exon sequences are linearly arranged, and divided by often much longer, non-coding intron sequences. Initial transcription of individual gene sequences generates a long pre-mRNA sequence that contains both exons and introns.

Subsequent removal of intronic sequences is termed 'splicing' and is mediated by the spliceosome complex[84, 85]. Spliceosome complexes are composed of several specialized RNA molecules (U1-6) and other protein subunits that recognize several key consensus nucleotide sequences within introns: the 5' splice junction; branching point; 3' splice junction. The recognition and binding of these sequences by the components of the spliceosome initiates a series of ATP catalyzed reactions that culminate in looping out of the intron segment and ligation of two adjacent exons[84]. The relative abundance of splicing factors, the relative length of the pre-mRNA, as well as the precise location of the splice junctions and branching points all affects splicing efficiency and outcome. Thus, these features allow alternative splicing of various transcripts, which can result in skipping of certain exons, retention of introns or truncation of exons in some cases. These changes to the coding sequence can lead to extensive changes in polypeptide sequence, generating protein isoforms with dramatically differing functionalities. Hence, this mechanism greatly increases the information storing capacity of genomic sequences.

1.8.2. Modification of Poly A Cleavage Sites:

In addition to alternative splicing, mRNA sequences require further processing to ensure their stability after nuclear export, as well as to allow recruitment of translation machinery. One such modification in eukaryotic cells depends on the location of cleavage and Polyadenylation sites. Normally, a cleavage site sequence resides between the defined consensus sequence (AAUAAA) and a GU rich region downstream[84, 86]. These sequences are bound by the multi subunit complexes CPSF (Cleavage and Polyadenylation Specificity Factor) and CstF (Cleavage Stimulation Factor), which recruit the cleavage and Poly A elongation machinery to the 3' end of the transcript. After Polyadenylation begins, other transcript specific factors bind the poly A tail and affect downstream fate. The presence of multiple polyadenylation sites per transcript, combined with the relative concentrations of CPSF and CstF lead to variability in C-terminal length of multiple transcripts, altering their function[84, 86]. For example, it has been shown that miR- 206 mediates repression of Pax3 protein expression in most satellite cells of the body, with the exception of several muscle groups in the trunk[87]. This repression is mediated by

miR- 206 interactions with two target sites located in the 3' UTR of the longest Pax3 transcripts. However, the 3'UTR is significantly shortened by alternative polyadenylation cleavage in satellite cells of the trunk, resulting in robust expression of Pax3.

1.8.3. mRNA localization:

mRNA localization is extensively used by cells to ensure protein production close to the site of need. When pre-mRNA processing is finished, transcripts are exported to the cytoplasm in the form of messenger ribonucleoprotein particles (mRNP). Depending on the protein content of the mRNP, certain transcripts can be either actively shuttled to specific compartments within the cell or passively diffuse until they are captured by relevant target complexes or free ribosomes[85]. For example, it was demonstrated that *β-actin* and *prox1* mRNAs are localized to the apical domain of radial precursors in the brain[88]. This localization is mediated by the specific mRNP factors Stau2, Pum2 and DDX1, as their genetic removal *in vivo* resulted in mislocalization of *prox1* mRNA and premature differentiation of the progenitor cells.

1.8.4. RNA Editing:

The most common type of RNA editing that occurs in eukaryotic cells is the deamination of adenine to produce inosine, which is catalyzed by a group on enzymes called ADARs (Adenosine Deaminases Acting on RNA)[84, 85]. These enzymes recognize double stranded pairing of the sequence to be edited and a complementary segment elsewhere on the mRNA. The three dimensional conformation of the mRNA dictates if the ADAR should edit the sequence. RNA editing can have major effect on the function of specific proteins. A strong effect was demonstrated for the glutamine receptor gene GluR-B, a CNS Ca2+ ion channel[84, 85]. RNA editing leads to conversion of a glutamine residue to arginine and alters the channel's Ca2+ permeability, leading to change in its current conductance and thus signalling role.

1.8.5. Control of mRNA stability:

The presence of the 5' Cap and the 3' poly-A tail of the processed mRNP molecule traditionally confer mRNA stability. Poly A shortening begins immediately upon transcript

export to the cytoplasm, and continues until a critical length of less than 25bp is reached. At this point, the mRNA may be 'decapped', resulting in loss of the 5' cap and immediate degradation from the 5' end; or transcript degradation may continue at the 3' end. As both the deadenylase and the translation machinery require association with the 5' and 3' end of the mRNA, there is often direct competition and cross inhibition between the two pathways, which is regulated by additional association of transcript specific factors that can either slow or accelerate these processes[84].

The effects of alternative splicing and 3' UTR processing can often affect mRNA stability by introducing a premature termination codon, which can trigger Nonsense Mediated mRNA Decay (NMD) in various physiological and pathological contexts. The processes that regulate NMD for various mRNAs can be transcript and context specific, but it was shown to always require recruitment of members of the UPF protein family. Indeed, UPF1, UPF2 and UPF3 are considered as the core regulators of NMD in all eukaryotes, with UPF1 being involved throughout all steps of NMD[89]. However, it was also shown that UPF1 binding is transient and non-specific towards the 3' UTR regions of most mRNAs, indicating that additional processes dictate initiation of NMD. NMD serves as a mechanism of cellular quality control, ensuring that aberrant mRNA products are rapidly removed from the cellular environment. However, NMD also plays a role in physiological gene regulation, as it was demonstrated that removal of NMD factors triggers extensive accumulation of unusual splice variants of various unrelated genes[89]. In particular, it was shown that various members of the SRSF (Ser/Arg Rich splicing factors) and HNRNP (heterogeneous nuclear ribonucleoproteins), which act as activators and repressors of mRNA splicing, are subject to self-regulation by NMD. Another example of physiological NMD based regulation was demonstrated in neural stem cells, where ablation of UPF1 led to inhibition of TGFβ signalling that triggered neural cell proliferation and precocious differentiation during embryonic development[90].

1.8.6. MicroRNA (miR) Mediated Repression of Translation:

The human genome produces over 1000 miRs, which are believed to post-transcriptionally control at least a third of all protein coding genes[84]. The relatively recent identification of miRs is attributed to their small size of roughly 21 nucleotides[91]. miRs are generated from small hairpin RNA, which loops to form short double stranded structures that get recognized by the miR processing effectors *Drosha* and *Dicer*. In higher eukaryotes, miRs associate with other RNA binding proteins; specifically members of the Argonaute (AGO) proteins, to form the RNA induced Silencing Complex (RISC)[91]. miRs contain 2-8 nucleotide long seed sequences, which allow specific targeting of RISC complexes to complementary control sequences in the 3' UTR of multiple mRNAs. While extensive complementation of the RISC complex may induce mRNA cleavage and degradation, it was found that translational repression is the more common outcome of RISC induced mRNA silencing.

Inhibition of 5'-Cap dependent translation initiation was demonstrated to be the most common mechanism of miR activity. Normally, the m^7GpppN 5' cap of transcripts is recognized by the translation initiation factor eIF4E, which mediates an interaction of two other factors: eIF4G and eIF3, and recruits the 40S ribosomal subunit to the mRNA. eIF4G also interacts with PABP1, a poly A binding protein that induces the typically observed circular structure of actively translated mRNA, which further enhances activity of eIF4E[92]. Evidence shows that miR mediated recruitment of RISC to the 3' UTR significantly disrupts the interaction between eIF4G and eIF4E, which is achieved by homologous imitation of the eIF4E Cap-binding site in AGO proteins. In addition, GW182 recruitment by RISC mediates deadenylation of the 3' end of certain mRNAs, eventually targeting them to degradation[91]. Other modes of miR mediated negative regulation have been proposed. However, a more recent demonstration that certain mRNAs contain AU Rich Elements (AREs) in their 3' UTR makes their repression reversible in certain cellular contexts. These elements help recruit other groups of RNA binding proteins, such as the ELAV family member HuR, that can either directly compete with miR mediated RISC complexes, inducing their displacement, or inhibit RISC associated recruitment of deadenylation factors[93].

1.8.7. mRNP granules and their function:

Post-transcriptional control of gene expression has been demonstrated at all levels of mRNA stability, subcellular localization and translation. In mammalian cell, two main types of mRNP granules are extensively studied: the P-body, which contains mRNA decay machinery and degrades transcripts; and stress granules, commonly formed in response to various forms of cellular stress and require additional regulatory mechanisms[83]. Aggregation of mRNPs into stress granules is generally hypothesized to be a protective mechanism during cell stress. Endoplasmic Reticulum overload by unfolded proteins is a common hallmark during cellular stress induced responses. Accumulation of nascent mRNA into stress granules and their subsequent translational repression therefore helps alleviate this stress by limiting the number of new peptides that enter the ER, as well as allow resident chaperons to better resolve unfolded proteins[94]. Closer analysis of stress granule contents shows that they are enriched with components of the translation machinery. Indeed, the presence of eIF4, eIF3 and eIF2 as well as 40S ribosomal subunits and poly A binding proteins are found within all stress granules, in addition to other variable components[83]. Crucially, most mRNAs within these granules are polyadenylated, indicating that decay does not occur.

1.8.8. Global Down Regulation of Protein Synthesis:

Furthermore, translation is often inhibited at the initiation phase through phosphorylation of eIF2α and inhibition of Methionin-tRNA recruitment to the start codon during cap mediated translation[94]. eIF2α phosphorylation as a response to downregulate global translation has been traditionally examined in the context of the unfolded protein response, also referred to as the integrated stress response in some literature. Four main kinases are responsible for downregulation of translation: Protein Kinase R (PKR) responds to presence of double stranded DNA during viral infection; General Control Nondipressible 2 (GCN2) responds to amino–acid starvation; Heme regulated inhibitor (HRI) responds to heme disregulation in the hematopoietic system and finally ER protein overload is mediated by PKR like endoplasmic reticulum kinase (PERK)[95, 96]. Phosphorylation of eIF2α causes stall at the initiation stage of translation. Normally, eIF2α forms a trimeric complex with the β and γ

subunit that binds a Met-tRNA and a GTP molecule. This complex is recruited to the start codon when it is recognized by the scanning 40S subunit of the ribosome. At the site, eIF5 mediated hydrolysis of the GTP molecule on eIF2γ provides the catalytic energy of attaching Methionine, assemble the 80S ribosome and signal the start of elongation. When liberated from the ribosome, the trimer complex of eIF2 recruits eIF2B, a guanine exchange factor that recycles the GDP molecule into a GTP. Thus, constant recycling of GDP to GTP in the eIF2 complex permits translation[92]. Phosphorylation of eIF2α on Serine 51 renders eIF2α into an allosteric inhibitor of eIF2B, thus reducing efficient eIF2 complex recycling and depletes available eIF2-Met-tRNA complexes. It is important to note however that eIF2α is normally ubiquitous in cells, and it is eIF2B that serves as the limiting factor in this biochemical process. When ribosomes cannot initiate translation, they initially stall, after which they continue scanning for additional AUG codons until they run off the mRNA[92]. When this occurs, transcripts become amenable to miRNA mediated silencing.

It is important to note however that eIf2α mediated translation attenuation does not result in complete arrest of translation, as eIF2-Met-tRNA- GTP complexes are still available[97]. In addition, it has been shown that a specific class of mRNAs that contain Upstream Open Reading Frames (uORFs) in their 5' UTR are in fact resistant to eIF2α phosphorylation. Furthermore, certain mRNAs, like the master regulator gene *ATF4*, enhance their protein expression during periods of high eIF2α. ATF4 is selectively translated during high phosphorylated-eIF2α conditions, and drives transcription of genes that either help resolve ER stress (XBP1) or activate a pro-apoptotic cascade (CHOP)[98]. Once proteostasis is achieved, homeostatic rates of protein translation must be restored, which is done via the dephosphorylation of eIF2α. This process is catalyzed through recruitment of Protein Phosphatase 1 (PP1) to eIF2α, which occurs constitutively but is also enhanced during stress. The recruitment of PP1 is mediated by two main subunits: the Constitutive Repressor of eIF2α Phosphorylation (CReP), and the stress induced Growth Arrest and DNA Damage inducible protein (GADD34). Nck1/2 also mediates these subunits interaction, which contain Src Homology type 2 and 3 domains, and are involved in signal transduction.

2. Hypothesis:

The UPR, together with other mechanisms that control proteostasis like the Heat Shock Response (HSR) and autophagy have been analyzed in the context of ageing and regeneration. Increasingly, it is becoming apparent that tight control of proteostasis is essential for proper tissue function and preservation[99]. Both embryonic stem cells and ASC were shown to tightly control protein homeostasis[100, 101]. In line with our understanding of miRNA function in satellite cells quiescence and the importance of mRNP granule formation and sequestration of Myf5, our group has focused on investigating proteostatic control in satellite cells and its potential role in the quiescent state. The above-described evidence indicates that post-transcriptional controls of gene expression may play a key role in satellite cell self-renewal and quiescence. Hence, we set out to formulate and thereafter investigate the following hypothesis, which postulates that: **Inhibition of eIF2α dephosphorylation delays satellite cell exit from quiescence and progression into the myogenic program.**

3. Materials and Methods:

3.1. Mice

Care and handling of animals were in accordance with the federal Health of Animals Act, as practiced by McGill University and the Lady Davis Institute for Medical Research. All mice were maintained on a C57BL/6 background on a standard rodent diet. For satellite cell isolation, 5-8 week old *Pax3*$^{GFP/+}$ and *Pax3*$^{GFP/+}$*; tg(actb-luc)* (Taconic) mice or 5-8 week old tmx treated *Pax7*$^{CreERT2/+}$*; eIF2a*$^{S51A/S51A}$*; tg(actb-eIF2a*^{fl} *-eGFP*) and *Pax7*$^{CreERT2/+}$*; tg(actb-eIF2a*^{fl}*-eGFP)* mice were used. For engraftment assays immunocompromised 5 to 7 week old *Foxn1*$^{nu/nu}$*; Dmd*$^{mdx-4cv/mdx-4cv}$ females and *Foxn1*$^{nu/nu}$; *Dmd*$^{mdx-4cv/Y}$ males (Jackson Laboratories) crosses were produced. For muscle regeneration, 6-8 week-old mice were anesthetized by isofluorane (CDMV) inhalation and 50ml of 10µM ctx (Sigma) was injected into the Tibialis Anterior (TA) muscle using a 1 ml syringe and 25 Gauge needle. At 21 days following injury, muscles were harvested for analysis by immunofluorescence. For satellite cell engraftment, host mice were anesthetized with rodent cocktail (ketamine (100mg/kg) xylazine (10mg/kg) and acepromazine

(3mg/kg)) and their right hind limbs were irradiated with 18 Gy of 180 kVp x-rays one day prior to engraftment.

3.2. Fluorescence-Activated Cell Sorting based Satellite Cell Isolation:

Mice were euthanized and pinned with their abdomen facing upwards on a sterile dissection board. Beginning with a small incision in the groin, skin was carefully pulled up while connective tissue was removed, detaching the skin from the underlying muscle. Abdomen and diaphragm muscles were harvested in a laminar flow cabinet and mechanically dissociated in a 100mm sterile petri dish with surgical scissors, until fine slurry consistency was achieved. Next, the sample was transferred to a 15 ml conical tube and resuspended in 10ml of F12 medium with 0.25% Trypsin (Life Technologies) and 0.25% CollagenaseD (Roche), after which it was placed on a nutating platform in a 37°C incubator. Digestion medium was replaced in intervals of 45 minutes; where digested material was collected and added to a 50ml conical tube with ice cold FBS to ensure enzyme inactivation. Digestion product was collected until no visible muscle tissue was left in the digestion tube. Next, tissue digest was filtered through 70-micron cloth and pelleted by centrifugation (20 minutes at 4°C and 623g). Following centrifugation, samples were re-suspended in 1 ml of collection buffer (90% F12, 10% FBS; [v/v]). The sample was additionally filtered through a 70-micron cloth and a 40-micron mesh cap into a 5 ml polypropylene tube. Propedium iodide, a membrane impermeable DNA binding agent that fluoresces when bound, was added to exclude dead cell populations (1µl PI/1ml sample). Samples were analyzed/sorted using a FACS Aria III cell sorter (BD Biosciences). Gating strategy included Forward and Side Scatter (FSC and SSC) to home on single, small and granular particles, followed by GFP positive, PI negative selection. A high yield sorting mask was applied to insure >95% purity.

3.3. Magnetic-Activated Cell Sorting based Satellite Cell Isolation:

For MACS based isolation, tissue was homogenized as described for FACS. Following pelleting, tissue was re-suspended in 1 mL cold PBS, and incubated in Red Blood Cell Lysis buffer (Sigma). Following by a wash in PBS and pelleting, non satellite cell populations were

depleted with the Satellite Cell Isolation Kit (Miltenyi) by negative selection. With this method, all non-myogenic cells were bound to magnetic beads marked by heterogeneous antibodies and then retained within a magnetic column inside a strong magnetic field. Eluted satellite cells were further purified from debris by positive selection with anti-Integrin α-7 MicroBeads (Miltenyi Biotec). Here, satellite cells were retained within the column while debris was eluted.

3.4. Single fibre isolation

Single fibres of Extensor Digitorum Longus (EDL) were isolated as follows. EDL muscle was surgically removed from 5-9 week old C57b6 mice. Individual EDL muscle bellies were incubated for 40 to 50 minutes in 0.2% collagenase D (Sigma) at 37°C on a hot plate to allow dissociation of perimysium ECM tissue. EDL bundles were subsequently placed in cell growth medium (DMEM+10% FBS) in 35 mm wells of a 6 well plate. A flame polished Pasteur pipette was used to triturate tissue and release single fibres into suspension. Single fibres were collected into a 2 mL Eppendorf tube and retained on a 37°C hotplate until fixation or change to culture medium.

3.5. Satellite cell and single EDL fibre culture:

Pure satellite cell preparations and isolated EDL fibres were cultured in 39% DMEM, 39% F12, 20% fetal calf serum (Life Technologies), 2% UltroserG (Pall Life Sciences), for the times indicated in each experiment. For cell culture, 35mm culture dishes were coated with 0.2% fish skin gelatin prior to seeding cells. When indicated, cultures were supplemented with 0.1% dimethyl sulfoxide (DMSO control, Sigma), 10 µM sal003 (Sigma), 1 µM Thapsigargin (TG, Sigma) 50 µM EdU (Life Technologies), 2.5-10 µM Guanabenz (Sigma), 10 µM Compound 1 to 12 (Lumb lab).

For engraftment of cultured cells, the following protocol was performed: Culture media was aspirated, and cells were washed with warm PBS. After PBS removal, cells were incubated with 100 µL 2.5% trypsin and left at 37°C for 1 minute. Cells were then collected in 400-µL of engraftment medium (F12+10%FBS [v/v]). Immediately prior to engraftment, donor cells were counted with a haemocytometer, with non-viable cells excluded by 0.4% Trypan Blue Stain (Gibco). Donor satellite cells were centrifuged for 20 minutes at 700 × g, 4°C and re-suspended

in 4 µL of engraftment medium per 10^4 cells. Engraftment into the TA muscle was done with a flame polished, autoclaved 5µl microcapillary pipette (Drummond).

3.6. Muscle tissue isolation and preparation for cryo-sectioning:

Tiabialis Anterior (TA) muscle was removed from one or both hind limbs of 5-9 weeks old mice. For snap freezing, TA was attached to a piece of cork with Tragacanth gum. Immediately after, the tissue was plunged into a bath of isopentane, previously chilled by partial submersion in liquid nitrogen. Prior to cryosectioning, tissues were mounted with Optimal Cutting Compound (OCT) (VWR). For GFP detection, prefixation and sucrose cryo-protection were required. Isolated TA muscles were fixed for 2 hours in 0.5% paraformaldehyde at 4°C and equilibrated in 20% sucrose at 4°C overnight. Tissues were briefly equilibrated in a 50:50 OCT: Sucrose solution and placed in custom made aluminum foil cryomolds with OCT and flash frozen in an isopentane bath.

Regardless of freezing method, tissues were mounted and sectioned in 10 µM thick sections at -25°C. Tissue sections were collected on warm Superfrost Coated glass slides (Fisher). Sections from snap frozen tissue were immediately fixed in formalin.

3.7. Immunofluorescence:

Immunofluorescence labeling of satellite cell cultures, single EDL myofibers and transverse sections of TA muscle was performed in a standardized fashion, with at least 3 PBS washes between buffer incubations. Cultured satellite cells were washed in PBS and fixed in 10%, neutral buffered formalin solution. Next, cells were washed in 50 nM NH_4Cl, 0.2% Triton permeabilization buffer, followed by 1 hour blocking in 0.2% Fish Skin Gelatine diluted in PBS, at room temperature. Primary antibody incubation was conducted overnight at 4°C, followed by 1-hour secondary antibody incubation at room temperature. All secondary antibody incubations included addition of 4',6-diamidino-2-phenylindole (DAPI) for visualization of cell nuclei. EDL myofibers were processed similarly, with an additional incubation in -20°C Methanol prior to permeabilization.

Labeling of TA sections was done as follows: Sections were washed in PBS and permeabilized in 0.1 % Triton X, 0.1M Glycine solution. Next sections were blocked in Mouse On Mouse (M.O.M) solution for 1 hour at room temperature, followed by primary antibody incubation overnight at 4°C. Antibodies were added to a 2% BSA, 2% horse serum mix in PBS for both primary and secondary antibody. Secondary antibody incubation followed for 1 hour at room temperature. In all experiments, 2-3 drops of Fluorescence mounting medium (Dako) were added prior to coverslip covering.

EdU was detected by Click-IT® Detection kits (Life Technologies) according to kit instructions. Apoptosis was detected by ApopTag Red In Situ Apoptosis Detection Kit (Millipore).

3.8. Immunoblotting:

Freshly isolated satellite cells were centrifuged lysed with 50 µL of lysis buffer or less (1 µL/1000 cells, 1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris) supplemented with cOmplete Protease inhibitor mixture (Roche). Alternatively, satellite cell cultures were scraped in lysis buffer on ice and centrifuged to remove debris. Total protein samples were prepared and were loaded into wells of NuPAGE NOVEX Bis-Tris Gels (Invitrogen) according to kit instructions. Detection was by ECL Plus Western Blotting Detection System (GE Healthcare), and blots were directly imaged on an ImageQuant LAS 4000 (GE Healthcare). Densitometry of immunoblots was performed with ImageJ. Relative expression levels were normalized to detected levels of housekeeping gene, β-tubulin.

Table 1 Immunodetection Reagents

Name	Company	Clonality	Host	Immunofluorescence	Western
Anti-Pax7	DHSB	Monoclonal	Mouse	1/100	NA
Anti-Pax7	Aviva Systems Biology	Polycloncal	Rabbit	NA	1/1000

Anti-eIF2a (Ser51)	Abcam	Monoclonal	Rabbit	1/100	1/1000
Anti-eIF2 Total	Cell Signaling	Monoclonal	Mouse	NA	1/1000
Anti-βTubulin	Millipore	Monoclonal	Mouse	NA	1/1000
Anti-Dystrophin	Pierce	Polyclonal	Rabbit	1/3000	NA
Anti-GFP	Life Technologies	Polyclonal	Rabbit	1/3000	NA
Anti-Ki67	BD Pharmingen	Monoclonal	Mouse	1/100	NA
Anti-MyoD	Dako	Monoclonal	Mouse	1/100	1/500
Anti-MyoD	Santa-Cruz	Polyclonal	Rabbit	1/100	NA
Anti-Myogenin	Santa-Cruz	Polyclonal	Rabbit	1/100	1/1000
Anti-TroponinT	Sigma	Monoclonal	Mouse	1/300	NA
Anti-Digoxigenin-Fluorescein	Millipore	NA	Goat	1/100	NA
Anti-Alexa Fluor 488	Thermo Fisher	Polyclonal	Rabbit/Mouse	1/500	
Anti-Alexa Fluor 594	Thermo Fisher	Polyclonal	Rabbit/Mouse	1/500	
DAPI	Waterborne	NA	NA	1/5000	

3.9. qPCR RNA Analysis

RNA was isolated from cell cultures and freshly isolated satellite cells with TRIzol reagent (Life Technologies) according to manufacturers instructions. RNA was reverse transcribed with Superscript III reverse transcriptase (Life Technologies) and levels of mRNA were measured using SYBR Green on a 7500 Fast Real Time PCR System (Applied Biosystems).

Table 2 Primers

Primers used:	Forward	Reverse
Pax7	5'-AGGCCTTCGAGAGGACCCAC-3'	5'-CTGAACCAGACCTGGACGCG-3'
MyoG	5'-CAACCAGGAGGAGCGCGATCTCCG-3'	5'-AGGCGCTGTGGGAGTTGCATTCACT-3'
Actb	5'-AAACATCCCCCAAAGTTCTAC-3'	5'-GAGGGACTTCCTGTAACCACT-3'

3.10. Bioluminescence Imaging:

For live animal bioluminescence imaging (BLI), D-luciferin (Gold Biotechnology) was administered by two 100µL contralateral intraperitoneal injections to give a final dose of 1,5mg/kg. 20 minutes after D-luciferin administration, animals were anesthetized prior to initiation of procedures and kept on a warm (32°C) platform. Imaging was performed on a weekly basis (Day 0,7,14 and 21) with an IVIS Spectrum *in vivo* imaging system (Perkin Elmer). Data was acquired and analyzed according to complimentary software instructions.

3.11. Bioluminescence Based Low- Throughput Screening:

For low throughput screening 200 isolated *Pax3*$^{GFP/+}$*; tg(actb-luc)* satellite cells were seeded in 200 µL *of* satellite cell culture medium (see above) with added drug treatment, in a black with clear bottom 96-well tissue culture plate. Cells were cultured for 7 days, and

bioluminescence signal was measured once a day from day 4 to 7 of culture. 10 µL of D-Luciferin (1.5mg/mL) was added to each well 20 minutes before imaging.

4. Results:

4.1. Phosphorylation of eIF2α as a translational control of satellite cell quiescence and self-renewal:

Our group used a transgenic mouse model to selectively ablate the phosphorylation site at serine 51 of eIF2α, thus ablating control of global protein synthesis in the mouse satellite cells. By doing so, we demonstrated the need of protein synthesis control in maintaining the quiescent and undifferentiated state of satellite cells *in vivo*. These results are described below to provide context for the experiments further described in this manuscript, as we then decided to ask if this mechanism could be exploited to modulate satellite cell quiescent in *ex vivo* culture. We proposed that inhibition of eIF2α dephosphorylation would delay entry into the myogenic in conditions that favour differentiation. A lack of flox alleles for the *GADD34* and *CReP* genes precluded an *in vivo* approach such as was done with the S51A mouse. Therefore, we elected to use the small molecule inhibitor of GADD34 and CReP, Sal003, to modulate P-eIF2α *ex vivo*.

Initial analysis of quiescent satellite cells on single myofibers and FACS isolated $Pax3^{GFP/+}$ cells has shown accumulation of phosphorylated eIF2α (P-eIF2α) in the quiescent satellite cells, while its rapid downregulation in culture coincided with activation of the myogenic program as indicated by detection of MyoD. Concomitant analysis of UPR branch genes showed high levels of activated PERK (P-PERK), ATF4, CHOP and Bip protein in the quiescent state. It has been shown that the balance of signaling within this pathway can maintain proteostasis or alternatively initiate apoptosis (Supplementary figure 1).

To gain more insight into the mechanistic role of P-eIF2α, our group resorted to using a genetic model of P-eIF2α ablation. In this model, Serine 51 is replaced by an Alanine residue, which ablates eIF2α. Homozygous mice are perinatal lethal, and therefore require the presence

of a rescue transgene, composed of a floxed, wild type ORF of eIF2α followed by a GFP reporter after the 3' flox site. This model was crossed to a $Pax7^{CreERt2}$ mouse line, insuring inducible Cre recombinase expression and P-eIF2α loss only in adult satellite cells of tamoxifen injected mice. Cells that lost their ability to express wild type eIF2α begin expressing GFP, which allowed isolation of $eIf2\alpha^{-/-}$ satellite cells (S51A) and facilitated tracing their fate *in vivo*. After 5 days of tamoxifen treatment, P-eIf2α, protein synthesis rates, *in vivo* localization and status of the myogenic program were measured in S51A cells and compared to wild type satellite cells. As expected, P-eIf2α decrease has led to increase in protein synthesis rates (as measured by OPP incorporation), impaired mRNP granule formation, and accumulation of Myf5 and MyoD protein together with translocation to the interstitial space; all hallmarks of activated satellite cells. As P-eIF2α has been reported to enhance translation of genes containing uORF, our group has hypothesized that stemness genes would be preferentially translated in high P-eIF2α conditions (Supplementary Figure 2).

Parallel analysis of wild type satellite cell transcriptome and proteome data identified multiple genes whose transcripts were present but were not translated. Further comparison to a database of transcripts selectively translation during P-eIf2α conditions identified multiple transcripts previously implicated with regulation of stemness, many of which contained uORFs (Supplementary Figure 3).

Having observed spontaneous activation of the myogenic program, our group went to further study the long-term impact of P-eIF2α ablation. Ten days after terminating tamoxifen treatment, our group has detected newly formed, centrally nucleated fibers that expressed high levels of GFP. Remaining GFP positive and MyoD or Pax7 positive cells were still proliferating and giving rise to fibers, as BrdU labeling was detected in nuclei of nascent myofibers and single cells. At 3 weeks (21 days) post P-eIF2α inactivation, no GFP positive, Pax7 positive satellite cells were detectable in the niche of uninjured S51A mice. Cardiotoxin injury of S51A muscle showed complete regeneration, as all fibers were centrally nucleated and GFP positive, but rare Pax7 positive satellite cells were exclusively GFP negative. Loss of self-renewal was further confirmed by engraftment of S51A cells into irradiated nude mouse TA. S51A cells gave rise to clusters of GFP fibers within the graft area, but no GFP positive, Pax7 positive

satellite cells were detected 3 weeks post engraftment in contrast to cells from similar mice that retained their eIF2α$^{+/+}$ homozygosity (Supplementary Figure 4).

We chose to test our hypothesis in an *ex vivo* model of satellite cell differentiation. Primary satellite cells cultured *ex vivo* are prone to rapidly proliferate and progressively differentiate to give rise to terminally differentiated myocytes, which then give rise to multinucleated myotubes. This is a suitable model for our hypothesis, as we show that P-eIF2α is significantly decreased after 3 days in culture, when satellite cells express MyoD and are highly proliferative. To achieve efficient inhibition of eIF2α dephosphorylation, and therefore maintain high levels of P-eIF2α, we chose to use a small molecule compound described in the literature[102, 103]. Sal003, a derivative of the better-characterized Salubrinal, is reported to be a potent and specific inhibitor of eIF2α dephosphorylation. Sal003 inhibits both CReP and GADD34 mediated recruitment of PP1 to eIF2α, and therefore was appropriate for the experiments at hand[103].

4.2. Sal003 Maintains High Levels of P-eIF2α in Cultured Satellite Cells:

Pax3$^{GFP/+}$ satellite cells were isolated by FACS and cultured with 10 μM Sal003. We first chose to examine the effect of Sal003 on P-eIF2α levels and the myogenic program in satellite cells after 4 days in culture. This time point is appropriate because the majority of cells in culture have downregulated Pax7 and gained MyoD and/or MyoG expression, indicating that

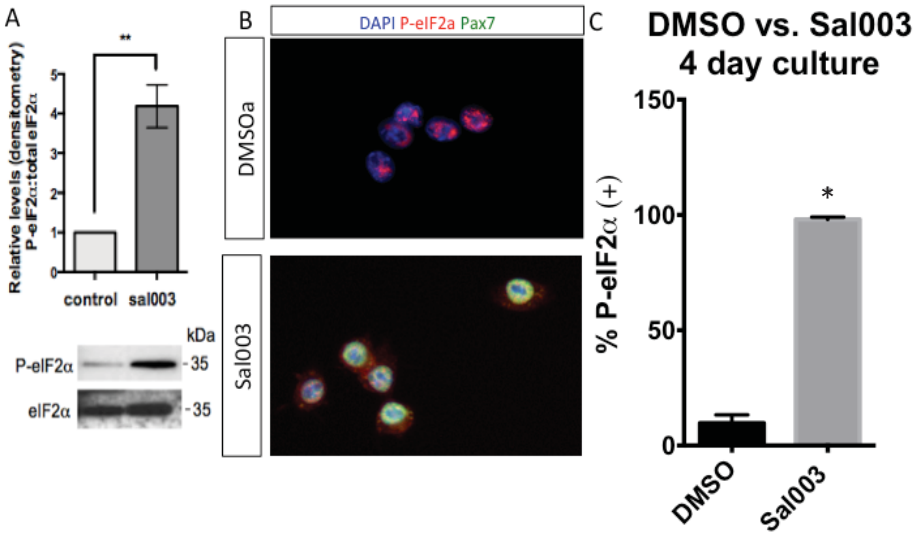

Figure 4.1 Sal003 treatment maintains high P-eIF2α in 4 day cultures of satellite cells. (A) Validation of P-eIF2α by western blot conducted in collaboration in our lab [Zismanov et al,Cell Stem Cell. 2016 Jan 7;18(1):79-90].(B) Representative image of P-eIf2α (red) and Pax7 (Green) in 4 day cultures of satellite cells in the presence of DMSO and Sal003. (C) Quantification of data represented in A. Significant results represented with a * at p<0.05.

they have committed to terminal differentiation and lost their stem cell status[51]. We used western blot and immunofluorescence to detect P-eIF2α. Significant maintenance of P-eIF2α in Sal003 treated cultures (Figure 4.1A) was observed, compared to DMSO (vehicle) treated cells. Immunofluorescence further showed that P-eIF2α remains diffused in the cytosol in Sal003 treated cultures, compared to few localized foci in control cultures (Figure 4.1B and C). Basal levels of P-eIf2α are an expected to occur in all cells and might represent specific foci that require translational regulation during the differentiated state.

4.3. Sal003 Treatment Increases Pax7+; MyoD- Satellite Cells After Four Days in Culture:

Next, we sought to understand how the myogenic program was influenced by Sal003 treatment. We used immunofluorescence for Pax7 and MyoD, and quantified the proportion of cells that were Pax7+,MyoD- , which would represent a self-renewed satellite cell population;

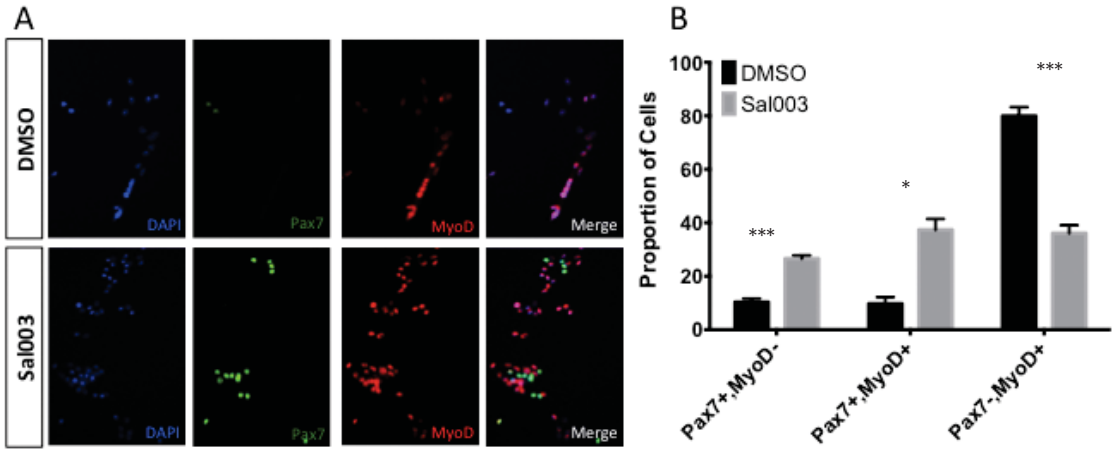

Figure 4.2 Quantification of Pax7 and MyoD expression in Sal003 treated satellite cells after 4 days in culture. (A) Representative immunofluorescence images of satellite cell colonies after 4 days in culture. Pax7 is green, MyoD is red. (B) Quantification of self-renewal [Pax7+,MyoD-], Activation [Pax7+, MyoD+] and differentiation [Pax7-,MyoD+] in Pax3$^{GFP/+}$ cells treated with Sal003 at Day 0 of culture. Significant results represented with a * at $p<0.05$.

Pax7+,MyoD+ which would represent activated, transiently amplifying myoblasts and; Pax7-, MyoD+ which would represent cells that have committed to differentiate. Previous reports in the literature showed that the proportion of Pax7+, MyoD- reserve cells after 4 to 5 days of normal culture was approximately 10%, compared with more than 75% of cells being Pax7-, MyoD+ [31]. In our experiment, we observed similar proportions in our control cultures, but a significant change in our Sal003 treated plates (Figure 4.2A). The proportion of Pax7+,MyoD- reserve cells has tripled to approximately 30%, while the proportions of Pax7+, MyoD+ and Pax7-,MyoD+ cells have significantly decreased(Figure 4.2B). Concurrent analysis by western blot confirmed that levels of Pax7 remained elevated in Sal003 cultures 4 days into culture, compared to significant decrease in controls (Figure 4.3). The opposite effect was observed for expression of Myogenin, a marker of commitment to terminal differentiation. Analysis of mRNA expression by qPCR confirmed decreased transcription of MyoG, and therefore

delayed progress of the myogenic program. This also excludes the possibility that MyoG protein production was limited by P-eIF2α despite transcript accumulation. Intriguingly, Pax7 transcripts were not significantly upregulated, despite detection of increased protein levels, possibly indicating that Pax7 protein expression is preferentially maintained in conditions of high P-eIF2α.

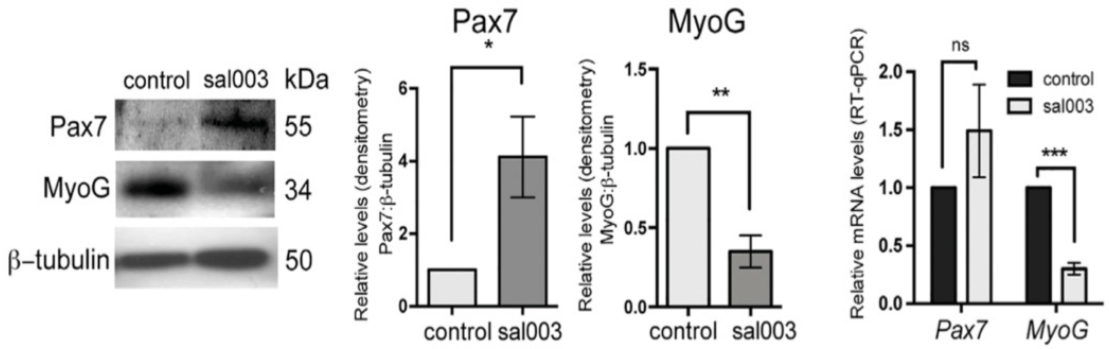

Figure 4.3 From Zismanov et al,Cell Stem Cell. 2016 Jan 7;18(1):79-90. Western blot and qPCR quantification of Pax7 and Myogenin. Left – representative images of Pax7 and MyoG, normalized to tubulin. Middle – densitometry based quantification of Pax7 and MyoG protein expression. Right- qPCR quantification of Pax7 and MyoG mRNA.

4.4. Sal003 Promotes Satellite Cell Cycling:

The increased numbers of Pax7+, MyoD- cells in 4 day cultures with Sal003 prompted us to ask if self –renewal has occurred, or if differentiation is merely delayed by maintenance of high P-eIF2α levels. First, we used the traditional cell proliferation marker Ki67 to detect cycling cells (Figure 4.4A). Ki67 is localized in the cell nucleus at all stages of the cell cycle, while absent in quiescent cells.

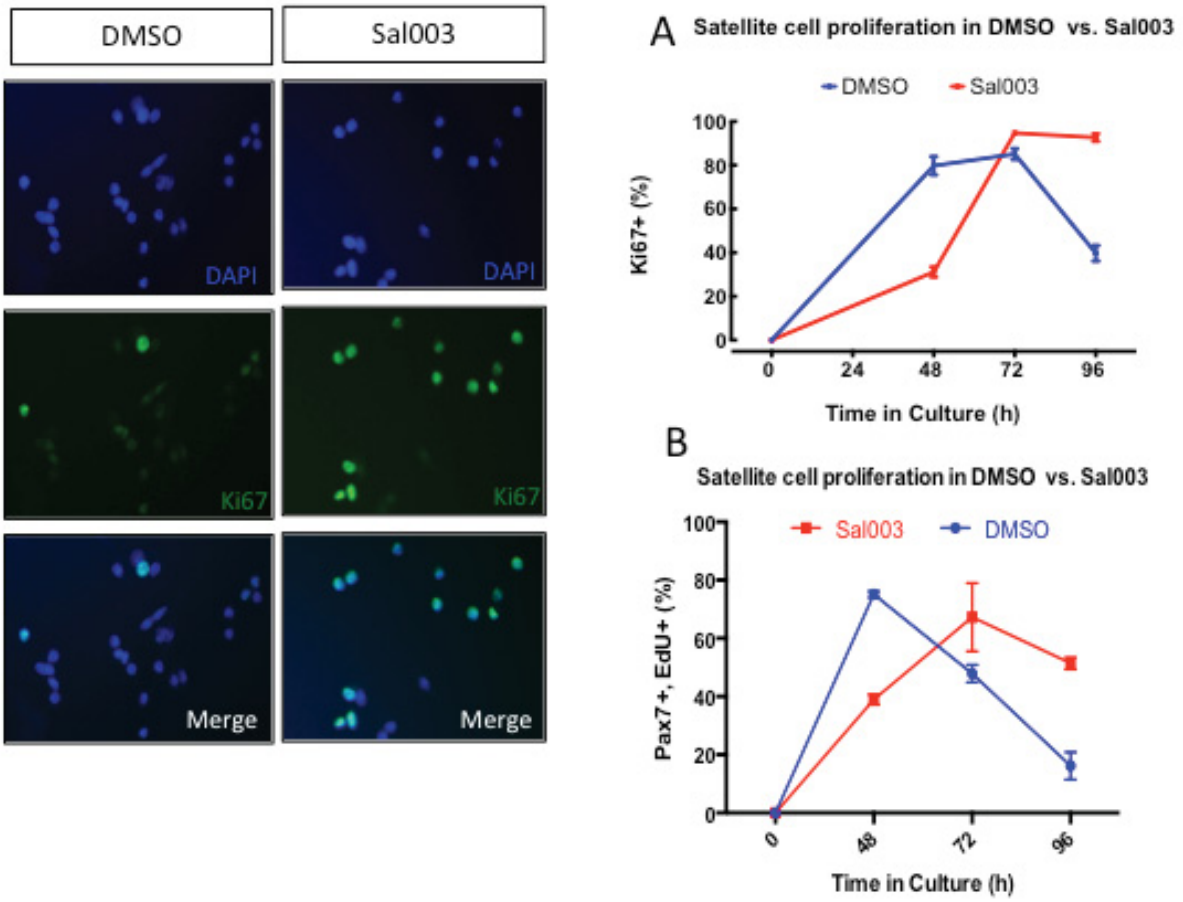

Figure 4.4 Cell cycle analysis of Sal003 treated satellite cells throughout 4 days of culture. Left- Representative images of Ki67 immunostaining on 4 day cultured satellite cells. (A) Quantification of ki67 staining from D0 to day 4. (B) Quantification of EdU incorporation after a 4-hour pulse throughout 4 days of culture.

Analysis of our control plates showed that Ki67 was significantly upregulated during Day 2 and 3, corresponding with exit from quiescence and proliferation of the transiently amplifying muscle progenitors. By day 4, more than 60% of cells downregulate Ki67 and exit the cell cycle, which corresponds with the high proportion of Pax7-, MyoD+ cells that commit to differentiation. In contrast, Sal003 treated cells show delayed re-entry into the cell cycle, and robust proliferation even after 4 days of culture (Figure 4.4A). As Ki67 protein may be affected by modulation of protein translation by Sal003, we also conducted EdU pulsing to quantify the number of cycling cells. The observed trend was similar to Ki67 labeling (Figure 4.4B). Comparison of colony sizes at day 4, where all myonuclei originate clonally from one stem cell, shows no significant difference in the average number of cells per colony (Figure 4.5A).

4.5. Sal003 Treatment Does Not Induce Cell Death:

In addition, we examined apoptosis in cultures treated with Sal003. Treatment with Salubrinal has been previously shown to be cytoprotective in stressed cells[103].

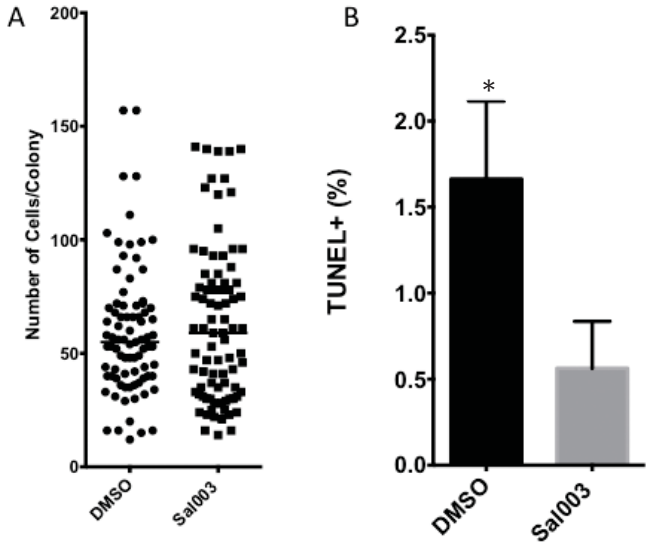

Figure 4.5 Absolute growth and cell death rates are not significantly impacted by Sal003. (A) Quantification of cells per satellite cell colony. (B) Quantification of TUNEL positive nuclei in DMSO and Sal003 treated cultures. Significant results represented with a * at $p<0.05$.

Nevertheless, as we showed that quiescent satellite cells express high levels of CHOP, a pro-apoptotic factor further upregulated in high P-eIF2α conditions, we reasoned that we may favour the pro-apoptotic pathway by maintaining high P-eIF2α. We used a commercial kit to detect cells undergoing late apoptosis through the TUNEL method. Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling allows detection of fragmented DNA within cells that are undergoing late stages of apoptosis. Microscopic analysis of satellite cell cultures detected little to none apoptotic cells in control cultures, and further less in Sal003 treated cultures, which is agreeable with Sal003's cytoprotective properties (Figure 4.5B).

Due to the canonical outlook of the ISR pathway, we went back and hypothesized that genetic removal of eIF2α phosphorylation site in the S51A mouse might impair cell survival during injury or *ex vivo* culture[98]. To address cell death *in vivo*, we used TUNEL staining on transverse cryosections of TA muscle from Pax7^{CreErt2}; eIF2a$^{S51A/S51A}$; tg[actb-eIF2α -GFP] or eIF2α $^{+/+}$; tg[actb-eIF2α -GFP]. Sections were stained for GFP and fluorescent TUNEL detection, and double stained cells were quantified early after

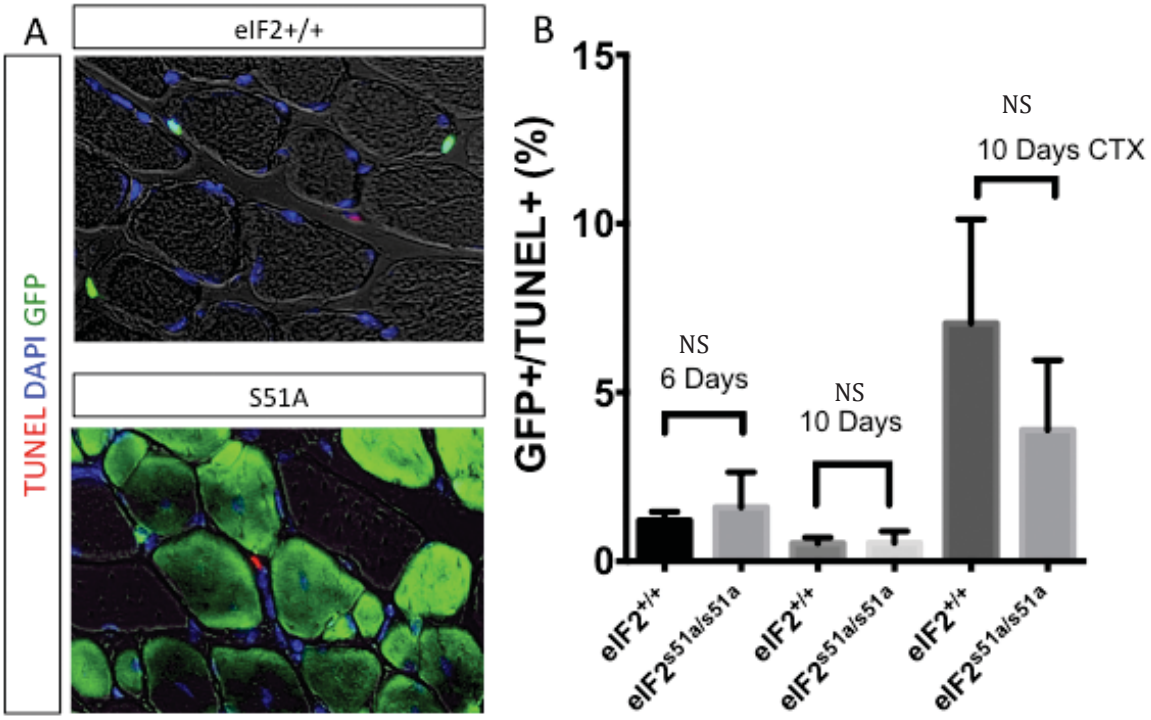

Figure 4.6 Investigation of Apoptosis *in vivo*. (A) Representative images of Apoptotic nuclei in muscle of wild type and S51A mice. (B) Quantification of average apoptotic nuclei per section of muscle. Significant results represented with a * at $p<0.05$

tamoxifen mediated excision of the eIF2α rescue transgene; 10 days after tamoxifen injection as well as 10 days post tamoxifen and cardiotoxin mediated muscle injury (Figure 4.6A). In all instances, no significant increase in TUNEL+, apoptotic cells was detected (Figure 4.6B). A similar approach was applied to *ex vivo* cultures of wild type and S51A satellite cells at four days of culture. Quantification of TUNEL + nuclei did not show a significant difference in late stage apoptosis between wild type and S51A in regular culture medium (Figure 4.7A and B). However treatment with Thapsigargin, a Sarco/Endoplasmic

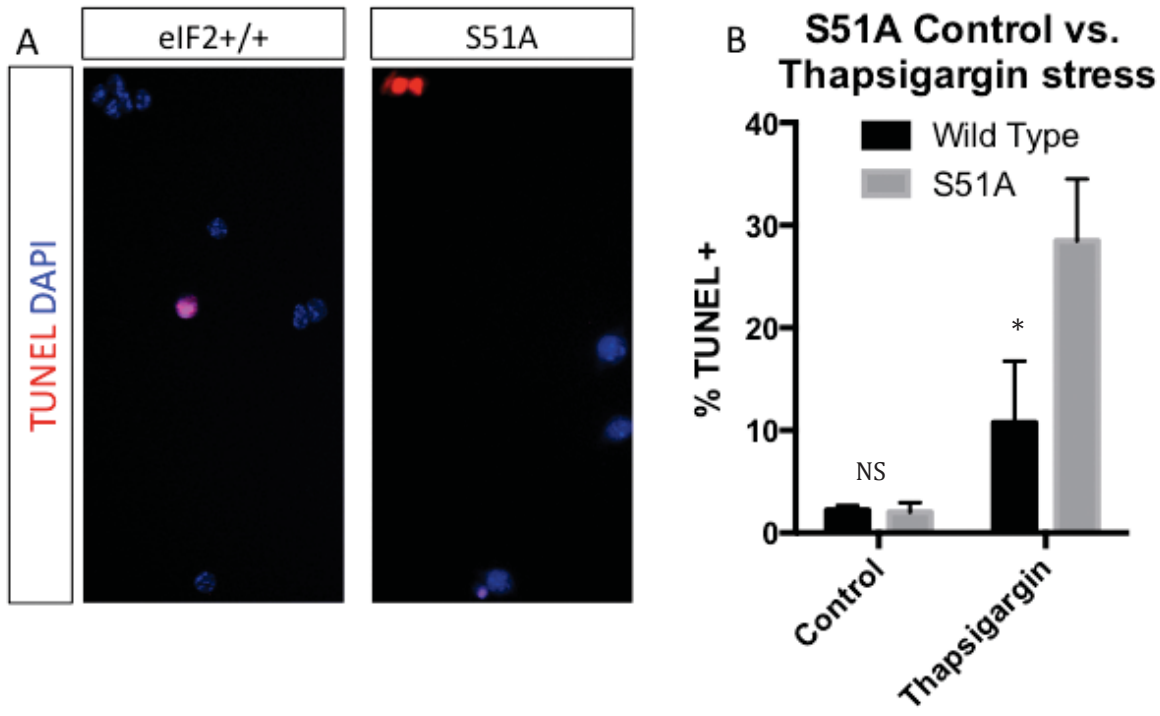

Figure 4.7 Investigation of apoptosis in vitro. (A) Representative image of apoptotic nuclei in cultures of wild type and S51A satellite cells at 4 days. (B) Quantification of Apoptotic nuclei in Control and Thapsigargin treated cells. Significant results represented with a * at $p<0.05$

Reticulum Ca^{2+} ATPase inhibitor commonly used to induce high levels of stress, showed significant increase in apoptosis of S51A satellite cells. Combined, these results show that loss of eIF2α phosphorylation does not impair satellite cell survival capacity in conditions of homeostasis or physiological injury, but plays a role in responses to cytotoxic stress.

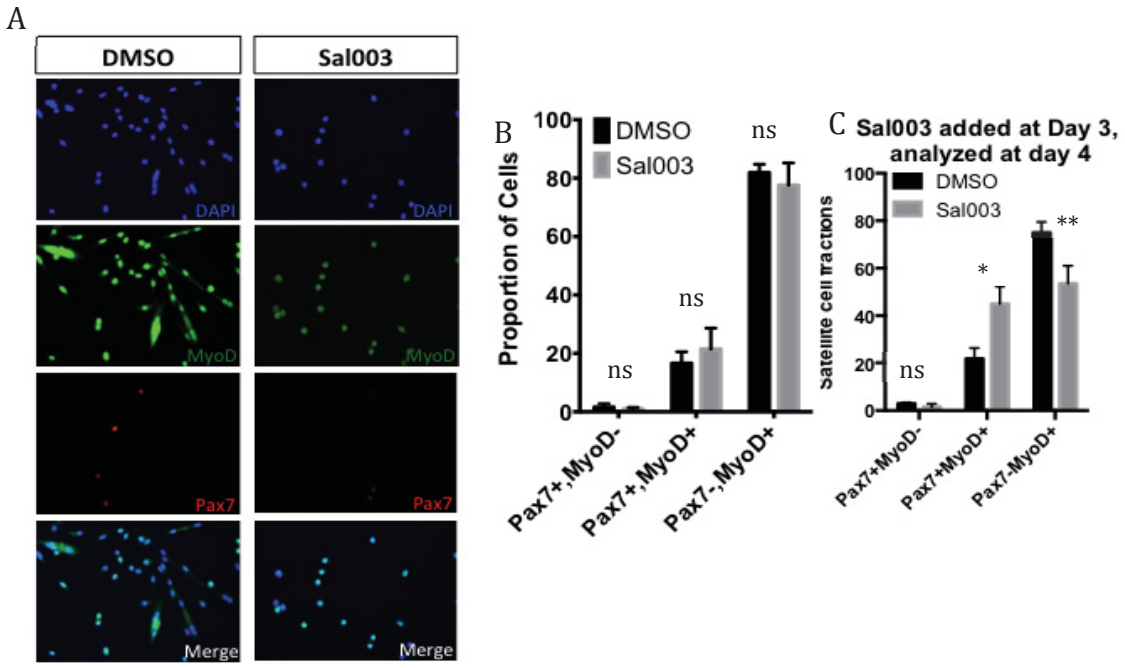

Figure 4.8 Confirmation of Sal003 effect through P-eIF2α. (A) Representative images of S51A satellite cells in culture after 4 days. (B) Quantification of self-renewal [Pax7+,MyoD-], Activation [Pax7+, MyoD+] and differentiation [Pax7-,MyoD+]in S51A cells. (C) Quantification of self-renewal [Pax7+,MyoD-], Activation [Pax7+, MyoD+] and differentiation [Pax7-,MyoD+] in Pax3$^{GFP/+}$ cells treated with Sal003 at Day 3 of culture. Significant results represented with a * at $p<0.05$

4.6. Sal003 Cannot Inhibit Myogenic Program Activation in the absence of P-eIF2α:

To test if the observed increase in Pax7+, MyoD- cells is P-eIF2α dependent; we resolve to repeat the above-mentioned experiments in satellite cells from S51A mice (Figure 4.8A). In both control and Sal003 conditions, the fraction of Pax7+, MyoD- cells remains unchanged, and significantly decreased compared to wild type cells (Figure 4.8B). Moreover, addition of Sal003 to satellite cell culture at day 3, and not at day 0, does not promote retention of Pax7+,MyoD- cells in wild type cells (Figure 4.8C). This indicates that high intracellular levels of P-eIF2α are required for preservation of Pax7+, MyoD- cells when satellite cells are cultured *ex vivo*, and that Sal003 cannot reverse progression throughout the myogenic program when eIF2α phosphorylation is lost or downregulated.

4.7. The Effect of Sal003 On The Myogenic Program Is Transient and Reversible:

Next, we decided to examine how Sal003 treatment affects satellite cell activity at later time points. We treated satellite cell cultures with 10 μM Sal003 upon seeding in a subset of plates, while also adding a second dose after 3 days of culture. By 5 days of culture, the majority of cells upregulate Myogenin and Troponin T, and fuse with other cells within their proximity to form multinucleated myotubes. In our experiment, we observed formation of large, polynucleated myotubes in cultures treated with Sal003, compared to

control (Figure 4.9A). Fusion index quantification indicated a significant increase in the number of myonuclei per myotube, which demonstrates an increase of clonal progenitors in Sal003 cultures (Figure 4.9B). Addition of Sal003 at day 3 only did not affect fusion index results, which correspond with our argument that P-eIF2α loss is unidirectional in *ex vivo* culture (Figure 4.9C and D). Finally, culture treatment with Sal003 at Day 0 as well as at day 3 of culture significantly impairs myotube formation in culture and expression of Myogenin and Troponin T (Figure 4.9E and F). Together, these findings demonstrate that

Sal003 exerts a robust inhibitive effect on satellite cell ability to progress through he myogenic program.

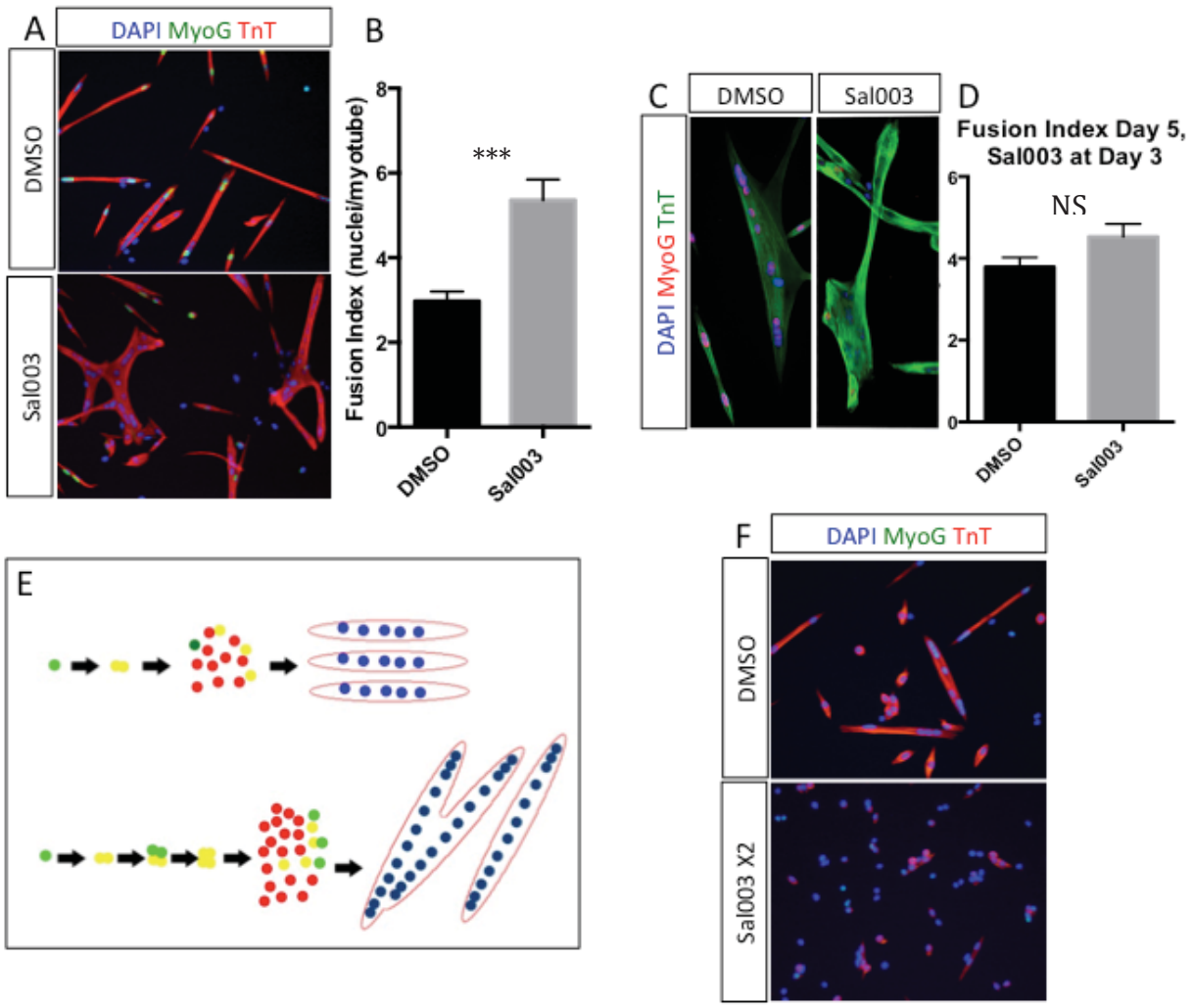

Figure 4.9 Long-term treatment of Satellite Cells with Sal003. (A) Representative images of 5-day satellite cell cultures treated with Sal003 at seeding time. (B) Fusion Index of cells from (A). (C) Representative images of 5-day satellite cell cultures treated with Sal003 at Day 3 of culture only. (D) Fusion Index of cells from (C). (E) Schematic representation of potential mechanism leading to increase in fusion index in Sal003 treated cultures. DMSO top, Sal003 bottom. (F) Representative images of satellite cells treated with Sal003 at seeding time and again at day 3 of culture. Significant results represented with a * at $p<0.05$

4.8. Utilization of a Non-Invasive Functional Assay of Satellite Cell Engraftment:

The observed delay in satellite cell entry into the myogenic program has prompted us to consider whether they retain stem cell potential while expanding *ex vivo*. To test this hypothesis, we chose to test their engraftment capacity and myogenic potential. We chose to adopt a recently described method of tracking graft survival *in vivo* by bioluminescence detection[14]. In our model, we crossed $Pax3^{GPF/+}$ mice to a transgenic mouse line that expresses constitutive levels of firefly luciferase from a transgene composed of β-actin promoter elements and the cloned ORF of *p. pyralis* luciferase. This model allowed us to isolate pure populations of GFP+, Luciferase (luc)+ satellite cells for immediate engraftment or for pre-engraftment expansion in culture. We also chose immunocompromised, $Foxn1^{nu/nu}$; $Dmd^{mdx\text{-}4cv}$ mice as appropriate hosts for our engraftment experiments[14]. Homozygous mutations in Foxn1 block development of a functional thymus, and thus inhibit proper generation of T cell populations. Thus, nude mice exhibit impaired acquired immune response functionality, which is slightly compensated by a potentiated innate immunity through enhanced natural killer cell activity[104]. To better visualize muscle regeneration, we crossed nude mice to the mdx mouse line. Mdx mice harbour a spontaneous nonsense mutation in the 53^{rd} exon of the *DMD* gene resulting in a truncated dysfunctional gene. Lack of functional dystrophin expression in muscle of these mice recapitulates the molecular and physiological phenotypes of Duchene's dystrophy, although mice do not develop pathological symptoms until very old age[105]. Furthermore, the constant cycle of tissue degeneration followed by regeneration in mdx muscle provides engrafted satellite cells with additional cues to activate. In addition, we chose to irradiate host muscle prior to engraftment, to ensure ablation of endogenous satellite cells that may out compete engrafted cells for their position in the niche.

The presence of the Luc gene allowed us to localize the engrafted cells as well as their differentiated progeny with the IVIS Bioluminescence imaging system. It is important to note however that differentiated satellite cells fuse to form new, or repair existing, muscle fibres and therefore signal is detected from single cells and also myofibers. Nevertheless, we could track

satellite cell survival and growth over the span of three weeks, after which we extracted muscle for more precise histological analysis.

4.9. Sal003 Treated Satellite Cells Maintain High Levels of Engraftment:

We chose to compare the engraftment capacity of 10000 freshly isolate (FI) satellite cells to the equivalent number of cultured cell. While freshly isolated cells served as a positive, "gold standard", control for assessing satellite cell self-renewal and muscle regeneration; we also choose to engraft 1600 freshly isolated cells. The later number corresponds to the initial number of satellite cells we calculated as required to give rise to 10000 cells after 4 days in culture in Sal003. Since satellite cells lose their capacity to self-renew in culture, we assumed that 1600 freshly isolated cells would be roughly equivalent to 10000 Sal003 cultured cells in their regenerative capacity.

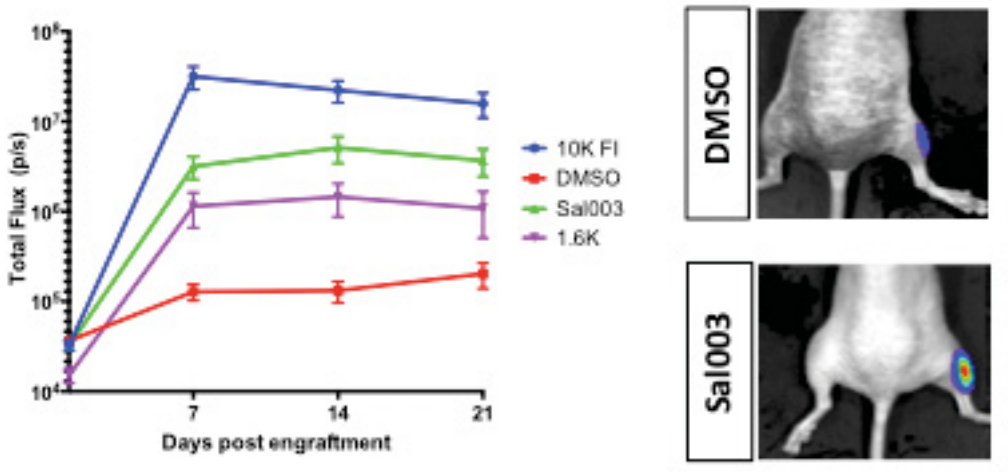

Figure 4.10 Bioluminescence Signal Quantification. (Left) Bioluminescence signals were quantified over 21 days for grafts of: 10000 Freshly isolated satellite cells (FI), 1600 Freshly isolated satellite cells (1.6K), 10000 DMSO cultured myoblasts, and 10000 Sal003 treated myoblasts. (Right) Heat map representation of bioluminescence signal.

As expected, all grafts showed roughly equivalent luminescence signal one day after engraftment, indicating that initial numbers of viable cells were equivalent upon injection. Over time, measured luminescence was strongest in grafts of FI cells, followed by Sal003. Grafts of 1600 freshly isolated cells were not as robust; indicating that cultures expanded in the presence of Sal003 contained more engraftment-suitable cells. As expected, DMSO grafts failed to

produce significant signal, potentially due to the high number of differentiated, non cycling myocytes present within the engrafted population (Figure 4.10)[14, 106].

After 21 days, luminescence signal has stabilized and plateaued in all engraftment conditions, signifying the end of satellite cell regenerative activity and return to homeostasis. As mentioned above, fibre derived bioluminescence signal could not be decoupled from satellite cell derived signal, therefore requiring more in depth analysis by immunohistochemistry. Engrafted muscles were cryosectioned, and consecutive sections were stained with anti-dystrophin or anti-GFP antibodies in conjunction with anti-Pax7 staining (Figure 4.11A and C). Detection of naturally occurring dystrophin positive fibres, termed revertant fibres, in mdx muscle has been previously reported to occur at low frequency in the mdx^{4CV} mouse line (Figure 4.11A). Therefore, any clustering of dystrophin positive fibres in engrafted muscle would be considered donor derived, and indicate regenerative capacity of donor cells. Similarly,

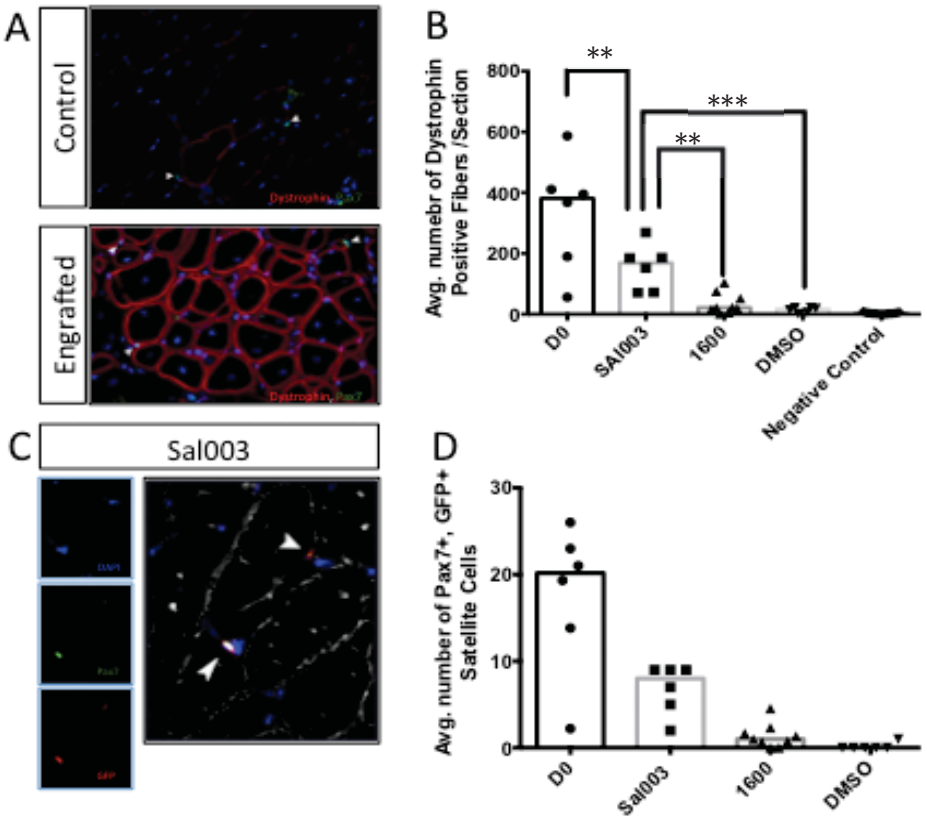

Figure 4.11Quantification of Dystrophin and GFP in satellite cell grafts. (A) Representative images of Dystrophin expression in non-engrafted (top) and engrafted (bottom) muscle of nude; mdx muscle. (B) Quantification of Dystrophin fibres in muscle grafts 21 days post engraftment. (C) Representative images of donor satellite cells in grafts of Sal003 treated cells. (D) Quantification of GFP fibres in muscle grafts 21 days post engraftment. Significant results represented with a * at $p<0.05$

detection of GFP positive, Pax7+ cells would differentiate donor cells from endogenous, surviving satellite cells.

In our analysis, we detected the largest number of dystrophin positive fibres as well as the largest number of donor satellite cells at the canonical satellite cell position in grafts of FI cells (345 fibres, 18 Pax7+, GFP+ cells respectively). Of note, donor satellite cells were localized to areas of dystrophin positive fibres, indicating that self-renewal occurred within the regenerating graft and those satellite cells do not migrate outside the graft area. The next highest number observed was in grafts of Sal003 treated cells, where significant numbers of dystrophin positive fibres were detected with adjacent Pax7 positive, GFP positive cells. In comparison, fewer fibres were detected after engraftment of 1600 satellite cells, while virtually no regeneration as observed in grafts of DMSO cultured cells. In addition, almost no Pax7 positive, GFP positive satellite cells were detected within grafts of DMSO treated or 1600 FI grafted cells, indicating that the majority of cells gave rise to fibres without undergoing self-renewal (Figure 4-11B and D). Overall, this experiment supports the notion that Sal003 mediated maintenance of P-eIF2α allows expanding satellite cells to retain their regeneration capacity.

4.10. Engrafted Cells From Sal003 Cultures Retain Myogenic Potential after Re-isolation:

Finally, our observation of GFP positive, Pax7+ cells in the histological satellite cell position did not provide direct evidence of these cells myogenic capacity. To demonstrate that donor cells were capable of de novo muscle generation post engraftment, we resolve to re-isolate these cells from engrafted muscle and culture it *ex vivo*. After having cultured isolated cells for 6 days, we have been able to detect Troponin T positive myotubes with Myogenin positive myonuclei (Figure 4.12).

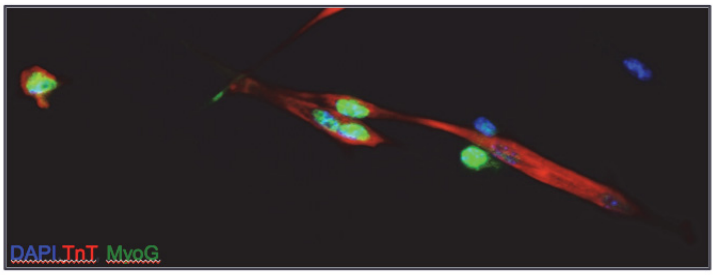

Figure 4.12 Re-isolated satellite cells in culture. Engrafted muscles were reprocessed for satellite cell isolation, and GFP positive cells were isolated from muscles using FACS, plated and cultured for 6 days.

4.11. Investigation of Additional Small Molecule Modulators of P-eIF2α:

The ensemble of results obtained by our group has been recently published in the literature under Zismanov, Chichkov, Collangelo et al. The positive outcomes of Sal003 treatment on satellite cell self-renewal and engraftment capacity have pushed us to try and investigate other modulation of the UPR affects satellite cell activity. We first set out to investigate Guanabenz, a clinically approved inhibitor of α2-adrenergic receptor, which was demonstrated in the literature to selectively inhibit eIF2α dephosphorylation through

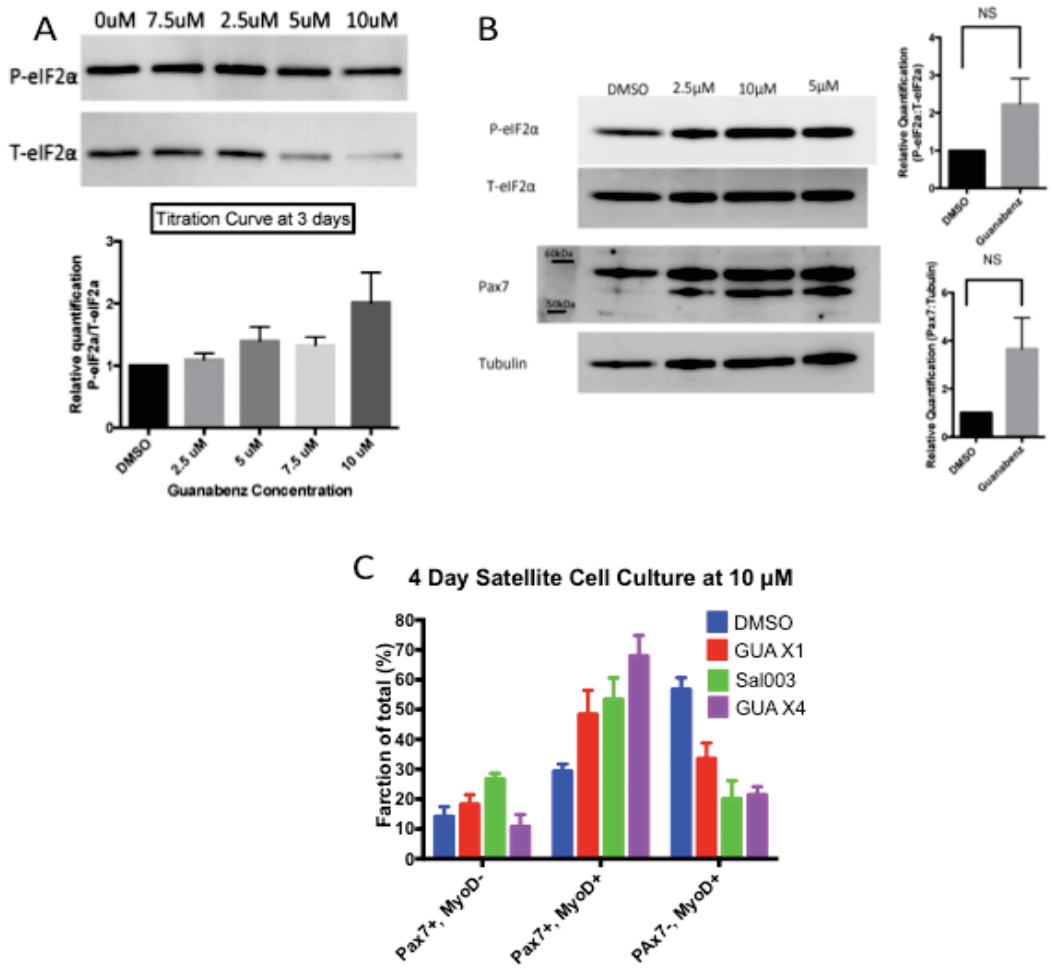

Figure 4.13 Characterization of Guanabnez and its effects of the myogenic program. (A) Titration curve of Sal003 treatment on levels of P-eIF2α. (B) 10 μM Guanabenz maintains high P-eIF2α and pax7 in satellite cells cultures treated daily. (C) Quantification of self-renewal [Pax7+,MyoD-], Activation [Pax7+, MyoD+] and differentiation [Pax7-,MyoD+] in Pax3$^{GFP/+}$ cells treated with Guanabenz at 10 μM, 10μM repeatedly, and Sal003 for 4 days of culture. Significant results represented with a * at p<0.05

inhibition of PP1-GADD34, but not PP1-CReP. We first sought to test the effect of Guanabenz treatment on eIF2α phosphorylation in 3-day satellite cell cultures, a single

treatment of Guanabenz showed a gradual increase in phosphoprotein levels when normalized to the total protein levels in the cell (Figure 4.13A). We established that 10 μM, similar to Sal003 was the maximal concentration that allowed cell growth. We then proceeded to measure P-eIF2α and Pax7 levels in satellite cells cultured for 4 days in daily 10 μM dosage of Guanabenz. Repeat dosage shows an increase in Pax7 and P-eIF2α levels at 4 days in culture, although great variability was observed (Fig. 4.13B). Concurrent immunofluorescence analysis of single and repeat Guanabenz dosage compared to a single dose of Sal003 shows that increased Guanabenz treatment increases the Pax7+ MyoD+ myogenic progenitor pool, but does not allow retention of a significant proportion of Pax7+, MyoD- cells, unlike Sal003 (Figure 4.13C). Due to the lack of significant benefit over Sal003, we chose to continue our investigation with Sal003 and its derivatives.

4.12. Further Development of Sal003 Derivative for Enhanced Inhibition of eIF2α Dephsphorylation:

Therefore, we have decided to continue investigating the molecular role Sal003 plays in satellite cell self renewal. Gadd34 and CReP inhibition have long been hypothesized as the direct targets of Sal003, which prevents their recruitment of Protein Phosphatase 1C to eIF2α. A recent X-ray crystallography analysis of GADD34 shed more light on its function, but failed to identify any direct interactions between it and Sal003 or Guanabenz. We've therefore decided to focus our recent efforts on collaboration with JP

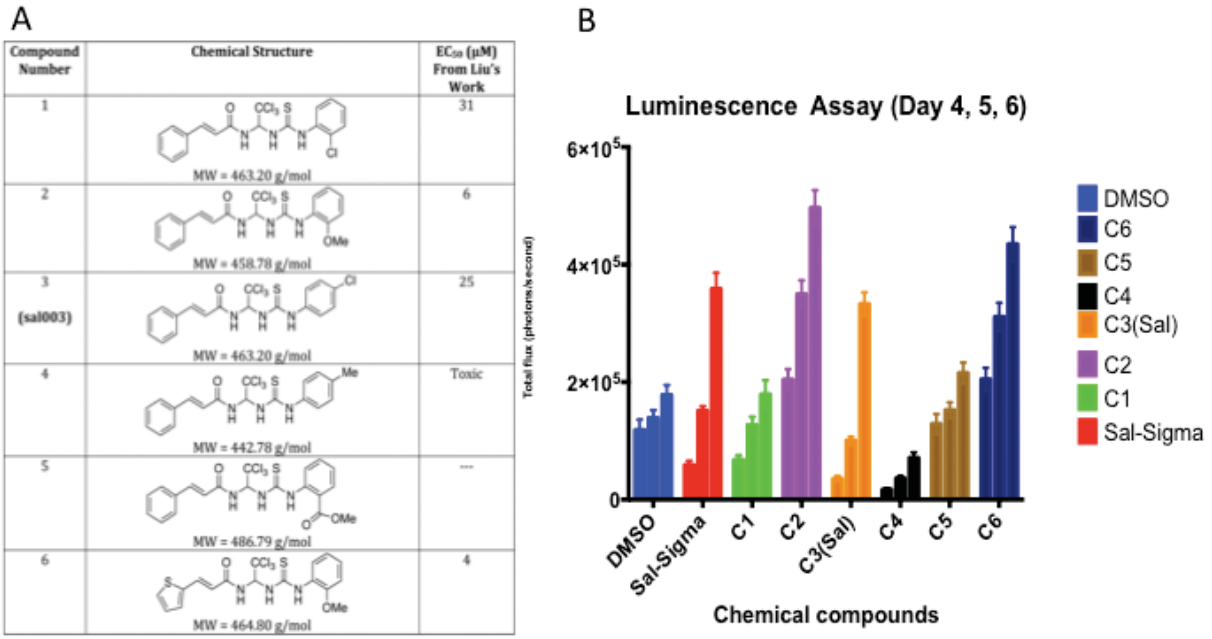

Figure 4.14 Screening of Sal003 derivatives for better efficacy. (A) Representative table of six previously synthesized Sal003 derivatives and their effective concentrations. (B) Bioluminescence signal throughout day 5,6 and 7.

Lumb's chemistry group at McGill; to generate Sal003 derivatives effective at lower, nM, concentration while simultaneously shed more light on Sal003's biochemical targets. We established a low-throughput system to screen compounds based on their ability to expand cell growth as measured by photon flux from cultured $Pax3^{GFP/+}$; actb-Luc^{+} satellite cells. Immunofluorescence Pax7 and MyoD quantification will than be used to examine lead candidates for their effect on cell differentiation. First, we sought to synthesize Sal003 with comparable quality to commercially available product as well as a series of already established derivatives generated characterized elsewhere (Figure 4.14A)[107]. Although

these derivatives were tested in cardiomyocyte stress conditions, their relative potency was maintained when satellite cells were treated and left to expand in their presence. Specifically, compounds 2 and 6 demonstrated linear increase in signal, which corresponds with their low reported effective concentrations. Commercial and synthetic Sal003 (C3)

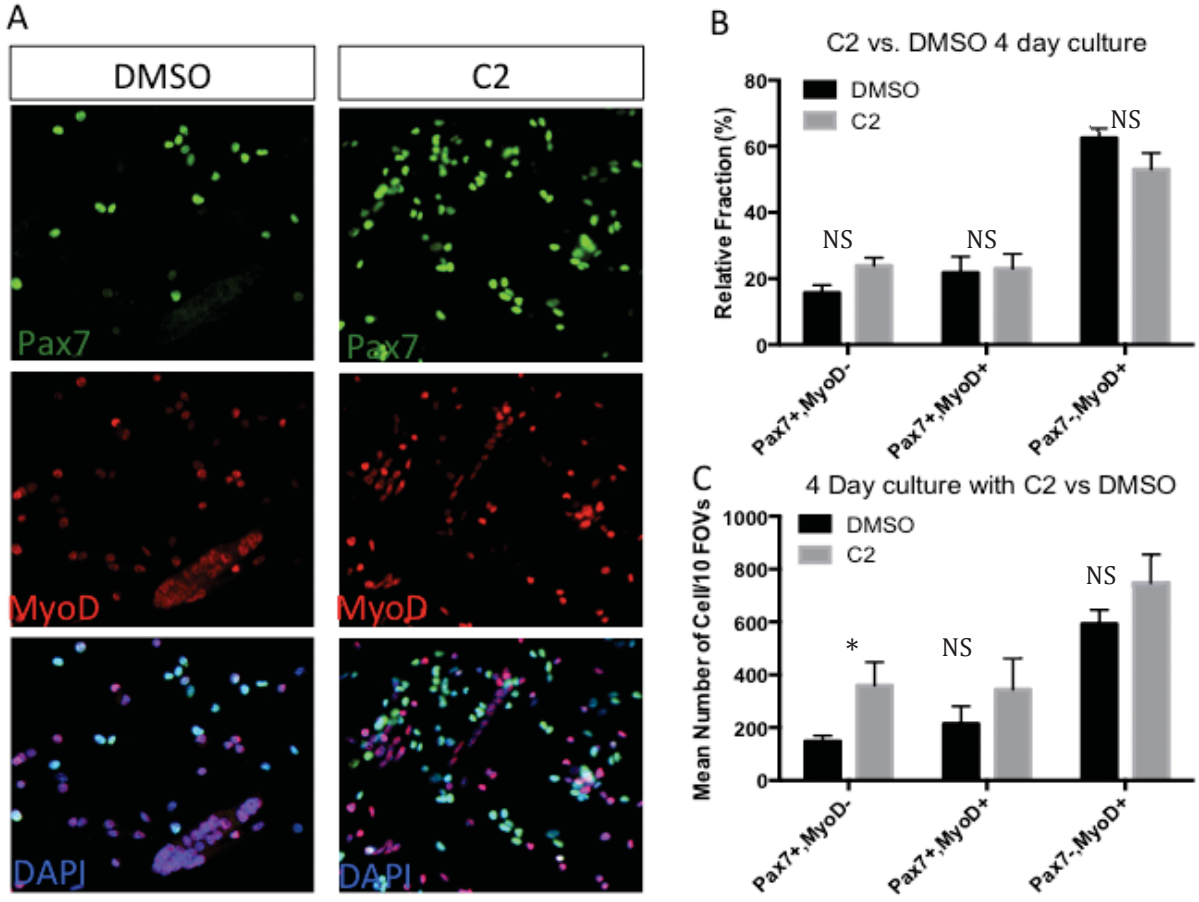

Figure 4.15 Examination of Compound 2 (C2) effect on the myogenic program. (A) Representative immunofluorescence images of satellite cell colonies after 4 days in culture. Pax7 is green, MyoD is red. (B) Quantification of self-renewal [Pax7+,MyoD-], Activation [Pax7+, MyoD+] and differentiation [Pax7-,MyoD+] fractions in Pax3$^{GFP/+}$ cells treated with C2 at Day 0 of culture. (C) Quantification of absolute numbers of self-renewal [Pax7+,MyoD-], Activation [Pax7+, MyoD+] and differentiation [Pax7-,MyoD+] in Pax3$^{GFP/+}$ cells treated with C2 at Day 0 of culture. Significant results represented with a * at $p<0.05$

showed similar activity levels, including a marked delay in expansion compared to other compounds (Figure 4.14B). We chose C2 for further immunofluorescence-based analysis due to its robust effect on cell proliferation (Figure 4.15A). While the data we obtain did not indicate significant variation in cell populations, closer examination of absolute cell growth showed a marked increase in Pax7+, MyoD- cells (Figure 4.15B and C). Further interrogation of these molecules would be required.

5. Discussion:

The significance of translational control of gene expression was exhaustively demonstrated in the context of cellular stress responses. In our published manuscript, we address other roles of translational control and propose a model mechanism that may alternatively regulate the quiescent state and self – renewal capacity of adult skeletal muscle stem cells. We first demonstrate that quiescent satellite cells contain high levels of P-eIF2α compared to their differentiating progeny. We then demonstrate that genetic removal of the phosphorylation site at Ser51 of eIF2α induced increases rates of protein translation and pushes quiescent satellite cells to re-enter the cell cycle and activate the myogenic differentiation program *in vivo*. Subsequently, we demonstrate that loss of P-eIF2α abolishes satellite cell self-renewal without affecting their regenerative potency. We also used polysome profiling to demonstrate multiple transcripts are regulated by P-eIF2α mediated translational control. Following these observations, we chose to investigate how maintaining P-eIF2α pharmacologically would affect satellite cell homeostasis. Specifically, we reasoned that forced maintenance of high P-eIF2α levels would permit expansion of satellite cells that retain regenerative capacity.

5.1. The Effect of Sal003 on Satellite Cells *Ex Vivo*:

The *ex vivo* culture of primary satellite cells is a widely accepted model of the myogenic program[60, 72, 76, 78, 100, 106, 108, 109]. It has been extensively documented that satellite cells, similarly to other ASC, are quickly depleted when grown in culture, as they rapidly activate the myogenic program and generate a pool of proliferating myoblasts[60, 72, 76, 78, 100, 106, 108, 109]. Indeed, pharmacological inhibition of eIF2α dephosphorylation by Sal003 demonstrated that P-eIF2α is maintained at high levels in satellite cell cultures (Fig. 4.1). Contrary to our expectations, we observed robust cell proliferation in Sal003 cultures of FACS isolated primary satellite cells. Deeper investigation revealed that a higher proportion of proliferating Sal003 treated satellite cells maintain high expression of the satellite cell marker Pax7, and low expression of the differentiation markers MyoD and MyoG (Fig 4-2). Analysis of

cell proliferation rates in Sal003 cultures demonstrated initially slow entry into the cell cycle, followed by rapid and continuous proliferation at later time points (Fig4-3). These observations correspond well with previous reports that Pax7+, MyoD- cells in culture exhibit slow rates of division, which accelerate when the myogenic program is activated[51]. In addition, it may be possible that slower rates of division may correspond with initial self-renewal events in culture corroborating the increased rates of Pax7+, MyoD- cells.

5.2. Potential Post-Transcriptional Regulation of Cell Cycle Mediators:

Slow rates of division were identified in the HSC compartment as well, where it was determined that HSC divide slowly upon reconstitution of the HSC population in the compartment, followed by increase in proliferation rates as differentiation is induced[110]. It is possible that Sal003 treatment pushes satellite cells to undergo self-renewal by modulating the ratio of cell cycle effectors within the cells. For example, the CDK inhibitor $p27^{Kip1}$ regulates cell cycle progression by inducing G_1 arrest in a dose dependent manner[111]. IGF1-mediated transcriptional downregulation of this gene correlates with rapid increase in proliferation of satellite cells *in vivo*. However, it was also shown that $p27^{Kip1}$ is also subject to extensive translational control, as it contains a uORF that is normally translated[112]. It is therefore possible that high conditions of P-eIF2α promote expression of high levels of p27, ensuring cell cycle arrest during quiescence. As dosage of p27 decreases, cells become prone to enter the cell cycle and proliferate. As we show that rapid downregulation of P-eIF2α occurs in the satellite cells, one can therefore assume that downregulation of p27 protein would occur much faster that loss of its mRNA, through initiation of translation at the uORF. Conversely, Sal003 treatment may maintain longer expression of $p27^{Kip1}$ through initiation at the true start codon, resulting in slower entry to the cell cycle. Furthermore, variable doses of p27 may play different roles in the satellite cell cycle, and interact with other cell cycle modulators to play a role in the choice between myogenic commitment and self-renewal. In HSCs, p27 ablation demonstrated no detrimental effect on self-renewal and viability, but greatly affected proliferative capacity of differentiating progeny[113]. We propose further investigation into the post-transcriptional control of $p27^{Kip1}$, with emphasis on translational control, in the satellite cell.

5.3. Post-Transcriptional Repression of the Myogenic Program and Promotion of Stem Cell Genes:

We propose that maintenance of high P-eIF2α may support a cellular microenvironment that favours other mechanisms of post-transcriptional control in the quiescent satellite cell. For example, we confirmed that Myf5, Dek and MyoD transcripts are not translated in the quiescent satellite cell[62]. As both Myf5 and Dek expression is inhibited by miR mediated translational repression, it is possible to speculate that prolonged treatment of Sal003 may prevent derepression of these transcripts and delay myogenic activation[60, 80]. Similarly, a high P-eIF2α environment may facilitate MyoD transcript degradation by TTP mediated mRNA decay in Sal003 treated satellite cells[54].

Repression of global translation may also help prolong stem cell related gene profile in cultured satellite cells. The phosphorylation of eIF2α greatly reduces the efficiency of translation initiation at the first detected start codon of an mRNA, leading to continued scanning and leaky initiation in mRNAs with uORFs or alternative initiation at downstream codons[114]. It is generally estimated that up to 48% of all mRNAs contain either individual or overlapping uORFs in their 5' UTR, with evidence to evolutionary selection in specific genes. Similarly, it has been shown that mRNAs with multiple in-frame, downstream start codons are also subject to negative selection and are particularly enriched in genetic sequences of transcription factor proteins. This indicates that even small variability in N-terminal sequences can provide various isoforms with functional differences[115]. For example, we demonstrate that the satellite cell marker Pax7 is translated equally well in conditions of high and low P-eIF2α[62]. Close inspection of the 5' coding sequence of Pax7 mRNA reveals multiple alternative AUG codons located within close proximity to each other (data not shown). It is possible to speculate that Pax7 may resist global repression of translation through a mechanism of alternative initiation. A similar mechanism was previously described for SOCS3 expression in conditions of cellular stress[116]. In addition to avoiding global translational repression, SOCS3 activity and stability were enhanced due to loss of a N-terminal ubiquitination site[116].

5.4. Modulation of P-eIF2α is Not Detrimental in Homeostatic Conditions:

As part of our investigation, we have also examined the effect of Sal003 treatment on rates of cell death culture. It is well documented that unresolved cellular stress promotes P-eIF2α mediated upregulation of pro-apoptotic genes. Specifically, its has been shown that during ER stress, PERK mediated phosphorylation of eIF2α promotes selective transcription of the master transcription factor ATF4, which in turn upregulates the pro-apoptotic factor CHOP[98]. Intriguingly, it was shown that CHOP is capable of repressing myogenic differentiation in C2C12 myoblasts through interaction and inhibition of MyoD enhancer elements[117]. If conditions of high P-eIF2α persist, elevated levels of CHOP initiate an apoptotic signalling cascade. In our broad study, we show that quiescent satellite cells express high levels of ATF4 and CHOP, as well as the cytoprotective chaperon Grp78. To eliminate the possibility that Sal003 treatment induces apoptosis through prolongation of P-eIF2α, we used TUNEL staining to measure rates of apoptosis in culture (Fig. 4.5) and total cell numbers/colony. We saw no difference in cell death rates and total cell numbers between Sal003 and control treated cultures. Indeed, we observed a slight cytoprotective effect in Sal003 cultures, in accordance with previous reports[103]. We also confirmed that modulation of protein synthesis does not affect cell survival during homeostatic conditions by examining cell death rates in S51A cells (Fig. 4.6 and 4.7).

5.5. The effect of Sal003 Treatment on the Myogenic Program is P-eIF2α Dependent:

Since our approach involved utilization of a poorly characterized inhibitor of P-eIF2α dephosphorylation, we could not exclude the possibility that off-target effects of Sal003 treatment contribute to the observed effect. We therefore elected to treat S51A satellite cells, which cannot maintain eIF2α phosphorylation, with Sal003. S51A satellite cells fail to self-renew in culture, as evidenced by the marked absence of Pax7+, MyoD- reserve cells[62]. Therefore, restoration of a reserve cell population would indicate an off-target effect, which would require further investigation. However, we could not observe any increase in Pax7+, MyoD- cells in our culture system (Fig. 4.8A and B). Similarly, we wanted to examine if Sal003 treatment may exert additional effects on self-renewal when added at a later time point, when P-eIF2α is already

downregulated. As expected, Sal003 treatment at Day 3 could not induce increase in self-renewing satellite cells, as most cells committed to the myogenic program, similar to previous reports[51, 106].

Our findings agree with previous reports that demonstrate that other ASCs may regulate their rates of protein synthesis to maintain their stem cell phenotype. A recent study in HSCs demonstrates that a perturbation of homeostatic translation rate by either increasing or decreasing synthesis negatively affects HSC function[100]. Specifically, genetically mediated upregulation of protein synthesis rates through conditional ablation of *Pten* leads to activation, proliferation, and depletion of HSCs[100]. Conversely, genetically induced decrease in protein synthesis was shown to impair HSC proliferation and differentiation, resulting in reduced repopulation and colony forming capacity[100]. These phenotypes are strongly recapitulated when P-eIF2α is downregulated in the S51A genetic model, and closely resemble behaviour observed in satellite cells that were treated with Sal003 *ex vivo*.

5.6. Translational Control Promotes Proteostasis in Non-Cycling Cells:

PERK - P-eIF2α mediated translation attenuation was demonstrated as a key mechanism of homeostatic control in pancreatic β-cells[118]. Specifically, loss of P-eIF2α was suggested to contribute to increase in ROS through disregulation of various genetic pathways, as well as perturbed proteostasis[118]. The notion that translational control can help maintain stem cell homeostasis has been also presented in the context of ageing, as it was proposed that accumulation of reactive oxygen species (ROS) is detrimental to long term stem cell maintenance. Indeed, it has been shown that HSC depletion can occur when ROS homeostasis is perturbed and ROS are allowed to increase. In addition, it was shown that non-cycling somatic and stem cells, including satellite cells, express a wide variety of genes that maintain a strong antioxidative environment that get diminished when these cells differentiate. High accumulation of ROS damages DNA and protein, and poses particular risks for stem cells, as accumulation of misfolded protein throughout long periods of quiescence can lead to cytotoxicity[99]. In quiescent satellite cells, detection of high levels of antioxidant enzymes such as Sulfiredoxin, Gluthathion Peroxidase 3 and Thioredoxin Reductase 1, confers them enhanced resistance against ROS accumulation.

Examination of Sal003 treated satellite cell cultures demonstrated that the effect of Sal003 remained transient, as cell cultures that underwent single treatment upon plating demonstrated robust formation of multinucleated myotubes (Fig. 4.9). This could be due active removal of Sal003 from satellite cells, as it was found that they express high levels of multidrug resistance genes such as *Abcb1a, Abca5* and *Abc9,* which pump xenobiotic out of the cell, as well as members of the Cytochrome p450 family[47]. In addition, it is possible that Sal003 stability is not maintained in aqueous solution. Fusion index measurement in cultures showed marked increase in nuclei per myotube, indicating an expansion of progenitor cells per clonal myogenic colony (Fig 4.9E). This observation, that Sal003 maintains Pax7+, MyoD- cells at earlier time points leads to the argument that initial treatment with Sal003 expands the muscle progenitor pool in culture. To examine this, we treated satellite cell cultures at day 3 only, when the Pax7+, MyoD- reserve cells cannot be restored, and observed no difference in fusion index of myotubes at day 5. Conversely, if we treat satellite cells with Sal003 in 3-day intervals we observe no formation of multinucleated myotubes.

Another factor that may influence these observations is our lack of understanding of the precise mechanism that drives phosphorylation of eIF2α *in vivo*. In our manuscript, we detect high levels of PERK signalling in the quiescent satellite cell. However, we did not demonstrate how PERK activation is induced. From the literature, PERK activation is induced by its homodimerization and subsequent autophosphorylation which can be induced in response to hypoxia mediated ER stress[119]. Indeed, hypoxia mediated activation of the UPR can provide a reasonable mechanism of P-eIF2α upregulation, as it was demonstrated that *ex vivo* culture in hypoxic conditions favours satellite cell self-renewal[61]. The PERK-eIF2α branch of the UPR was shown to protect slow cycling cells within the hypoxic tumour environment from ROS induced cell death, as well as promote autophagy through a ATF4 dependent mechanism[119]. It is possible that immediate treatment of freshly isolated satellite cells prolongs these characteristics in culture, resulting in better self-renewal capacity. Likewise, we propose that the transient effects of Sal003 treatment can be attributed to the loss of positive eIF2α regulation, as our culture experiment were conducted in a normoxic (20% O_2) environment. It would be useful to reproduce some of our experiments in a hypoxic environment, where we

would expect Sal003 to have a more robust effect possibly through active maintenance of P-eIF2α by one of its kinases.

5.7. The Effect of Sal003 Treatment on Satellite Cell Engraftment:

The apparent increase in reserve cell populations in Sal003 treated cultures has prompted us to investigate whether they retain better engraftment capacity than regular *ex vivo* expanded myoblasts. As mentioned earlier, self-renewal and regenerative capacities are quickly depleted as satellite cells undergo repeated rounds of expansion *ex vivo*. These became evident in a dozen of clinical myoblast transplantation trials conducted throughout the 1990's. As discussed above, these and other challenges significantly hindered cell-based therapies[16, 26, 106, 120].

In our study, we use non-invasive bioluminescence techniques to examine how Sal003 treatment affects *ex vivo* expanded satellite cell progeny's ability to engraft into irradiated muscle of dystrophic, immunocompromised hosts. We observe marked improvement in survival and expansion of Sal003 treated cells, compared to vehicle treated cells (Fig 4.10). Histological analysis of the engrafted muscle shows that Sal003 treated cells possess better regenerative potential, as evidenced by increased numbers of centrally nucleated, dystrophin positive cells (Fig. 4.11A and B). A higher level of donor derived Pax7+ nuclei detected adjacently to myofibers also indicates increased self-renewal upon engraftment (Fig. 4.11C and D). Finally, we demonstrate that Sal003 treated satellite cell progenies retain myogenic potential multiple weeks after engraftment, as demonstrated by our re-isolation and *ex vivo* culture of such cells (Fig. 4.12). Taken together, our data demonstrate that Sal003 treatment improves engraftment capacity of *ex vivo* expanded satellite cells, likely through a P-eIF2α mechanism.

While stem cell therapies have lagged behind gene therapies in the field of muscle disorders, several examples of successful use of localized myoblasts transplantation have been reported: For example, autologous myoblast transplantation into the cricopharyngeal muscles of 12 patients was reported to improve symptoms of Oculopharyngeal Muscular Dystrophy in a

Phase I/II study. The treatment proved to be safe and tolerable in all cases, resulting in halt of functional degradation in swallowing metrics[19]. Another example was demonstrated in treatment of Stress Urinary Incontinence (SUI). In this context, myoblasts were injected to improve the tone and strength of several striated muscles that control urethral function. Thickening of the urethral wall and increase in closing strength were observed in treated patients, however onset of improvement could be delayed by up to six months[16].

These small studies demonstrate a proof of concept for small-scale satellite cell engraftment procedures, which can be enhanced by pharmacological by Sal003 or its chemical derivatives. As evidenced from the above trials, quality of life can be improved in many patients that suffer from minor losses of muscle tone.

5.8. Investigation of Guanabenz:

After demonstrating that P-eIF2α modulation can influence satellite cell activity in culture, we set out to investigate other small molecules that were reported as potent UPR modulators. We began our investigation by characterizing the effect of Guanabenz, a pharmacological inhibitor of β2-adrenergic receptor that was shown to also inhibit eIF2α dephosphorylation through specific inhibition of GADD34[121]. We chose to focus on Gunabenz because of its well-defined chemical and pharmacological properties, as it is already used for hypertension treatment in humans[121]. Knowledge of such characteristics such as it mechanism of action, structure-function relationship and effective concentrations *in vivo* would serve as a better starting point for derivation of a molecule with UPR specific properties. By using the same methods that we applied in our Sal003 analysis, we demonstrate that Guanabenz exerts a less powerful effect on satellite cells, as a much higher dosage of the drug is required to maintain P-eIF2α and delay the myogenic program (Fig. 4.13).

It has been recently reported that phosphorylation of eIF2α plays an important role in preservation of embryonic stem cell pluripotency[101]. In their report, *Friend et al.* argue that dephosphorylation of eIF2α requires the constitutive subunit CReP, rather than GADD34, to maintain the stem cell condition. Since Guanabenz has been demonstrated to

specifically inhibit GADD34, it possible that its addition is insufficient to maintain a high level of eIF2α phosphorylation similar to what can be achieved with Sal003. Further dissection of the relative functions of GADD34 and CReP in P-eIF2α equilibrium may be conducted with novel derivatives of Guanabenz.

5.9. Development of a Screening Strategy for Sal003 Derivatives:

The effect of Sal003 was first identified in a large-scale biochemical screen of cytoprotective compounds[103]. Since then, the Sal003 treatment was primarily used as a tool compound to demonstrate the effects of prolonged eIF2α phosphorylation in cultured cells. The effective concentration of the drug was reported to be between 1-100μM, which is within range of its effect in satellite cells. However, no detailed reports of systemic administration of the drug in rodents have been reported[102]. The biochemical properties of the drug also remain obscure, as no direct biochemical interaction between Sal003 and any of its presumptive targets have been reported[122]. The knowledge gap presented here made it difficult for us to form a dosage regime for *in vivo* testing of the drug.

An additional challenge remains in translating our results to the human context. While mouse physiology and genetic background recapitulate human phenotypes in multiple contexts, significant differences may still occur. Therefore, we propose future replication of our study in human satellite cells. A reliable human satellite cell protocol was recently published, which would permit preliminary analysis of Sal003 effects in human cells *in vitro*[123]. In addition, engraftment capacity of cultured human satellite cells can also be assed by their transplantation into SCID/NOD, immunocompromised mice. Donor tissue can be easily distinguished by immunohistochemistry based on immunoreactivity to human Lamin A/C[124].

We therefore chose to continue our investigation by investigating de-novo generated Sal003 derivatives. We applied the same methodology to investigate the effect of Sal003-derived compounds on cell growth (Fig. 4.14), as well as Pax7 and MyoD expression (Fig. 4.15). The Luciferase screening step allows rapid detection of Sal003 derivatives that would affect satellite cell growth kinetics. We would expect exponential increase in entry to the myogenic program. Lead compounds would be selected for further investigation with

double marker immunofluorescence labeling. We have encountered several challenges during our low-throughput screening process.

First, the rarity and fragility of satellite cells reduces their yield and renders their isolation difficult and prevents significant scale up of our screening methodology. Therefore, we are obliged to conduct our analysis with low numbers of cells, which do not expand significantly in culture. Equipment sensitivity does not allow signal quantification from single live cells, limiting our flexibility further. Overall, this reduces statistical power and makes determination of result significance difficult. Second, immunofluorescence microscopy precludes analysis of more that three markers at a time, limiting our scope of investigation and obscuring any differences that may arise due to population heterogeneity within individual cell colonies. Third, immunofluorescence analysis is a manual, labour intensive process, and therefore is not scalable to large-scale analysis of phenotypic differences between more than five culture conditions.

To address these issue we propose adoption of multi-parametric technology, which would greatly enhance data output and speed. For example, multi-parametric flow cytometry could be adopted to analyze more than two markers at time. Protocols for adherent cell analysis, as well as intracellular marker analysis are currently available[125]. As this method is more precise, it can also produce more statistically pertinent results that fluorescence imaging. The downside of this approach for skeletal muscle is that it cannot examine terminally differentiated myotubes, as their quick death upon trypsinisation is unavoidable.

For detailed imaging based analysis, a significant increase in work speed can be achieved by implementation of automatic image analysis protocols. While multiple types of commercial software are available, free community derived alternatives such as CellProfiler are available[126]. In particular, CellProfiler provides a user-friendly interface and an extensive support network that can help users tailor their protocols for their individual needs.

6. Conclusion and Outlook:

This work presents additional evidence to support our group's finding that eIF2α phosphorylation is a key regulator of stem cell homeostasis. We demonstrate that Sal003 treatment induces self-renewal of satellite cell progeny in *ex vivo* culture, and that Sal003 treatment promotes better engraftment capability in treated cells. We also investigate Guanabenz as a potential modulator of the UPR in satellite cells, and conclude that its effect requires further investigation. We therefore resolve to investigate novel derivatives of Sal003 and their effect on the satellite cells' myogenic program.

We believe that discovery and characterization of better Sal003 derivatives would help us achieve better therapeutic effect in future clinical testing of such drugs.

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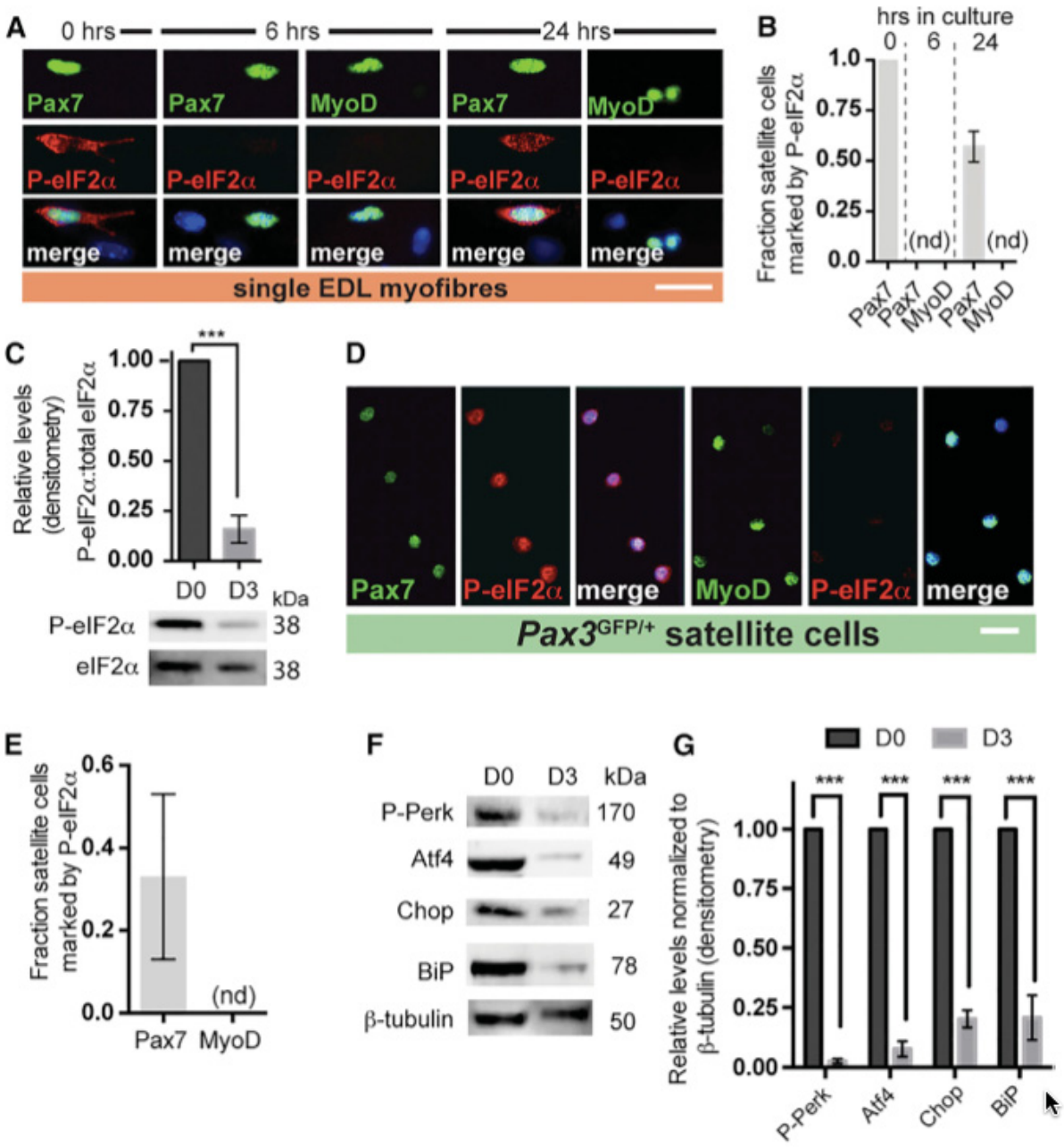
Supplemental Figures:



Supplemental figure 1-eIF2α is Phosphorylated in Quiescent Satellite Cells.

From *Zismanov et al,Cell Stem Cell. 2016 Jan 7;18(1):79-90*

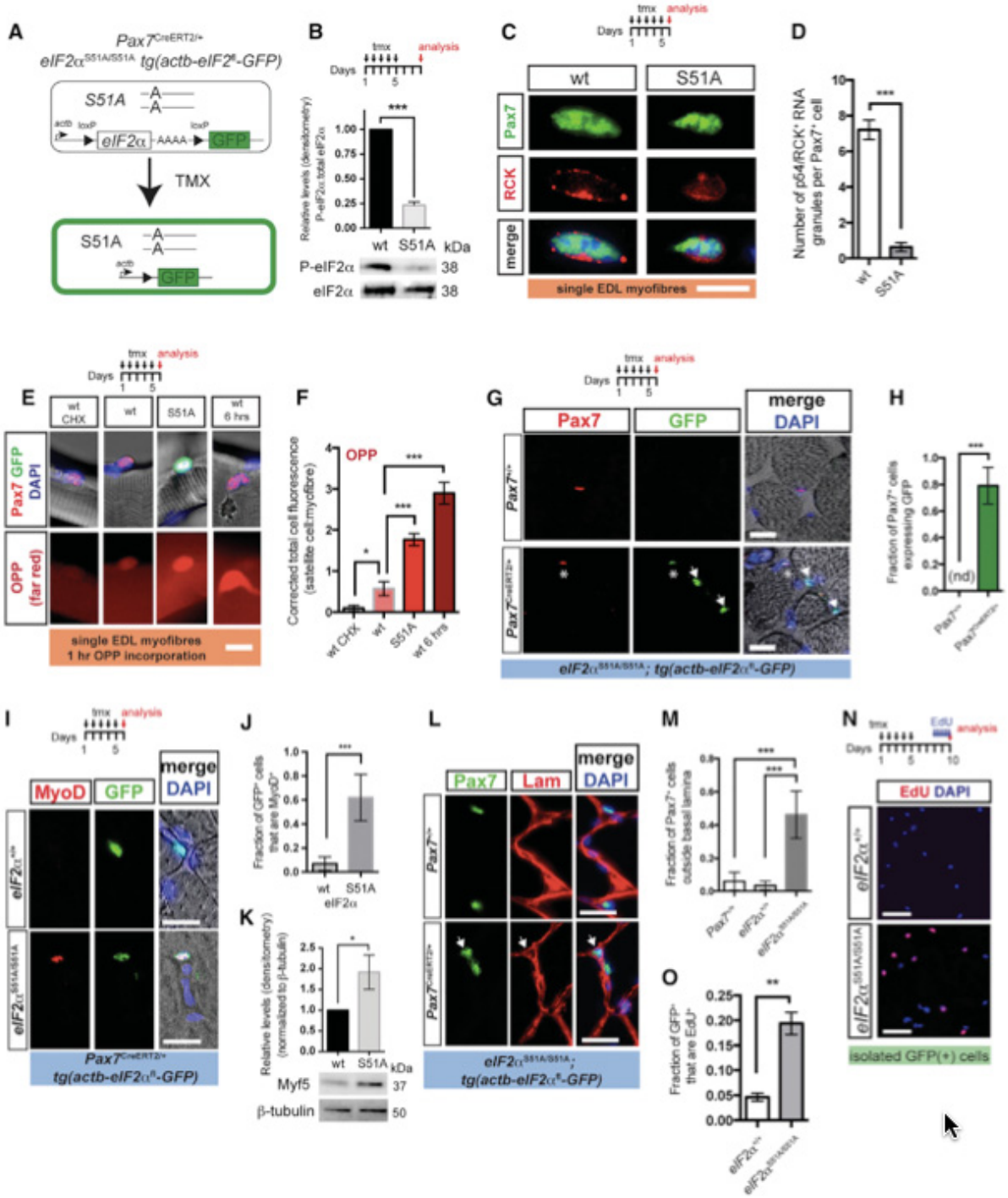
(A) Immunostaining Pax7 (green) or MyoD (green) and P-eIF2α (red) on newly isolated (0 hr) and cultured (6, 24 hr) EDL myofibers from wild-type mice. Lower panels show merged images with DAPI.

(B) Fraction of $Pax7^{+}$ and $MyoD^{+}$ nuclei on single myofibers that show immunofluorescence for P-eIF2α after 0, 6, and 24 hr of culture. (C) Immunoblotting against P-eIF2α and total eIF2α (eIF2α) from cell lysates of newly isolated satellite cells (D0) and after 3 day culture (D3). Relative levels of P-eIF2α, normalized to total eIF2α are reported, with a representative immunoblot shown.

(D) Immunostaining for Pax7 (green), MyoD (green), and P-eIF2α (red) after 3 day culture of satellite cells. Merged images with DAPI are shown.

(E) Quantification of satellite cell nuclei expressing Pax7 or MyoD and P-eIF2α after 3-day culture of satellite cells. (F) Representative images of Immunoblotting against P-Perk, Atf4, Chop, BiP, and b-tubulin from cell lysates of newly isolated satellite cells (D0) and after 3-day culture (D3).

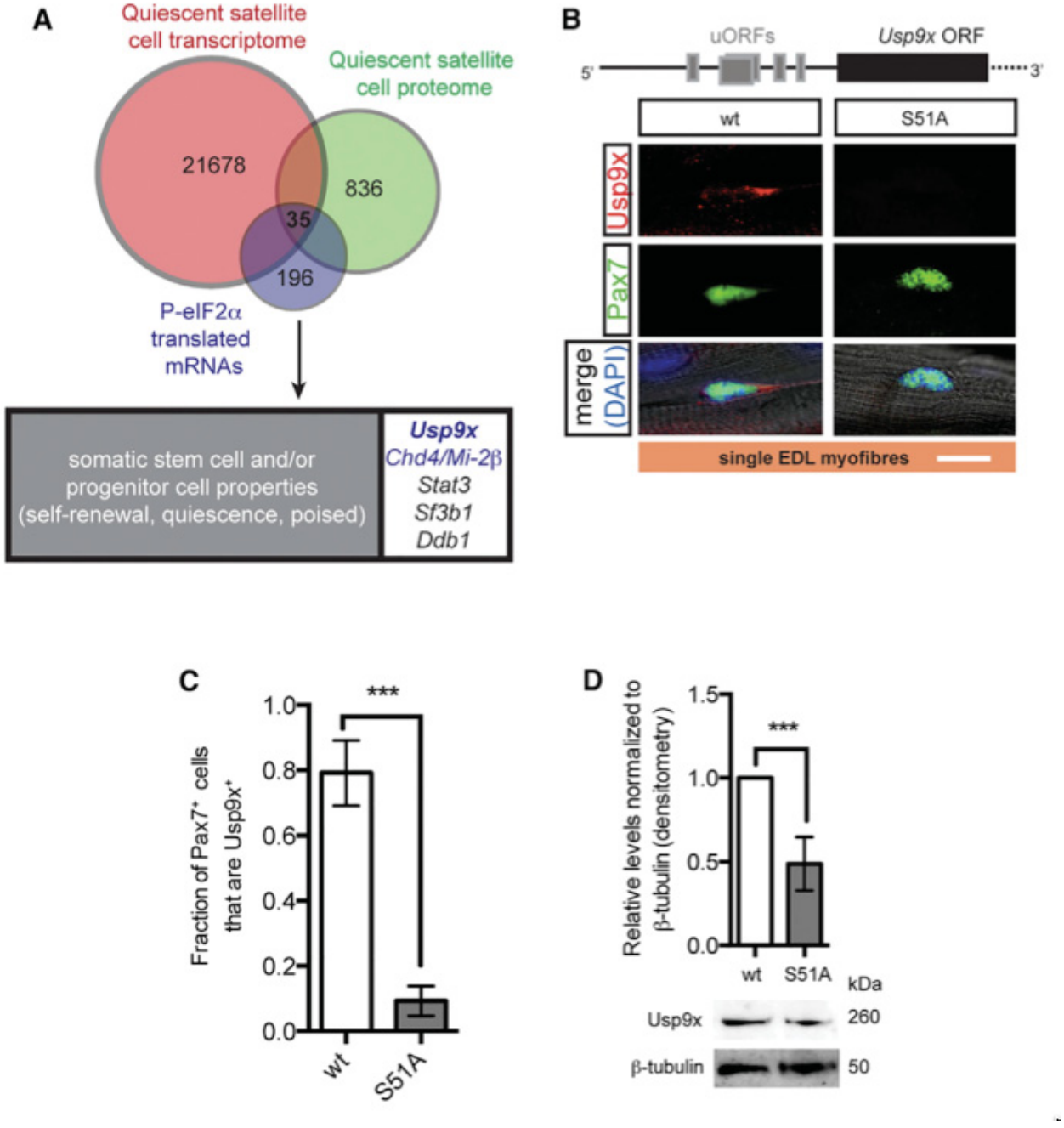
(G) Relative levels of P-Perk, Atf4, Chop, and BiP, normalized to b-tubulin, are indicated. All values indicate mean (n R 3) ± SEM. ***p < 0.001; nd, not detected. Scale bars represent 20 mm.



Supplemental figure 2 Satellite Cells Unable to Phosphorylate eIF2α Enter the Myogenic Program *in vivo*.

From *Zismanov et al,Cell Stem Cell. 2016 Jan 7;18(1):79-90* (A)

A serine to alanine switch at position 51 (S51A) prevents eIF2α phosphorylation. Mice homozygous for this allele are not viable and are rescued by a transgene with wild-type eIF2α under the control of CMV enhancer and chicken b-actin promoter (actb). The wild-type eIF2α is flanked by two loxP sites and positioned upstream of a GFP reporter (green). Crossing this line with a *Pax7*$^{CreERT2/+}$ allele, followed by tmx administration, permits the conditional expression of homozygous eIF2a S51A and GFP (green) in Pax7 satellite cells. (B) Immunoblotting for P-eIF2α and total eIF2α (eIF2α) of cell lysates from newly isolated GFP^{+} cells from muscle of *Pax3*$^{GFP/+}$ (WT) and tmx-treated *Pax7*$^{CreERT2/+}$, *tg(actb-eIF2α*^{fl}*-GFP), eIF2α*$^{S51A/S51A}$ (S51A) animals. The tmx regime and day of analysis are shown. Relative levels of P-eIF2α, normalized to total eIF2α, are indicated, with representative immunoblots. (C) Immunostaining for Pax7 (green) and p54/RCK (RCK, red) on isolated EDL myofibers from tmx-treated WT (*Pax7*$^{+/+}$; no Cre) and S51A (*eIF2α*$^{S51A/S51A}$) mice. Lower panels show merged images with DAPI. (D) Numbers of p54/RCK^{+} granules per Pax7-positive satellite cell in (C). (E) Immunostaining for Pax7 (red) and GFP (green), combined with detection of OPP (far red) on EDL myofibers from tmx-treated WT (*Pax7*$^{+/+}$; no Cre) and S51A (*eIF2α*$^{S51A/S51A}$) mice. EDL myofibers were also cultured for 6 hr (right panels) or in the presence of cycloheximide (CHX, left panels). Upper panels show merged images with DAPI, overlaid on brightfield to show myofibers. (F) Rates of protein synthesis, reported by total cell OPP fluorescence in (E). (G) Immunostaining for Pax7 (red) and GFP (green) on transverse sections of TA muscle after 5 daily doses of tmx (indicated). Right panels show merged images with DAPI, which are overlaid on brightfield images of transverse fiber sections. Asterisk indicates a Pax7^{+}GFP^{+} satellite cell. Arrows indicate position of GFP^{+} cells between muscle fibers. (H) Fraction of Pax7^{+} nuclei that show immunofluorescence for GFP indicated in (G). (I) Immunostaining MyoD (red) and GFP (green) on transverse sections of TA muscle after tmx treatment. Right panels are merged images with DAPI, overlaid on brightfield to show myofibers. (J) Fraction of GFP^{+} cells that are MyoD^{+} in (I). (K) Immunoblotting against Myf5 and b-tubulin from cell lysates of newly isolated GFP^{+} cells from muscle of *Pax3*$^{GFP/+}$ (WT) and tmx-treated *Pax7*$^{CreERT2/+}$, *tg(actb-eIF2α*^{fl}*-GFP), eIF2α*$^{S51A/S51A}$ (S51A) animals. Relative levels of Myf5 normalized to b-tubulin are indicated, with representative immunoblots shown. (L) Immunostaining Pax7 (green) and Laminin (Lam, red) on transverse sections of TA muscle after tmx treatment. Right panels show merged images with DAPI. Arrows indicate position of satellite cells outside basal lamina. (M) Fraction of Pax7^{+} nuclei outside the basal lamina of myofibers after tmx treatment indicated in (L). (N) Representative images of EdU^{+} satellite cells isolated from tmx-treated *Pax7*$^{CreERT2/+}$, *tg(actb-eIF2α*^{fl}*-GFP)*, *eIF2α*$^{+/+}$, or *eIF2α*$^{S51A/S51A}$ and deposited on slides by cytospin. (O) Fraction of EdU^{+} satellite cells isolated from tmx-treated mice, as indicated in (N). Scale bars represent 50 mm except in (C) and (N) where they represent 10 mm and 20 mm, respectively. All values indicate mean (n R 3) ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001; nd, not detected.



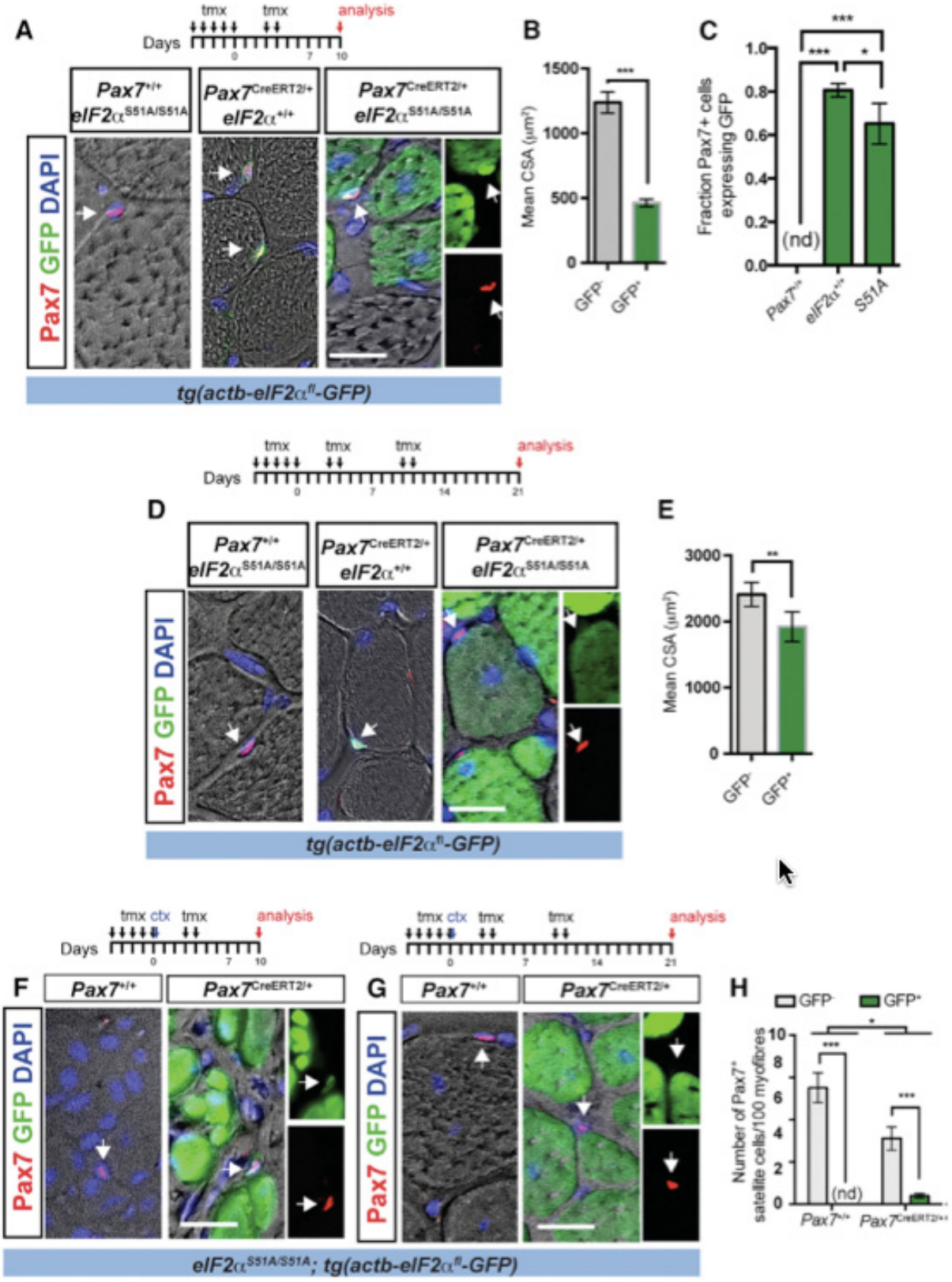
Supplemental figure 3 Selective mRNA Translation during eIF2α Phosphorylation

From *Zismanov et al,Cell Stem Cell. 2016 Jan 7;18(1):79-90.*

(A) Venn diagram of the quiescent satellite cell transcriptome (red) and proteome (green) with mRNAs that are selectively translated by P-eIF2α (blue). 35 genes common to each of the datasets are indicated of which 5 are further identified as regulators of stem and/or progenitor cells. *Usp9x* and *Chd4* transcripts are highlighted (blue) because they have uORFs in the 5^0 UTR.

(B) Immunostaining Pax7 (green) and Usp9x (red) on EDL myofibers isolated from tmx-treated wild- type (WT) and S51A mice. Lower panels show merged images with DAPI. Scale bar represents 10 mm. Structure of *Usp9x* transcripts are shown with five uORFs.

(C) Fraction of Pax7^{+} cells that show immunofluorescence for Usp9x indicated in (B). Values indicate mean (n R 3) ± SEM. ***p < 0.001. (D) Immunoblotting against Usp9x and b-tubulin from cell lysates of newly isolated GFP^{+} cells from muscle of tmx-treated *Pax7*$^{CreERT2/+}$, *tg(actb- eIF2a*^{fl}*-GFP), eIF2a*$^{+/+}$ (WT), and *eIF2a*$^{S51A/S51A}$ (S51A) animals. Relative levels of Usp9x normalized to b-tubulin are indicated, with representative immunoblots shown.


A
tmx
tmx
analysis
Days
0
7
10
Pax7+/+ eIF2αS51A/S51A
Pax7CreERT2/+ eIF2α+/+
Pax7CreERT2/+ eIF2αS51A/S51A
Pax7 GFP DAPI
tg(actb-eIF2αfl-GFP)
B
Mean CSA (μm²)
1500
1000
500
0
***
GFP-
GFP+
C
Fraction Pax7+ cells expressing GFP
1.0
0.8
0.6
0.4
0.2
0.0
***
*
(nd)
Pax7+/+
eIF2α+/+
S51A
tmx
tmx
tmx
analysis
Days
0
7
14
21
D
Pax7+/+ eIF2αS51A/S51A
Pax7CreERT2/+ eIF2α+/+
Pax7CreERT2/+ eIF2αS51A/S51A
Pax7 GFP DAPI
tg(actb-eIF2αfl-GFP)
E
Mean CSA (μm²)
3000
2000
1000
0
**
GFP-
GFP+
tmx ctx
tmx
analysis
Days
0
7
10
F
Pax7+/+
Pax7CreERT2/+
Pax7 GFP DAPI
tmx ctx
tmx
tmx
analysis
Days
0
7
14
21
G
Pax7+/+
Pax7CreERT2/+
Pax7 GFP DAPI
eIF2αS51A/S51A; tg(actb-eIF2αfl-GFP)
H
GFP-
GFP+
Number of Pax7+ satellite cells/100 myofibres
10
8
6
4
2
0
*
***
***
(nd)
Pax7+/+
Pax7CreERT2/+

Supplemental figure 4 Activated S51A Satellite Cells Contribute to New Muscle Fibers, but Not to Self-Renewal *In Vivo*

From *Zismanov et al,Cell Stem Cell. 2016 Jan 7;18(1):79-90* .

(A) Immunostaining Pax7 (red) and GFP (green) on transverse section of uninjured TA muscle. Days of tmx administration (black) and analysis (red) are shown. Arrows indicate the position of $Pax7^{+}$ nuclei. Magnified images (right) are provided. Scale bar represents 20 mm.

(B) Mean cross section area (CSA) of $GFP^{!}$ and GFP^{+} myofibers of uninjured TA muscle, shown in (A), 10 days after tmx administration. Values indicate mean (n R 500 myofibers from three in- dependent mice) ± 95% confidence interval (CI); ***p < 0.001.

(C) Fraction of $Pax7^{+}$ satellite cells, which are positive for GFP, shown in (A), 10 days after tmx administration. Values indicate mean (n R 3) ± SEM. *p < 0.05, ***p < 0.001; nd, not detected. (D) Immunostaining Pax7 (red) and GFP (green) on transverse section of uninjured TA muscle. Days of tmx administration (black) and analysis (red) are shown. Arrows indicate the position of $Pax7^{+}$ nuclei. Magnified images (right) are provided. Scale bar represents 20 mm.

(E) Mean CSA of $GFP^{!}$ and GFP^{+} myofibers of uninjured TA muscle, shown in (C), 21 days after tmx administration. Values indicate mean (n R 200 myofibers from three independent mice) ± 95% CI. **p < 0.01.

(F and G) Immunostaining Pax7 (red) and GFP (green) on transverse section of TA muscle (F) 10 and (G) 21 days after ctx injury. Days of tmx administration (black), ctx injury (blue), and analysis (red) are shown. Arrows indicate the position of $Pax7^{+}$ nuclei. Magnified images (right) are pro- vided. Scale bars represent 20 mm.

(H) Numbers of $Pax7^{+}$ satellite cells, per 100 myofibers that are negative (gray) or positive (green) for GFP, shown in (D), 21 days after tmx administration and ctx injury. Values indicate mean (n R 3) ± SEM. *p < 0.05, ***p < 0.001; nd, not detected.