MUSCLE STEM CELL FATE IS CONTROLLED BY THE MITOCHONDRIAL FUSION PROTEIN OPA1

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Abstract

During aging there is a decline in (MuSCs) and muscle regeneration, though the underlying reason is unknown. Interestingly, mitochondrial fragmentation is a common feature in aging, however, how this impacts MuSC function and maintenance has not been investigated. To address the effect of mitochondrial fragmentation in MuSCs, we generated a knockout mouse model using the Pax7CreERT2 inducible system to target deletion of the mitochondrial fusion protein Opa1 specifically within MuSCs (Opa1-KO). Analysis of MuSC function following muscle injury revealed a defect in the regenerative potential of Opa1-KO MuSCs. Moreover, following injury there was a substantial decrease in the number of MuSC in Opa1-KO animals with a concomitant increase in the number of committing cells, illustrating that loss of Opa1 drives MuSC towards commitment at the expense of self-renewal. Furthermore, loss of Opa1 in MuSCs alters the quiescence state, priming MuSCs for activation, as indicated by a reduction in quiescence-related genes, increased EdU incorporation, and enhanced cell cycle kinetics. To address the impact of mitochondrial dysfunction on muscle stem cell capacity, we generated a model of chronic Opa1 loss. Analysis of muscle stem cell function 3 months after Opa1 ablation revealed mitochondrial dysfunction and a defect in proliferation upon activation, leading to failed muscle regeneration. These data are the first to demonstrate a novel role for mitochondrial structure in the regulation of MuSC maintenance and regenerative capacity.

KEYWORDS: Muscle Stem Cells, Mitochondrial Dynamics, Opa1, Stem Cell Fate, Quiescence, Activation, Metabolism, Gene Expression, Muscle Regeneration, Aging.

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Abbreviations

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
CI	Complex I
CII	Complex II
CIII	Complex III
CIV	Complex IV
cMET	Tyrosine Protein Kinase Met (Hepatocyte Growth Factor Receptor)
CNF	Centrally Nucleated Fibers
CSA	Cross-Sectional Area
CTX	Cardiotoxin
CXCL12	C-X-C Motif Chemokine 12
CXCR4	C-X-C Chemokine Receptor Type 4
DNA	Deoxyribonucleic Acid
DPI	Days Post-Injury
DRP1	Dynamin-Related Protein 1
EDL	Extensor Digitorum Longus
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FGF2	Fibroblast Growth Factor 2
Fis1	Mitochondrial Fission Protein 1
GLCL	Glutamate-cysteine ligase catalytic subunit
GPX1	Glutathione Peroxidase 1

GSH	Reduced Glutathione
GSS	Glutathione synthetase
GTP	Guanosine Triphosphate
H&E	Hematoxylin and Eosin
HGF	Hepatocyte Growth Factor
HGF-A	Hepatocyte Growth Factor Activator
HIF2α	Hypoxia-Inducing Factor 2α
HMGB1	High Mobility Group Box 1
HSC	Hematopoietic Stem Cell
IF1	ATP Inhibitory Factor 1
IMM	Inner Mitochondrial Membrane
IMS	Intermembrane Space
iPSC	Induced-Pluripotent Stem Cell
Keap1	Kelch-Like ECH associated protein 1
KO	Knockout
MFF	Mitochondrial Fission Factor
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MiD49	Mitochondrial Dynamics Protein 49
MiD51	Mitochondrial Dynamics Protein 51
miR-31	MicroRNA 31
MRF	Myogenic Regulatory Factor
mRNA	Messenger RNA

mRNP	Messenger RNA with Bound Proteins
MTCH2	Mitochondrial Carrier Homolog 2
mtDNA	Mitochondrial DNA
mTORC1	Mammalian Target of Rapamycin Complex 1
mTORC2	Mammalian Target of Rapamycin Complex 2
MuSCs	Muscle Stem Cells
Myf5	Myogenic Factor 5
Myf6	Myogenic Factor 6 (Myogenic Regulatory Factor 4)
MyoD	Myoblast Determination Protein
MyoG	Myogenin
NAD+	Oxidized Nicotinamide Adenine Dinucleotide
Nrf2	Nuclear Factor Erythroid 2
NSC	Neural Stem Cell
OMM	Outer Mitochondrial Membrane
OPA1	Optic Atrophy 1
OXPHOS	Oxidative Phosphorylation
PAX7	Paired Box 7
PDH	Pyruvate Dehydrogenase
q-RT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
SLC7A11	Cysteine/Glutamate antiporter xCT

SOD1	Superoxide Dismutase 1
SOD2	Superoxide Dismutase 2
Spry1	Sprouty 1
TA	Tibialis Anterior
TCA	Tricarboxylic Acid Cycle
UCP2	Uncoupling Protein 2

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Chapter 1: Introduction

1.1 Overview of Skeletal Muscle

Skeletal muscle is one of the most abundant tissues in the human body and is known for its remarkable regenerative capacity[1-3]. Skeletal muscle is composed of many bundles of multinucleated muscle fibers. Each fiber is surrounded by two membranes called the endomysium and the basement membrane, which can further be subdivided into the reticular and the basal lamina[1,2,4,5]. Skeletal muscle myofibers consist of myofibrils containing the proteins actin, myosin, and troponin which are primarily responsible for the contractile properties of skeletal muscle[6,7]. Adult skeletal muscle encompasses a variety of different properties that allow for numerous different functions including movement, structural support, and whole-body metabolism and health[8-10].

1.2 Adult Skeletal Muscle Regeneration

Damage to skeletal muscle can occur through intense exercise, causing tears in existing fibers, or through more extensive injuries leading to myonecrosis and degradation of impacted myofibers[1,11-13]. Skeletal muscle is composed of post-mitotic cells, and therefore requires additional methods to mediate repair of existing damaged muscle[1,2,4]. The presence of resident adult skeletal muscle stem cells allows for the maintenance and reparation of skeletal muscle following destruction of muscle tissue[1,2,4]. Upon injury to skeletal muscle, necrotic fibers are degraded, and an inflammatory response is initiated, whereby macrophages assist in elimination of damaged myofibers and release growth factors to promote muscle repair[1,4,14]. Muscle regeneration through myogenesis commences with muscle stem cell activation, which involves the exit from quiescence and timely upregulation of myogenic factors, including Myf5 and MyoD to

promote myogenic progression[15-17] (Figure 1). Activated muscle stem cells go through the processes of migration and proliferation before committing to terminal differentiation and upregulating the MRFs MyoG and Myf6 (MRF4)[1,4,18-20]. Terminally differentiating myocytes then undergo fusion, where newly formed myofibers can fuse to existing fibers to repair injured sites, or fuse together to form new multinucleated myotubes[21-23]. The process of skeletal muscle regeneration results in not only newly formed functional skeletal muscle, but re-innervation, re-vascularization, and re-population of interstitial cells within the skeletal muscle niche[18] (Figure 1).

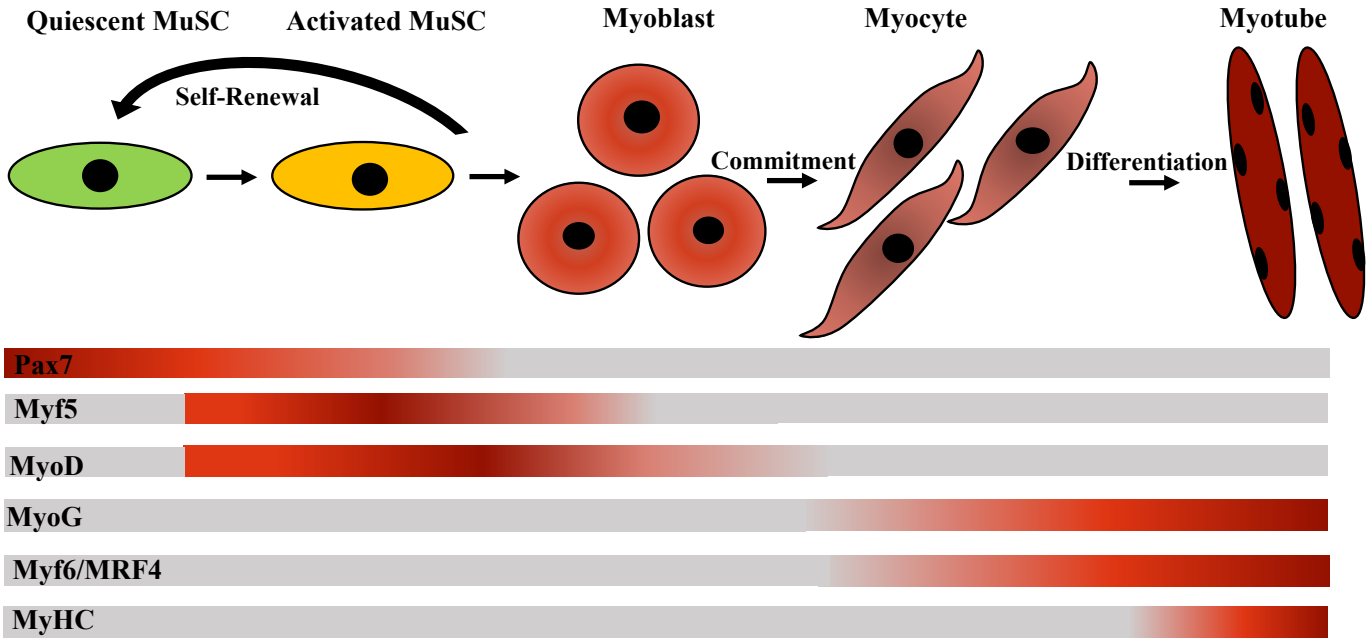

Figure 1. Adult Myogenesis and Expression of Myogenic Regulatory Factors (MRFs)
Quiescent MuSCs express high levels of Pax7, and upon activation up-regulate Myf5 and MyoD. Muscle stem cells can either self-renew and return to quiescence, allowing for re-upregulation of Pax7, or they can commit to a myoblast progenitor lineage, and continue to express Myf5 and MyoD. Myoblasts undergo differentiation into myocytes, where they express the MRFs MyoG and Myf6. Finally, myocytes fuse into multinucleated myotubes and expression myosin heavy chain.
Figure adapted from Dumont et al. (2015) *Comprehensive Physiology.*

1.3 Adult Skeletal Muscle Stem Cells

1.3.1 Overview of Adult Skeletal Muscle Stem Cells

The ability of skeletal muscle to consistently meet regenerative requirements is due to the presence of resident adult skeletal muscle stem cells (MuSCs), or satellite stem cells[1,2,19]. MuSCs are classified as adult stem cells due to their dual capacity to self-renew, maintaining a constant stem cell pool, and to commit to a progenitor lineage allowing for tissue reparation[1,20,24]. Historically, it was observed that adult MuSCs only give rise to a population of myogenic progeny and were therefore defined as unipotent[25,26]. However, recent studies have suggested that MuSCs represent a heterogenous population, and are able to differentiate into brown adipocytes, thus making them multipotent[25]. MuSCs reside between the basal lamina and the plasma membrane of myofibers and make up approximately 2-7% of total nuclei on a single myofiber[1,4,27]. The muscle stem cell niche is an orchestrated composition of vascularization, neuromuscular junctions, myotendinous junctions and interstitial cell populations that provide structural support and allow for the transmission of mechanical and chemical signals to muscle stem cells[4]. The niche therefore provides an environment that allows MuSCs to respond to the state of the external environment.

1.3.2 Muscle Stem Cell Quiescence

Under normal physiological conditions, adult skeletal muscle stem cells reside in state of dormancy, known as quiescence[1,2,28,29]. Stem cell quiescence is generally characterized by the absence of cell cycling, defined as a G_0 state[28]. Stem cells that occupy a quiescent state typically maintain low levels of metabolism, RNA content, and have reduced DNA damage, which allows them to sustain quiescence for prolonged periods of time while maintaining their integrity, until they are required for tissue regeneration[28,30,31]. Thus, the maintenance of quiescence requires

careful orchestration of many processes and is therefore highly regulated by both extrinsic and intrinsic factors[4].

1.3.3 Regulation of Muscle Stem Cell Quiescence

The environment surrounding the muscle stem cell, known as the niche, contains factors important for maintaining quiescence[4,20,32-36]. Specifically, Wnt4 released from the myofiber preserves quiescence by activating RhoA to restrict mobility of MuSCs, confining them to the niche and conserving quiescence[35]. Moreover, Oncostatin M, a member of the interleukin-6 family, is produced within myofibers and has been recently discovered as a potent inductor of MuSC quiescence[32]. The MuSC plasma membrane is home to many cell-surface markers which anchor the cell to the surrounding niche and mediate communication between the external and the internal environment, including CD34, Integrin α-7, VCAM and NCAM[20,34,37,38]. Notably, M-Cadherin (M-Cad) and N-Cadherin (N-Cad), which connect the MuSC surface to the myofiber are required to maintain quiescence[34]. In the absence of M-Cad and N-Cad, the adhesive junctions connecting the stem cell to the myofiber are broken and MuSCs display a phenotype consistent with early activation[34].

In addition to niche regulation, many intrinsic pathways are required to maintain muscle stem cell quiescence[4]. One of the most characterized pathways in the maintenance of MuSC quiescence is the Notch pathway[39-41]. Adult quiescent MuSCs express high levels of Notch and RBPJ, which control the expression of downstream targets Hes1, HeyL and Hey1 to maintain quiescence, while loss of RBPJ in MuSCs induces precocious activation of the myogenic program[40,29,39,41]. Notably, it was discovered that Hey1 inhibits recruitment of MyoD to myogenic

promoters, thus repressing myogenesis[42]. Another regulatory pathway that has been identified is the Sprouty1 (Spry1) pathway. Spry1 is critical for the regulation of Fibroblast Growth Factor-2 (FGF2). In the absence of Spry1, FGF2 signalling promotes exit from quiescence into a cycling state[29,43]. Furthermore, many studies have identified the p38/MAPK signalling pathway as an important regulator of quiescence by mediating communication between the extracellular niche and internal control of gene expression and epigenetic modifications[17,32,44-48]. In response to extrinsic signals from an injury, p38/MAPK becomes phosphorylated and can induce the transcription of MyoD, among other genes to promote MuSC activation[17,45].

Notably, many of the above-mentioned pathways mediate transcriptional control of myogenic genes[46,47]. Specifically, a group of basic helix-loop-helix transcription factors termed myogenic regulatory factors (MRFs)[49]. MRFs consist of MyoD, Myf5, Myogenin and MRF4 (Myf6) which are differentially expressed during the different stages of myogenesis. An important regulator of MRF expression is Paired Box 7 (Pax7) (Figure 1). Pax7 is the transcription factor that confines MuSCs to a myogenic lineage, is expressed in quiescent and activated MuSCs, and is also responsible for upregulating the transcription of other myogenic regulatory factors, including Myf5 which is both expressed upon MuSC activation[2,15,19,50,51]. These studies have demonstrated an intricate relationship between the extrinsic environment and internal signalling pathways to alter the state of MuSC quiescence.

In addition to transcriptional control through signalling pathways, studies have shown that quiescence is also regulated at the translational level[16,52,53]. Specifically, phosphorylation of eukaryotic initiation factor 1 (eIF2α) is required for quiescence, whereby inhibition of eIF2α

phosphorylation activates the myogenic program[52]. Furthermore, epigenetic alterations, through chromatin remodelling or post-translational modifications of histones, help maintain lineage and the state of quiescence in MuSCs[54,55]. A recent study demonstrated that quiescent MuSCs possess high amounts of the active marker H3K4me3, specifically at the transcriptional start sites of Pax7 and Myf5, indicating active transcription of the MRFs expressed in quiescent muscle stem cells[54]. Thus, MuSC quiescence is regulated by extrinsic and intrinsic factors at the transcriptional, translational and post-translational levels to ensure preservation of the muscle stem cell pool.

1.3.4 Depth of Quiescence in Muscle Stem Cells

As previously discussed, stem cell quiescence is described as a mitotically inactive, or G_0 state[28,56]. However, contemporary investigations have expanded our knowledge on the scope of cellular quiescence. A critical function of muscle stem cells is to rapidly exit quiescence in response to a stimulus, thus, in order to accomplish this muscle stem cells possess different depths of quiescence[28,57]. As briefly mentioned, this requires an intricate balance of epigenetic marks, gene expression, post-translational modifications, and regulation of integral pathways. Recent studies have demonstrated the ability of MuSCs to exist in two distinct states: classic "G_0" quiescence, and more shallow quiescence in which stem cells are primed for activation, termed "G_{Alert}"[28,57]. MuSCs identified as being in a G_{Alert} state were observed in muscle tissue that was distant, or contralateral to an injury and were described to be larger in size with increased mitochondrial metabolism and transcriptional activity compared to G_0 MuSCs[57]. The state of G_{Alert} allows MuSCs to rapidly respond to an additional injury, and enhance the activation and regeneration process, thus proving to be a beneficial mechanism[58]. These studies provide the notion that MuSC quiescence is a gradient that responds to physiological stimuli.

Since the discovery of the G_{Alert} state of muscle stem cells, there has been a number of pathways identified in the regulation of the shallow quiescent state[57-59]. It was initially identified that the activation of mammalian target of rapamycin complex 1 (mTORC1) was sufficient to transition MuSCs from G_0 to G_{Alert}[57]. G_{Alert} MuSCs had increased levels of phospho-S6, an indicator of mTORC1 activation. Furthermore, MuSC knockout of tuberous sclerosis 1 (TSC1), an inhibitor of mTORC1 signalling was sufficient to transition MuSCs into a G_{Alert} phenotype, while knockout of Raptor, a part of the mTOR complex did not confer any of the characteristics of the G_{Alert} phenotype, even in the presence of a remote injury[57]. This study demonstrated that activation of the mTOR complex 1 is a necessary intracellular signal for the G_{Alert} state of MuSCs, however in itself is not sufficient to explain the G_{Alert} state of quiescence.

Since the identification of the G_{Alert} state and its reliance on mTORC1 signalling, a few systemic pathways have been implicated in this adaptation of quiescence[58,59]. Skeletal muscle tissue that has received a localized injury has been shown to release many systemic and inflammatory factors[14,60-63]. It was identified that at the site of injury hepatocyte growth factor activator (HGFA) was circulated to surrounding muscle tissue, where it induces proteolytic cleavage of hepatocyte growth factor (HGF) into its active form[59]. Active HGF then binds to the cMET receptor on the surface of MuSCs, initiating the transition from G_0 to G_{Alert}[59]. Alternatively to the HGF-cMET axis, the fully reduced thiol form of high mobility group box 1 (HMGB1) has been demonstrated to transition MuSCs into G_{Alert} following injury[58] (Figure 1.2). Upon fracture injury, systemic fully-reduced HMGB1 forms a heterocomplex with the chemokine CXCL12, which subsequently binds the MuSC surface receptor CXCR4 to transition MuSCs into G_{Alert}[58].

Interestingly, treatment with exogenous HMGB1 alone was sufficient to shift MuSCs into G_{Alert}, indicating a substantial role for HMGB1 in the systemic regulation of the G_{Alert} state[58]. Notably, both the HGF-cMet axis and the HMGB1-CXCL12-CXCR4 pathway were found to be upstream of mTORC1, yet mTORC1 was still required for the G_{Alert} status[58,59] (Figure 2). The observation that these systemic factors, along with upregulation of mTORC1 signalling to transition deeply quiescent MuSCs into a primed state of quiescence is highly influential, however, what regulates the G_{Alert} state intracellularly beyond mTORC1 signalling remains elusive.

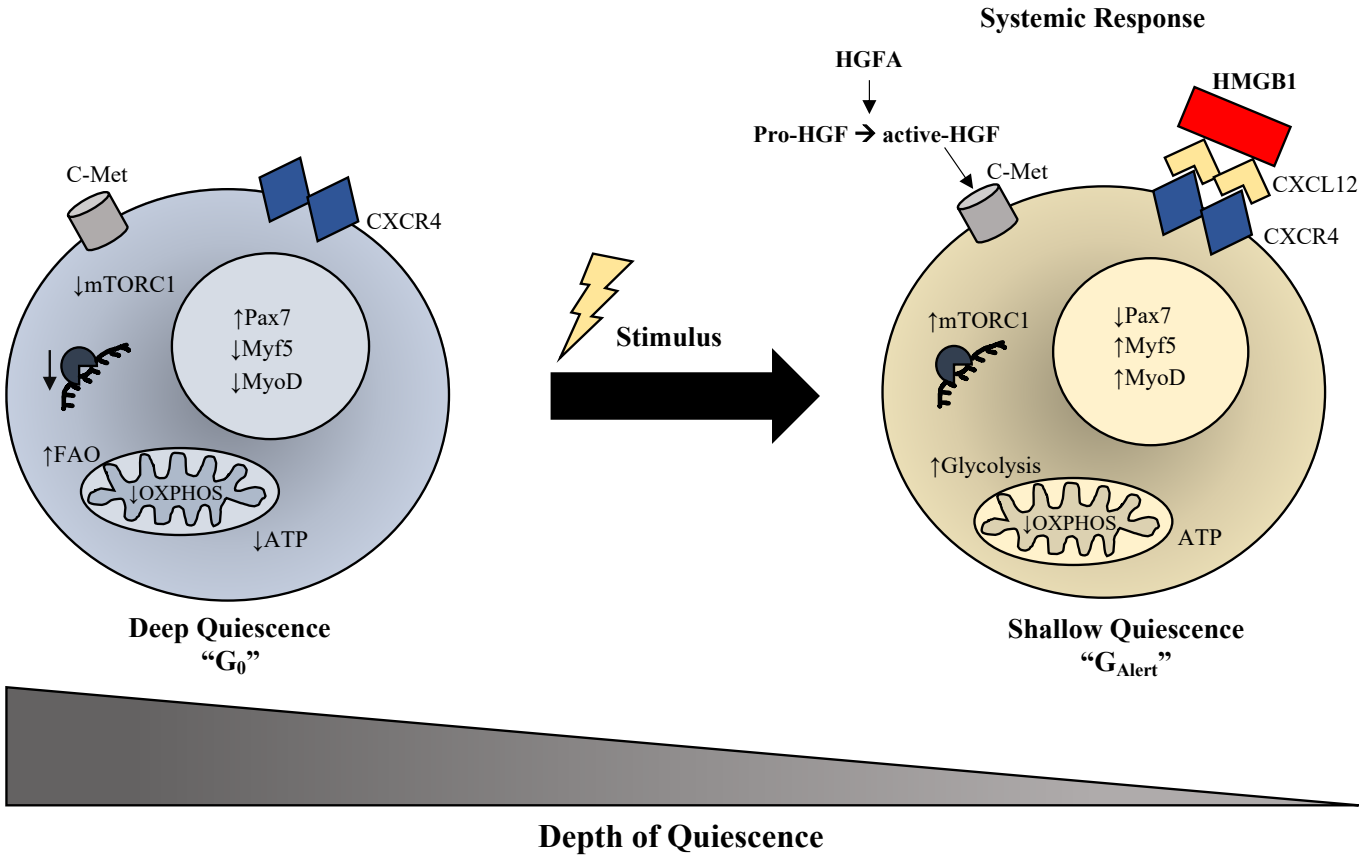

Figure 2. Depth of Quiescence in Muscle Stem Cells. Muscle stem cells in a deep quiescent state have unique gene expression and metabolic profile, which is altered as muscle stem cells reduce their depth of quiescence. Recent studies have demonstrated that upon an injury stimulus, a systemic response is initiated, which through multiple pathways can transition stem cells into "G-Alert" and alter the characteristics of quiescence.
Figure adapted from Rodgers et al (2017) *Cell Reports* and Lee et al (2018) *PNAS.*

1.3.5 Muscle Stem Cell Activation

Upon a necessary stimulus, such as injury or exercise, quiescent muscle stem cells are triggered to activate and enter the cell cycle to allow for reparation of damaged muscle tissue[1,4,17,34,64]. The process of activation prepares MuSCs for cell cycle entry and progression through the myogenic program towards differentiation[1,4,17,34,64]. In total, MuSC activation requires about 25-40 hours to exit G_0 and progress through G_1 of the cell cycle to prepare for first division[31,65]. Stem cell activation is an intricate process involving an array of cytokines and growth factors, including HGF, Wnt4, and FGF, among others,[14,35,43,59,66] which provide the required signals to transition quiescent MuSCs into an activated state[14,35,43,59,66].

A notable characteristic of early muscle stem cell activation is changes in gene expression[30,31]. The development of new techniques in the MuSC field has allowed researchers to identify an array of genes that are upregulated during the early stages of activation[30,31]. One study that utilized *in situ* fixation demonstrated major transcriptional changes in MuSCs that were fixed immediately, compared to MuSCs at 3 and 5 hours post-extraction[31]. Genes that were upregulated during early activation included rRNA maturation, cell cycle entry, and factors involved in the initiation of the myogenic program[31]. Interestingly, a second group using nascent RNA labelling concluded the same transcriptional profile, solidifying the large-scale transcriptional changes observed as MuSCs transition to activation[30]. Furthermore, the authors observed a down-regulation of genes involved in fatty acid oxidation, which is consistent with the metabolic switch that MuSCs undergo during activation[67].

In addition to large scale gene expression changes involving cell cycle regulation and cellular growth and maturation, muscle stem cells must also upregulate an array of transcription factors required for myogenic specification[12]. Of the myogenic regulatory factors, Myf5 is one of the first factors to be upregulated[16]. Myf5 is a direct target of the transcription factor Pax7, which initiates its transcription immediately during MuSC activation[15]. Furthermore, studies suggest that Myf5 mRNA is present in quiescent MuSCs but is sequestered in mRNP granules by miR-31 for rapid expression of Myf5 upon activation[16]. Another MRF that is promptly upregulated following MuSC activation is MyoD. Unlike Myf5, MyoD is not expressed in quiescent MuSCs, thus the expression of MyoD is an acceptable marker of MuSC activation[18]. Myf5 and MyoD, along with Pax7 are expressed during stem cell proliferation and downregulated upon commitment to differentiation[68].

1.3.6 Overview of Muscle Stem Cell Fate Decisions

Once stem cells are activated and enter the cell cycle to proliferate, they must then determine whether they will return to quiescence to replenish the stem cell pool, called self-renewal, or commit to myogenic differentiation[4,20]. The ability of muscle stem cells to either self-renew or commit is referred to as stem cell fate decisions. A dynamic balance in MuSC fate decisions must exist in order to preserve the stem cell pool while simultaneously supporting muscle regenerative requirements[1,2]. Thus, maintaining the ratio of MuSC self-renewal and commitment is a highly regulated process.

1.3.7 Regulation of Muscle Stem Cell Fate Decisions

Muscle stem cells preparing for mitotic division can divide either symmetrically or asymmetrically[20,36]. The orientation of division is defined by cell polarity, including the segregation of necessary proteins and factors, and by the orientation of the mitotic spindle[17,53,69]. Symmetric division occurs in a planar orientation along the basal membrane and results in two identical daughter cells that promote stem cell expansion[1,4,24]. In contrast, MuSCs that divide asymmetrically, in an apical-basal orientation will give rise to one daughter cell that will self-renew and one daughter cell that will undergo commitment[4]. Following asymmetric division, the daughter cell destined to commit maintains contact with the plasmalemma of the myofiber, while the self-renewing daughter cell maintains contact with the basal lamina, preserving interaction with the niche[53]. The orientation of the daughter cells is dictated by the PAR polarity complex, which is comprised of partitioning-defective 3 (PAR-3), PAR-6, and atypical protein kinase-3 (PKC)[17,53,70]. The PAR complex is uniformly distributed to one daughter cell and subsequently activates the p38/MAPK signalling cascade to induce transcription of MyoD, allowing for myoblast commitment and consecutive proliferation[17,44,46,47,61] (Figure 3).

1.3.8 Muscle Stem Cell Self-Renewal

Self-renewal relies on the ability of the stem cell to withdraw from the cell cycle and return to quiescence[17,53,69,71]. This is a coordinated effort requiring timely suppression of MRFs, and the re-activation of quiescence pathways[24,39,52,69,71,72]. First, in order for a stem cell to self-renew it must express an abundance of Pax7[71]. Pax7, in addition to confining MuSCs to the myogenic lineage and promoting cell growth and proliferation, is essential for repression of the terminal myogenic differentiation program[71,73]. In accordance with increased Pax7 expression upon self-renewal, one

study demonstrated that cells with higher levels of Pax7 expressed additional markers of stemness, and retained the template DNA strand, suggesting that cells with increased Pax7 expression are destined for self-renewal[65]. In addition to increased Pax7 expression, stem cells set to self-renew must re-integrate pathways involved in the maintenance of quiescence[52,74,75]. The re-expression of Sprouty1 is required for the inhibition of FGF2 and subsequent return to quiescence[74]. Similarly, up-regulation of the Notch pathway is required during muscle stem cell self-renewal[75]. Furthermore, in addition to maintaining quiescence, phosphorylation of eIF2α is also required for stem cells to self-renew, suggesting that there is similar regulation in quiescence and self-renewal[52] (Figure 3).

1.3.9 Muscle Stem Cell Commitment

Muscle stem cell commitment centres around the timely expression of key MRFs, specifically MyoD and Myf5. In MuSCs designated for myogenic commitment, Pax7 recruits a histone methyltransferase to the enhancer regions of Myf5 and MyoD, promoting transcription of the MRFs and designating the committed daughter cell[15,73,15,24] (Figure 3). Expression of MyoD is additionally regulated at the translational level. As previously discussed, activation of the p38/MAPK signalling cascade is required to progress myogenesis. The p38/MAPK signalling pathway further promotes MyoD expression by repressing the MyoD mRNA decay factor Tristestraprolin, thus advancing myogenic commitment[76]. Thus, the decision between self-renewal and commitment is tightly regulated process involving many coordinated factors to ensure a balance allowing both maintenance of the stem cell pool and effective muscle regeneration.

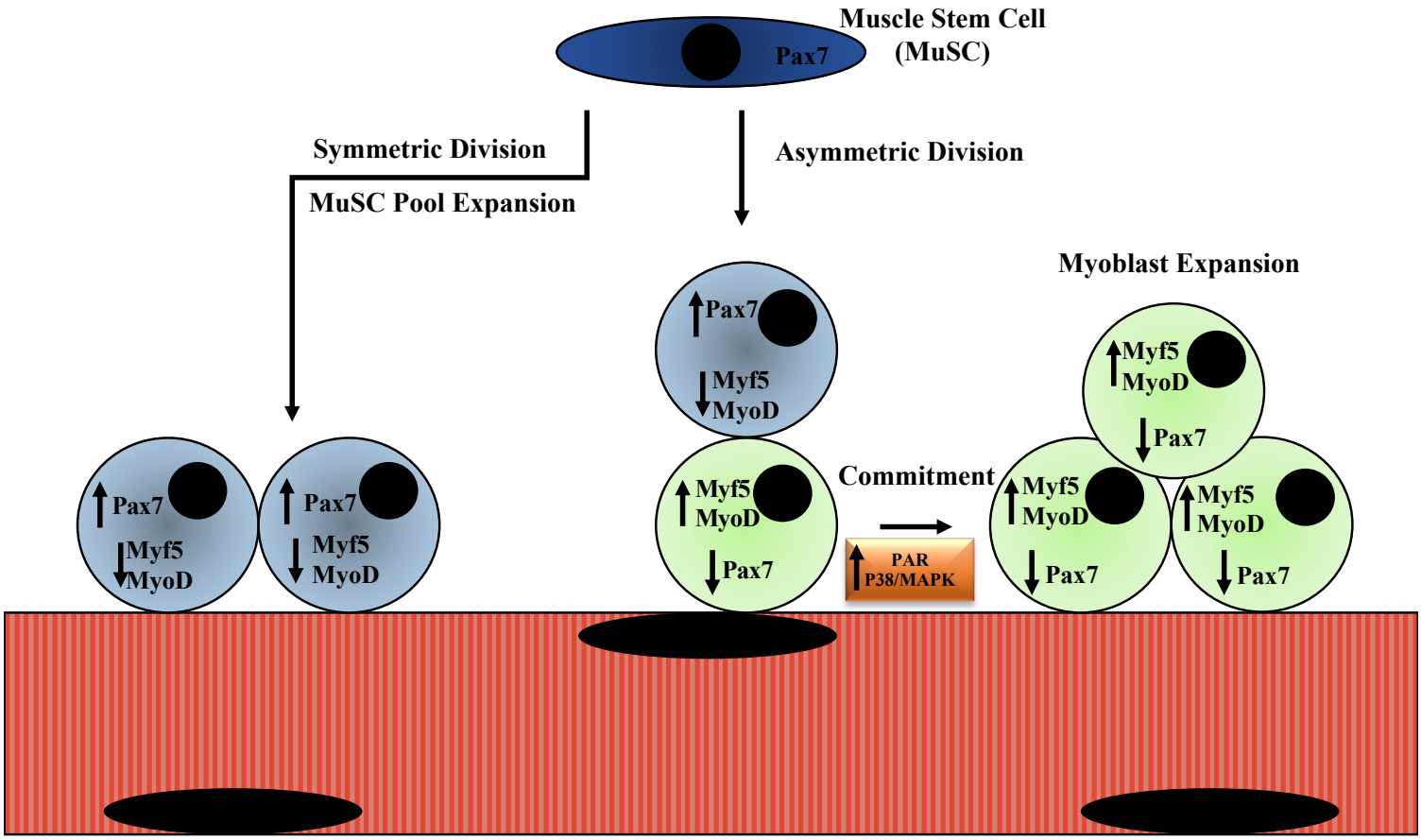

Figure 3. Regulation of Muscle Stem Cell Fate Decisions. Muscle stem cells have the option to self-renew and return to a quiescent stem cell or commit to myogenic differentiation as a myoblast. The process of muscle stem cell fate decisions is highly regulated and requires the coordination of stem cell orientation and gene expression regulation. Muscle stem cells destined to self-renew re-upregulate Pax7, while downregulating the myogenic regulatory factors MyoD and Myf5. Muscle stem cells committed to a myoblast fate maintain high levels of MyoD and Myf5, while downregulating Pax7. Orientation of Asymmetrically dividing muscle stem cells is coordinated by the PAR-Polarity complex.
Figure adapted from Dumont et al (2015) *Development.*

1.3.10 Muscle Stem Cell Fate and Function in Degenerative Diseases and Aging

As discussed, muscle stem cell fate is a highly regulated process involving intricate coordination of external and internal factors to ensure both maintenance of the stem cell pool through sufficient self-renewal, and formation myogenic progenitors for subsequent muscle repair and regeneration[43,53,77-81]. Dysregulation in muscle stem cell fate decisions can therefore be detrimental to muscle quality and function, and in fact is a common feature in conditions of muscle wasting and atrophy[43,53,74,78,80]. In aging and age-induced sarcopenia, dysfunctional muscle stem cells and abnormal stem cell fate can lead to a decline in the stem cell pool or stem cell senescence, thus impairing proper muscle repair and regeneration, leading to a decline in muscle mass and quality[43,80,81]. Interestingly, studies have demonstrated that geriatric mice contain up to 65% fewer functional muscle stem cells, where loss of MuSC function stems from DNA damage, reduced antioxidant capacity, and alterations in transcriptional and epigenetic landscapes[54,82-84]. Geriatric MuSCs exhibit unique epigenetic marks, as characterized by alterations in H3K4me3 and H3K27me3 markers of chromatin that have been documented to regulate many important factors for MuSC function. Furthermore, major transcriptomic changes are observed in aged MuSCs, where the majority of these genes include oxidative stress sensing genes, antioxidant genes, and genes involved in mitochondrial function, dynamics and metabolism, including oxidative phosphorylation and the tricarboxylic acid (TCA) cycle[54,80,85-88].

Moreover, a deregulation of intrinsic signalling pathways is another contributing factor promoting MuSC dysfunction. Studies have demonstrated that during aging, there is a reduction in Spry1/FGF2 signalling, which leads to a loss of quiescence and depletion of the MuSC pool[74,80]. Interestingly, a study that focused on neuromuscular junction (NMJ) degeneration during aging

identified lack of MuSC contribution as a prominent factor leading to NMJ deterioration, while overexpression of Spry1 improved MuSC function and attenuated NMJ degeneration[89,90]. Other studies have identified an increase in $p16^{Ink4A}$, which dephosphorylates Retinoblastoma protein (Rb), leading to loss of reversible quiescence and promotes an irreversible senescent state[91]. Additionally, an increase in p38/MAPK, the key signalling pathway in the regulation of commitment of asymmetrically dividing stem cells is upregulated upon aging, promoting commitment over self-renewal and leading to MuSC depletion[44]. Finally, another common feature observed in aging muscle is an increase in fibrotic tissue. Studies have demonstrated that dysregulation of MuSCs and the surrounding niche can lead to increased fibrosis in geriatric mice[43]. Notably, an increase in Wnt signalling during aging has been demonstrated to activate a fibrogenic program, promoting the formation of fibrotic tissue in muscle[92]. These studies highlight the contribution of muscle stem cells in maintaining quality muscle tissue, where a decline in functional muscle stem cells during aging leads to aged-induced sarcopenia and loss of muscle function.

Similarly, a dysregulation in muscle stem cell fate and function has been observed in muscular dystrophies and myopathies[53,79]. Interestingly, both aged-induced sarcopenia and Duchenne's Muscular Dystrophy (DMD) share common traits, including muscle atrophy and weakness, indicating a link between these diseases and their etiology[79]. Until recently, DMD was believed to be caused by myofiber weakness, though current studies have identified a critical role for MuSCs in the DMD phenotype[79,93,94]. In DMD, there is a loss of dystrophin protein, which leads to a deregulation of cell polarity, spindle orientation and a reduction in the number of asymmetric divisions that occur[53,70]. This causes over-expansion of the stem cell pool with a

concomitant decline in myogenic progenitors, leading to failed muscle regeneration[70]. Interestingly, similar extrinsic signalling factors identified in aging have also been implicated in DMD. In the *mdx* mouse model of DMD, there is a reduction in genes for myogenic potential and an increase in fibrosis related genes, which is mediated through the Wnt-TGFβ2 axis, leading to a fibrotic phenotype in DMD[92]. Thus, muscle stem cell fate and function is highly regulated, while dysregulation at the extrinsic or intrinsic level is a leading cause in muscle wasting and degenerative conditions. Despite our current understanding of regulation of muscle stem cell fate and function, a lot is still unknown about what controls the fate of MuSCs during muscle regeneration. Notably, recent advancements in the stem cell metabolism field have identified pivotal roles for mitochondria in the regulation of stem cell fate and function.

1.4 Mitochondria

1.4.1 Overview of Mitochondrial Metabolism

Mitochondria are the site of the major metabolic processes that occur to provide cells with means of energy supply for continual function. Mitochondria mainly generate adenosine triphosphate (ATP), through the process of oxidative phosphorylation (OXPHOS)[95]. Electrons are shuttled through a series of complexes, whereby protons are pumped out at each complex to then fuel ATP synthase by proton motive force to convert ADP into ATP[96]. In addition to the movement of electrons and protons across the complexes, the process of OXPHOS produces many by-products for biosynthetic pathways that are integral for cell function and health. As electrons pass through complexes I (CI) and III (CIII), reactive oxygen species (ROS) are produced, which is important for cell signalling, notably during differentiation[97,98]. Thus, mitochondria are not not

only essential for energy production, but also play an essential role in cell signalling and homeostasis.

1.4.2 Mitochondrial Dynamics

Mitochondria are highly dynamic organelles that constantly undergo structural modifications through fission and fusion, that alter overall length and cristae morphology[99-104]. The dynamic nature of mitochondria is an interesting and highly important phenomenon for the regulation of overall mitochondrial bioenergetic function and ultimately cell homeostasis[100,102,105-107]. The dynamic nature of mitochondria is regulated at both the macro- and ultra-structural levels by a series of intricately linked proteins.

1.4.3 Mitochondrial Fission and Fusion

Mitochondrial dynamics is controlled by a series of dynamin like GTPases that mediate fission and fusion. Mitochondrial fission, also known as mitochondrial fragmentation, is regulated by the activity of dynamin related protein 1 (DRP1), as well as additional factors that aid in the fission process, including mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (MFF), and mitochondrial dynamics proteins 49 and 51 (MiD49 and MiD51)[99,108,109]. Mitochondrial fission is initiated by recruitment of DRP1 from the cytosol to the outer mitochondrial membrane (OMM), where it associates with outer-membrane-bound MFF or Fis1 and forms oligomers surrounding the OMM[109-111]. Through GTP hydrolysis, DRP1 then constricts the OMM, causing mitochondrial fragmentation[99,110,111] (Figure 4). Mitochondrial fission is an important process that allows for the elimination of damaged or dysfunctional mitochondria. Mitochondrial fission allows for the segregation of damaged portions of mitochondria and

subsequent elimination through the process of mitophagy, therefore preserving the integrity of mitochondria[112].

Mitochondrial fusion is a two-step process involving both the outer mitochondrial membrane and the inner mitochondrial membrane (IMM) (Figure 4). Mitochondrial fusion begins with OMM tethering, which is mediated by Mitofusin 1 (MFN1) and Mitofusin 2 (MFN2)[100,101,113-115]. MFN1 and MFN2 localize around the area at which OMM fusion occurs, where they form oligomeric complexes to bring the outer membrane of two mitochondria together via GTP hydrolysis[99,114,115]. Although MFN1 and MFN2 both mediate OMM fusion, their functions are not redundant. MFN2, in addition to OMM fusion, is also responsible for mediating mitochondrial-endoplasmic reticulum (ER) contact[116]. Loss of MFN2 induces mitochondrial fragmentation, while loss of MFN1 impairs the formation of long tubular networks of mitochondria[117].

The second step is fusion of the IMM, which is predominantly performed by optic atrophy 1 (Opa1) (Figure 4). Opa1, in addition to IMM fusion, is also a master regulator of cristae[118-121]. When Opa1 is expressed, cristae are compact and metabolically efficient, however in the absence of Opa1, cristae undergo remodelling for cell death, linking Opa1 to cellular function[118,119,122-124]. Opa1 has eight isoforms which can undergo proteolytic cleavage to form long and short variants of the protein[125,126]. While long isoforms of Opa1 remain anchored in the IMM and the short forms of Opa1 are soluble in the intermembrane space (IMS), studies have demonstrated that both long and short isoforms of Opa1 are required for IMM fusion[127-129]. Thus, Opa1 is an important protein for the regulation of mitochondrial structure and ultrastructure to mediate mitochondrial and cellular function.

1.4.4 Functions of Mitochondrial Dynamics

Mitochondrial plasticity, meaning the constant adjustment in mitochondrial structure and cristae architecture is required for many aspects of mitochondrial health and cellular function[99,112,130]. Mitochondrial fission is intricately linked to mitochondrial quality control and cell death signalling under extreme stress conditions, while mitochondrial fusion regulates metabolic efficiency and allows for distribution of mitochondrial content, specifically mtDNA across the mitochondria[99-102,107,118,119,123]. Recently it was observed that complete mitochondrial adynamism, meaning the lack of both mitochondrial fission and fusion, was more detrimental to cellular health than the absence of fission or fusion alone[131]. Thus, both mitochondrial fission and fusion are uniquely involved in cell health, and a dynamic balance in both processes is required to maintain proper mitochondrial function.

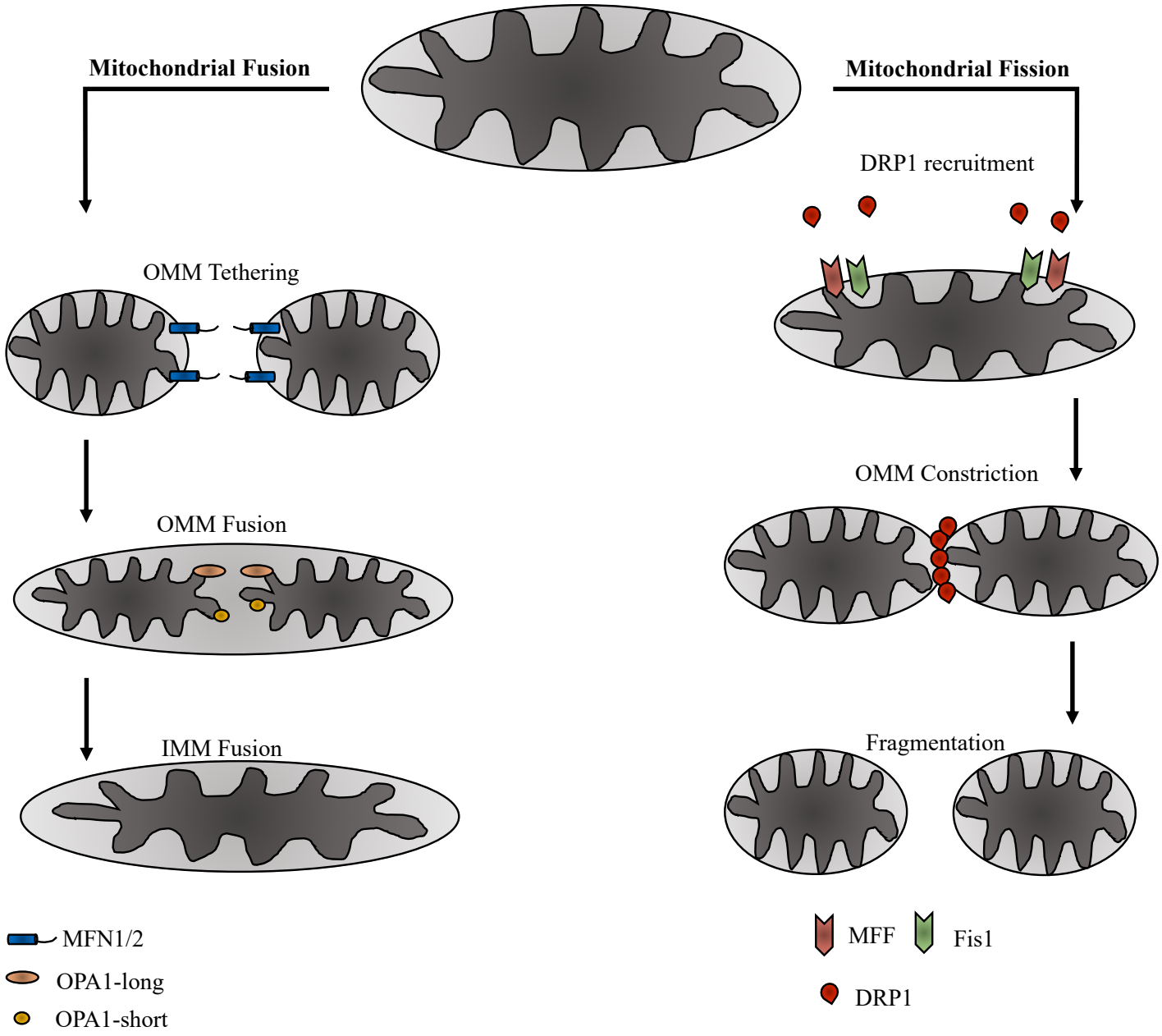

Figure 4. Mitochondrial Dynamics. Mitochondria constantly undergo cycles of fission, creating fragmented mitochondria, and fusion, forming elongated mitochondria. Mitochondrial fusion begins with outer mitochondrial membrane (OMM) tethering by mitofusins 1 and 2 (MFN1/2). Following OMM fusion, OPA1 isoforms mediate inner mitochondrial membrane (IMM) fusion. Mitochondrial fragmentation begins with cytosolic DRP1 recruitment to associate OMM proteins MFF and Fis1. DRP1 then surrounds the OMM, leading to membrane constriction and subsequent fragmentation.
Figure adapted from Baker et al (2019). *Mitochondrion.*

1.5 Mitochondria in Stem Cells

1.5.1 Metabolic Suppression in Stem Cells

Many stem cells rarely utilize mitochondrial oxidative respiration, and instead rely on glycolysis as the primary source of ATP, including hematopoietic stem cells (HSCs), neural stem cells (NSCs), and embryonic stem cells (ESCs), while MuSCs exhibit a shift from FAO to glycolysis upon activation, prior to differentiation[67,132-136]. It was previously conceived that stem cells maintain little-to-no mitochondrial metabolism as a consequence of low protein synthesis and general stem cell activity[99,137]. Although this notion is a logical assumption it has since been demonstrated that stem cells maintain low mitochondrial metabolism in order to preserve stemness[135,137,138]. Furthermore, OXPHOS is actively inhibited in stem cells through a range of mechanisms[139-145]. Upstream of OXPHOS, the mitochondrial carrier homolog 2 (MTCH2) which resides on the OMM suppresses OXPHOS function in stem cells, while loss of MTCH2 promotes mitochondrial respiration and leads to premature HSC differentiation[139-141]. Additionally, uncoupling protein-2 (UCP2) suppresses mitochondrial oxidative function by inhibiting glucose oxidation and promoting glycolysis through substrate shunting[142,143]. Furthermore, ATP-synthase activity is truncated by inhibitory factor 1 (IF1)[144,145]. Beyond inhibition of mitochondrial metabolic activity, there are also many mechanisms in place to suppress downstream by-products of OXPHOS, including ROS[137,146,73]. Stem cells exhibit high levels of HIF2α, and the forkhead box protein O (FOXO) family of transcription factors, which mediate levels of antioxidant factors, including, but not limited to, catalase, glutathione (GSH), glutathione peroxidase 1 (GPX1), catalase, thioredoxin, and superoxide dismutases (SOD1 and SOD2)[72,146.75,147,148]. Together, these studies indicate that mitochondrial metabolism is actively repressed in stem cells, thus inferring an important role for mitochondrial metabolism in stem cell differentiation.

1.5.2 Metabolic Remodelling in Stem Cells

During the differentiation process, mitochondria undergo drastic changes in their function and capacity[137,146,149]. As previously discussed, stem cells maintain low levels of mitochondrial respiration, including reduced ATP generation, low levels of mitochondrial metabolites, little to no mitochondrial biogenesis and maintain a primarily glycolytic phenotype[137,146,149]. Upon the initiation of a differentiation program, mitochondria drastically increase levels of ATP generation, mitochondrial content, and ETC complexes[137,146,149-151]. Most notably, stem cells undergo a metabolic switch during differentiation. Stem cells, which rely on glycolysis to meet metabolic demands transition to utilization of OXPHOS as the primary energy source upon differentiation[99,134,137,152] (Figure 5). Classically, this was believed to be a consequence of the increase in energy demands of post-mitotic cells, however several innovative studies have demonstrated that the metabolic switch is an active contributor to the differentiation program[95,151,153-155]. Studies performed on induced pluripotent stem cells (iPSCs) were the first to show that upon reversal to a stem cell state, the first genes to be upregulated are those involved in glycolysis[95,151], while inhibiting glycolytic activity stunts pluripotency[151]. Furthermore, suppression of OXPHOS through treatment with CCCP or Antimycin A, as well as induction of hypoxia in stem cells severely impairs differentiation[153-155]. Thus, these studies indicate that this switch in energy source is required for stem cells to commit to differentiation.

It has been observed that quiescent muscle stem cells primarily utilize fatty acid oxidation (FAO) and undergo a switch to a primarily glycolytic state upon activation[67,136]. This switch from FAO to glycolysis upon muscle stem cell activation was demonstrated to decrease levels of nicotinamide adenine dinucleotide (NAD+) and the SIRT1 deacetylase to subsequently elevate

levels of H4K16ac and thus transcription of genes involved in the myogenic program[67]. In addition to epigenetic regulation of MuSC activation through the NAD+/SIRT1 axis, studies have also demonstrated a pivotal role for Pyruvate Dehydrogenase (PDH) as the rate-limiting factor for the epigenetic regulation of MuSC activation and differentiation[136]. These influential studies therefore demonstrate that metabolism is an important regulatory factor of MuSC quiescence.

1.5.3 Mitochondrial Dynamics in Stem Cells

In the recent years, many studies have focused on mitochondrial morphology changes as stem cells progress through differentiation. Initially, studies observed that mitochondria in stem cells were underdeveloped, displaying a more fragmented phenotype with disordered cristae[134,156,157]. Upon terminal differentiation, mitochondria undergo dramatic alterations in biogenesis and morphology at the structural and ultrastructural level[150,158], though it was not until more recently that this change in morphology was determined to be essential for stem cell differentiation, and not a consequence of increasing energy demands (Figure 5).

Since the discovery of dynamic mitochondrial shape changes upon differentiation, a few ground-breaking studies have demonstrated that manipulating mitochondrial structure can alter the fate of stem cells[132,159,113,160]. In HSCs, loss of MFN2 leads to fragmented mitochondria and affects the maintenance of HSC lymphoid potential through altered mitochondrial-ER contact and calcium signalling[160]. Moreover, it was observed that modifying mitochondrial cristae ultrastructure in T-cells via loss of Opa1 altered FAO efficiency and impaired the ability of T-cells to adapt a memory T-cell fate[113]. Most notably, however, was the discovery that directing mitochondrial dynamics, by acute loss of MFN1/2, Opa1 or DRP1 could drastically alter the fate of NSCs during

neurodevelopment. This study implicated mitochondrial dynamics as an upstream regulator of the metabolic switch and ROS signalling which activated the Keap1-Nrf2 transcriptional program to elicit changes in nuclear gene expression to favour either self-renewal in DRP1-KO NSCs, or commitment/differentiation in MFN1/2-dKO and Opa1-KO NSCs[132]. Thus, the importance of mitochondrial dynamics in the regulation of stem cell fate and differentiation has been observed in a variety of processes, including hematopoiesis, inflammatory and immune responses, and in neurogenesis[132,160,113]. However, the role of mitochondrial dynamics on the adult muscle stem cell pool during muscle regeneration and myogenesis has yet to be studied.

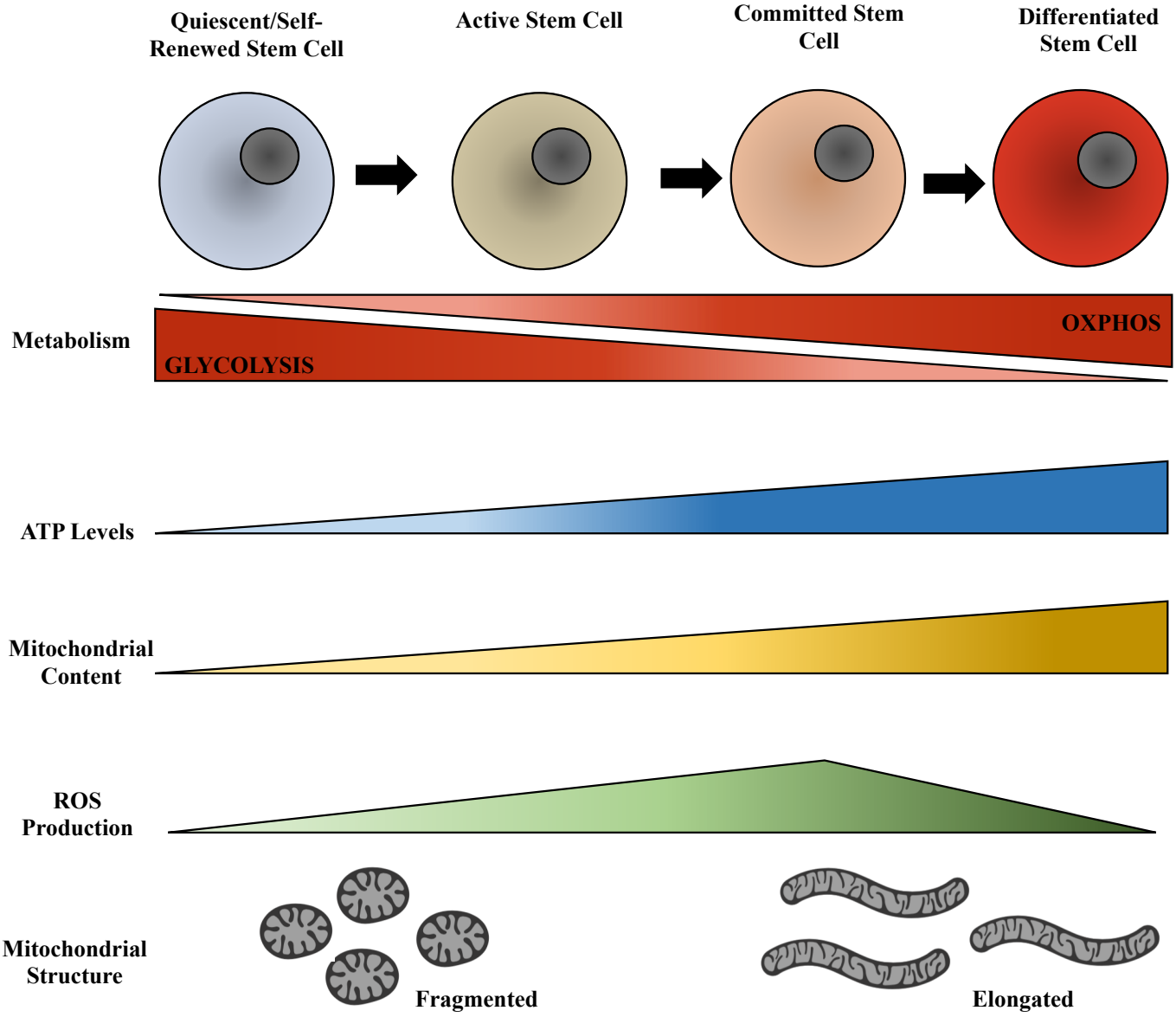

Figure 5. Summary of mitochondrial and metabolic events during stem cell differentiation. Stem cells undergoing differentiation exhibit a metabolic switch, in which the primary process for ATP production changes from glycolysis to oxidative phosphorylation (OXPHOS). During differentiation, stem cells also increase levels of ATP, mitochondrial biogenesis and reactive oxygen species (ROS) production for signaling. Lastly, there is a change in mitochondrial structure from fragmentation to elongation during stem cell differentiation.

1.6 Metabolic and Redox Signalling in Stem Cells

Morphological and metabolic alterations in mitochondria are required for stem cells to commit to differentiation[132,160,113], thus posing the question of how mitochondrial structure and metabolic changes can promote stem cell differentiation. An abundance of studies have identified metabolite and redox driven signalling pathways that induce stem cell differentiation[132,138,161,162] (Figure 5). Many studies have observed that as stem cells commit to differentiation, there is an accompanied increase in ROS levels and decrease in antioxidants[22,128,148,149]. Physiological ROS is required for cell processes, including gene expression, protein translation and protein to protein interactions[132,162-164], thus it is highly conceivable that ROS is a potent signalling molecule that mediates the necessary cellular adaptations as stem cells commit to differentiation. In fact, several studies have effectively proven this. Recent literature has demonstrated that reduction of ROS levels, by knock-out of the CIII Rieske Iron Sulfur Centers, or with antioxidants can halt the differentiation process[132,165]. Furthermore, it has been illustrated that increased ROS levels in NSCs, through changes in mitochondrial dynamics, act as a signaling molecules through the Nrf2 master redox regulator pathway (Figure 6). This study was the first to provide mechanistic evidence that redox signaling can regulate the transcriptional landscape of stem cells via the Nrf2 transcription factor, by suppressing self-renewal genes while activating neural commitment and differentiation genes to promote differentiation[132]. In addition to differentiation progression, physiological ROS can also mediate the self-renewal potential of stem cells. Critical studies have demonstrated that, in addition to an elongation of the mitochondrial network, levels of physiological ROS are substantially reduced in iPSCs and stem cells that execute self-renewal programs[152]. Furthermore, ROS has been shown to interact with the Notch pathway, a critical regulator of stem cell self-renewal and quiescence, through Nrf2 mediated upregulation of Botch,

an inhibitor of the Notch pathway[132]. Thus, ROS signalling is not only an important mechanism for the regulation of stem cell differentiation but can also act in response to mitochondrial dynamics to dictate stem cell fate[132]. However, whether ROS itself is able to elicit changes in nuclear transcription, or whether there are additional intermediate molecules that aid in this process remain to be discovered.

The longstanding relationship between ROS and the antioxidant glutathione has been of particular interest in recent years with regards to stem cell metabolism and signalling[107,166-168]. Reduced glutathione (GSH) is an abundant tripeptide thiol antioxidant that is synthesized in the presence of ROS[168]. Upon increased ROS levels, the cysteine-glutamate exchanger (xCT) SLC7A11 is upregulated, allowing entry of the glutathione precursors cysteine and glutamine into the cell, thus promoting glutathione synthesis[169]. Moreover, glutathione levels are linked to the Keap1-Nrf2 pathway, which has been established as an important transcriptional pathway that senses redox signalling in stem cells to promote differentiation[132,170-172]. Furthermore, several studies have identified reduced glutathione as an important regulator in cell cycle progression[173-177], while some studies have suggested that glutathione can promote differentiation[178-180]. The dynamic relationship between ROS and GSH to elicit redox signalling thus may be an important consideration in stem cell maintenance and differentiation.

In addition to ROS signalling, several metabolites can drive stem cell differentiation by modifying the epigenetic landscape[137,146,181]. The tricarboxylic acid (TCA) cycle intermediates α-ketoglutarate, Acetyl-CoA, and succinate are all involved in epigenetic alterations to determine stem cell fate[165,181]. Knockout of the CIII Reiske Iron Sulfur Centre (RISP-KO) elevated the TCA

cycle intermediates Acetyl-CoA, fumarate, succinate, α-KG, 2-hydroxyglutarate, which are known to induce changes in histone methylation and acetylation, thus altering the epigenetic landscape of HSCs[165]. Another study has demonstrated that human naïve and primed ESCs during early development have distinct levels of the metabolite S-adenosyl methionine, which is required for repressive histone methylation and control of ESC activation and differentiation[182]. Finally, studies conducted in muscle stem cells have identified NAD+ as an essential cofactor in the regulation of the metabolic switch during myogenic progression, whereby low levels of NAD+ and the histone deacetylase Sirt1 promote muscle gene transcription[67]. These studies have demonstrated that, in addition to the importance of mitochondrial dynamics and metabolism, OXPHOS-independent metabolites are also required for stem cell fate and differentiation.

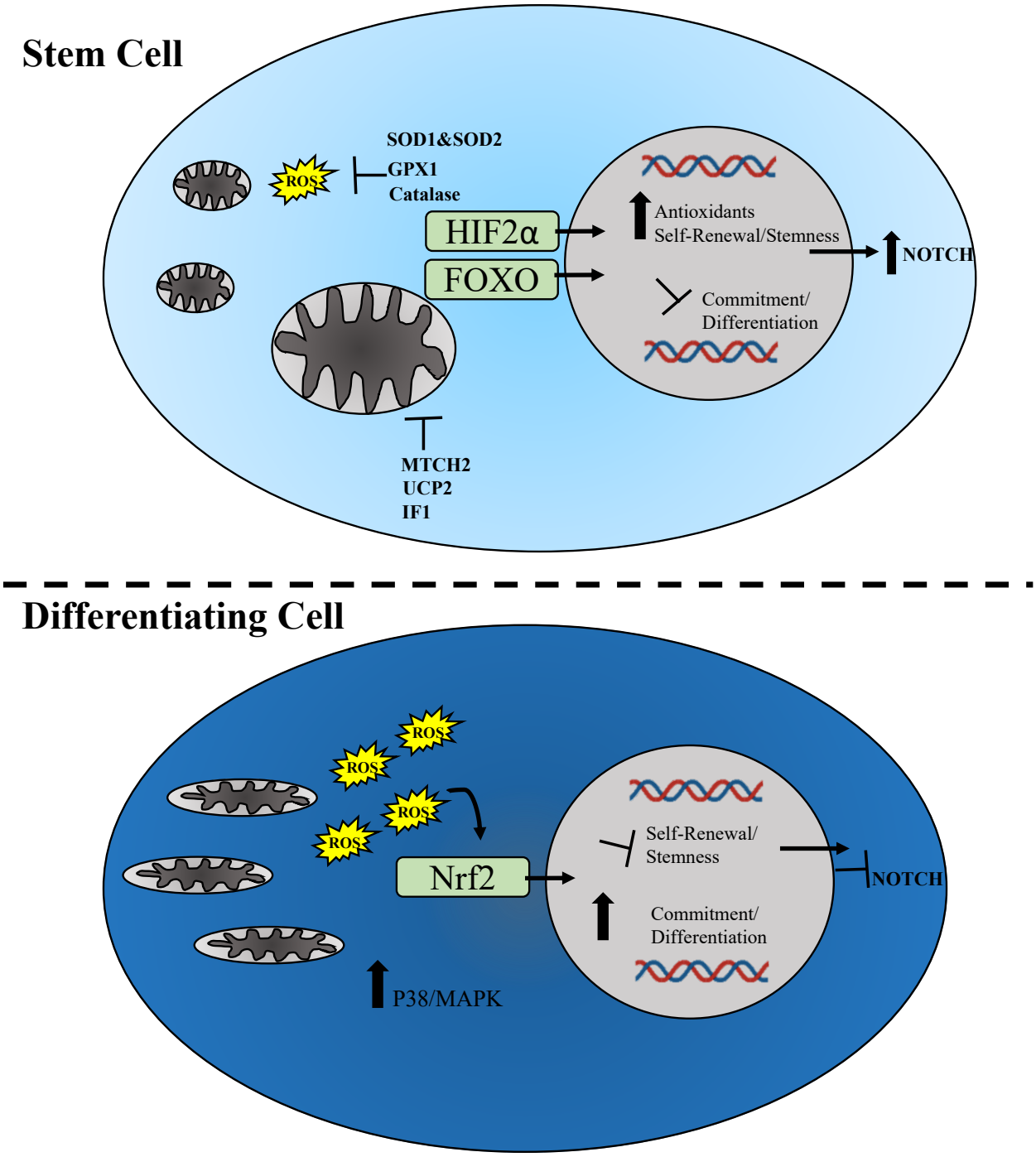

Figure 6. Summary of Metabolic Events during Stem Cell Commitment and Differentiation. In stem cells, mitochondria maintain low levels of reactive oxygen species through a range of antioxidant mechanisms to suppress redox signaling and subdue differentiation signals. In contrast, stem cells undergoing the process of commitment and differentiation down-regulate antioxidant responses, allowing for increased reactive oxygen species, which drives many pathways to promote differentiation, including the Nrf2 transcriptional program to downregulate self-renewal genes and increase commitment and differentiation gene expression. Figure adapted from Khacho and Slack (2017) *Antiox Redox Signalling*.

1.6.1 Mitochondrial signalling in disease and aging

Mitochondrial dynamics, metabolism and metabolite-based signalling are important in determining the fate and differentiation potential of many types of stem cells[132,160,113]. With aging and many degenerative diseases, there is often a decline in stem cell function, with a reduced capacity for tissue repair and stem cell maintenance. This leads to a reduction in the stem cell pool and ultimately tissue degeneration[77,183]. Notably, dysregulated mitochondrial function, including dynamics, metabolism and increased ROS are commonly observed during aging and degenerative diseases[184-186]. Multiple studies have demonstrated that aged or dysfunctional stem cells are accompanied by elevated ROS levels and dysregulated antioxidant and redox responses[187-191]. Chronic mitochondrial dysfunction can lead to accumulation of harmful ROS, generating DNA damage, protein carbonylation, lipid oxidation, and ultimately stem cell dysfunction[187-191]. Furthermore, research conducted in mice deficient in essential antioxidant response proteins, including the FOXO family of transcription factors, demonstrate an accelerated aging phenotype with characteristics of stem cell dysfunction[186]. Interestingly, studies have demonstrated that supplementation with N-Acetyl Cysteine (NAC), which promotes GSH synthesis can improve stem cell exhaustion through antioxidant mechanisms[164]. Likewise, addition of nicotinamide riboside (NR), the precursor to NAD+ can ameliorate some of the observed defects in aging stem cells by increasing the expression of genes involved in OXPHOS and the TCA cycle, thus improving mitochondrial function[87,175,192,193].

In addition to elevated oxidative stress accompanied by dysregulated redox and antioxidant defence, research has demonstrated an impairment in mitochondrial dynamics and metabolism in aged stem cells[184]. Prolonged mitochondrial fragmentation can act as a pathogenic factor, leading

to dysregulated metabolic function[77,194-196]. In fact, a recent study in germline stem cells demonstrated a shift in mitochondrial dynamics towards fragmentation, leading to a progressive stem cell loss[184]. Furthermore, impaired mitochondrial dynamics was implicated in MSC senescence through FGF signalling[197]. These studies pose an important role for mitochondria in continual stem cell function and longevity.

Chapter 2: Hypothesis and Aims

2.1 Rationale

Muscle degeneration resulting from a depletion of the muscle stem cell pool is a prevalent issue in many diseases and in aging. Dysregulated mitochondrial dynamics through aberrant fission and fusion events, and increased levels of damaging ROS are pathogenic factors leading to the depletion of the stem cell pool. However, research has demonstrated the importance of mitochondrial dynamics and physiological levels of ROS in regulating stem cell fate and differentiation. Thus, it is important to understand how dysregulated mitochondrial dynamics affects muscle stem cell fate and function during regeneration, and its impact on the longevity of the muscle stem cell pool.

In order to study the impact of impaired mitochondrial dynamics on the adult muscle stem cell pool, our lab developed and validated a tamoxifen-inducible conditional knockout of Opa1 in the Pax7+ MuSCs (Figure 7A-B). Using an established injury model, in which cardiotoxin (CTX) was injected into the tibialis anterior (TA) muscle and harvested at 4,7- and 21-days post-injury (DPI), previous members of our lab demonstrated that perturbing mitochondrial dynamics severely impairs muscle regeneration (Figure 7C, Chwastek, Girgis and Khacho (2018) Unpublished). Analysis of Hematoxylin and Eosin (H&E) stained muscle cross-sections from wild-type and Opa1-KO mice revealed a significant decline in both newly forming centrally nucleated myofibers (CNF) and the cross-sectional area (CSA) of these fibers, at 4,7- and 21-DPI, as well as a substantial loss in total TA muscle mass following regeneration (Figure 7D-G).

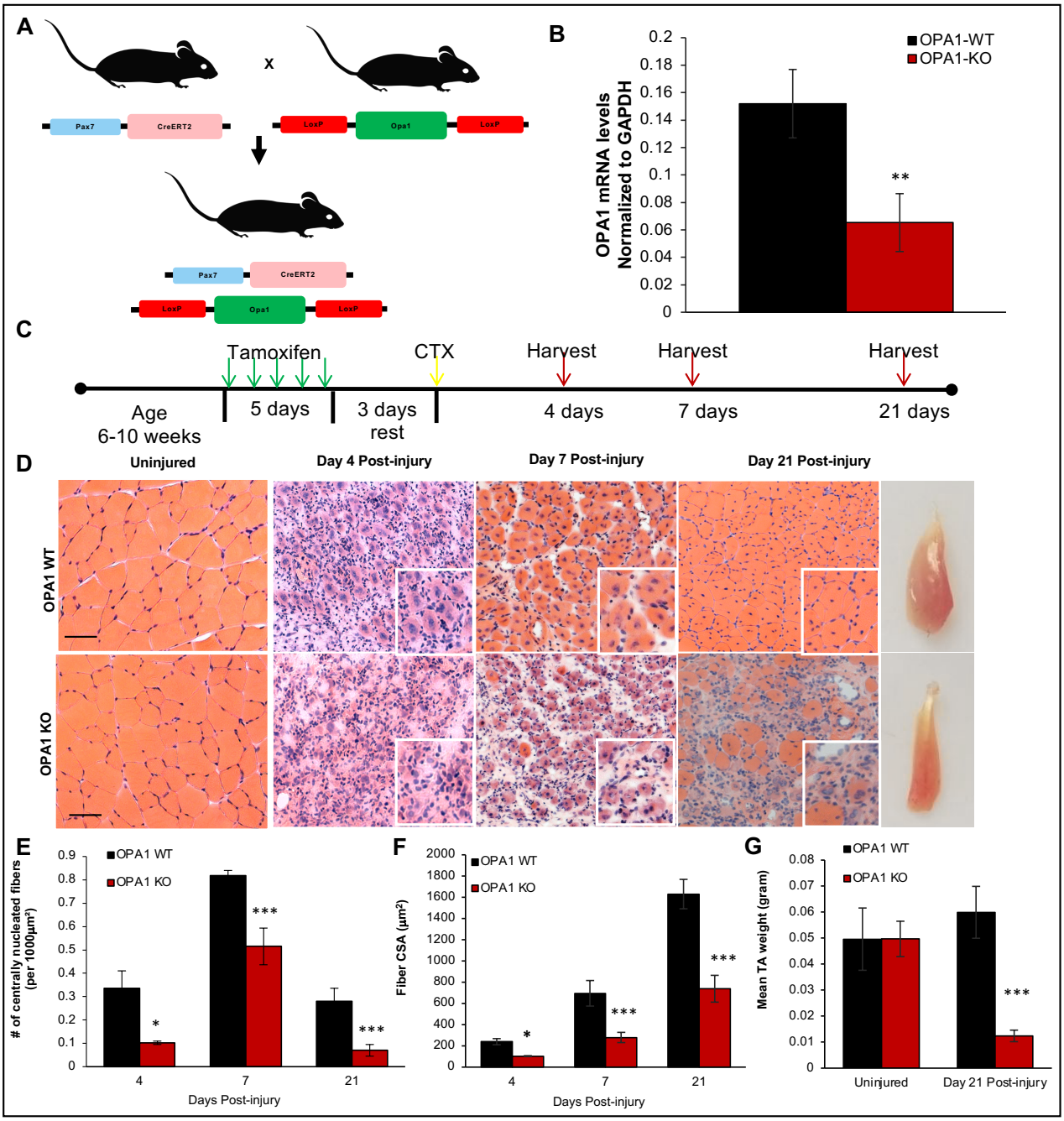

Figure 7. OPA1-KO Impairs Muscle Regeneration.

(A) Mouse Model

(B) OPA1 mRNA levels in OPA1-KO muscle stem cells compared to wild-type littermates. N=5 ± SD $p<0.001$ ***, students unpaired t-test.

(C) Injury Model Paradigm.

(D) Representative cross-sectional H&E Images of regenerating muscle at 4,7 and 21-days post-injury (DPI), and images of whole Tibialis Anterior (TA) muscles at 21 days post-injury.

(E) Quantification of the number of centrally nucleated fibers (CNF) at 4,7 and 21 DPI. (N=3) ± SD.

(F) Quantification of the cross-sectional area (CSA) of newly forming muscle fibers at 4,7 and 21 DPI. (N=3) ± SD

(G) Mass of whole TA muscles before injury and at 21 days post-injury (N=4) ± SD. Student's unpaired t-test, $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***

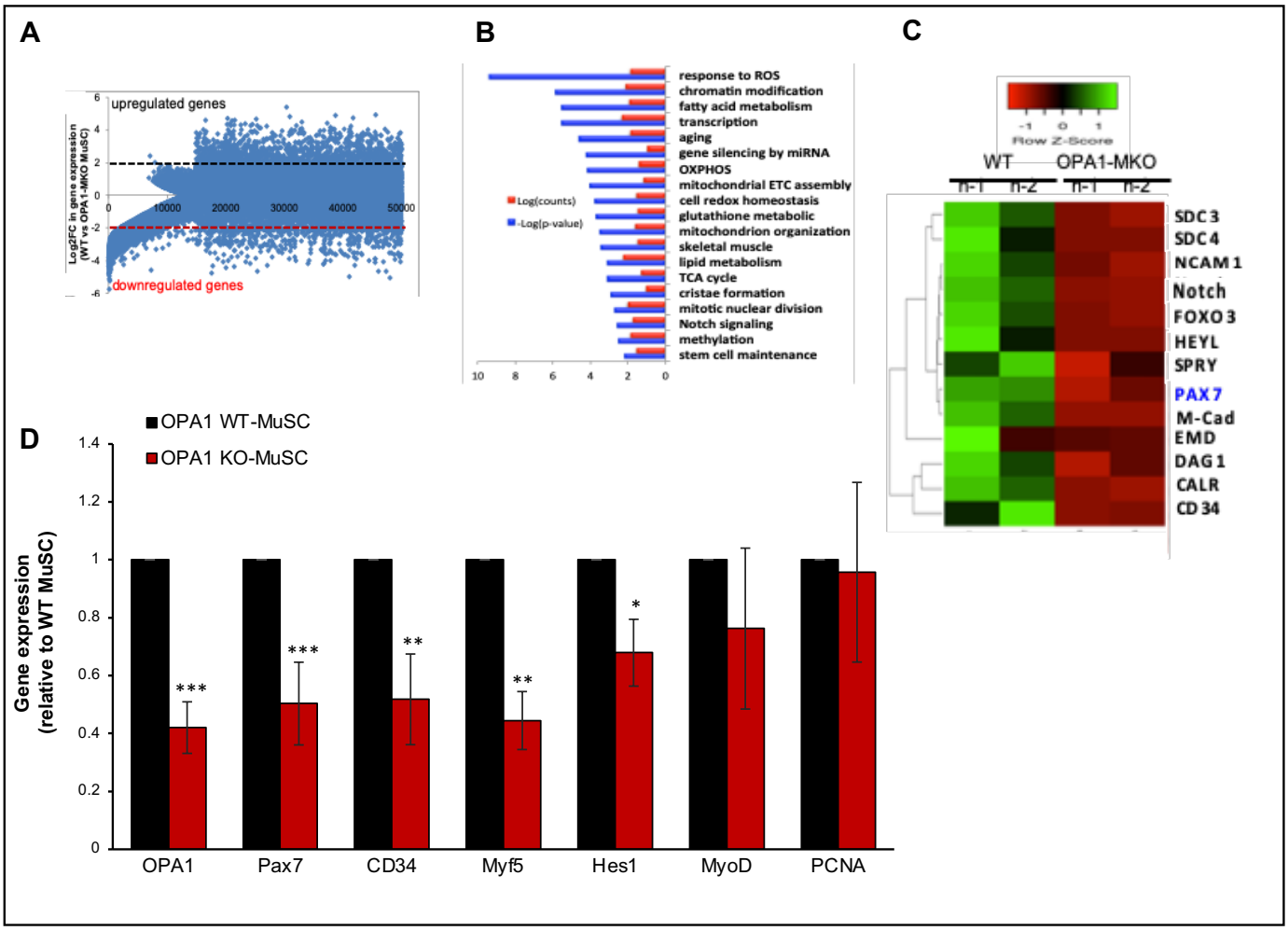

Figure 8. Gene Expression Profiling of OPA1-KO MuSCs.
(A) Distribution of gene expression from RNAseq data. N=2. Y-Axis represents fold-change of gene expression and X-Axis denotes the number of genes analysed by RNA-sequencing.
(B) Gene Ontology. N=2
(C) Heat-map depicting down-regulation of genes involved in quiescence and self-renewal in OPA1-KO MuSCs compared to wild-type littermates. N=2
(D) Validation of gene expression by RT-qPCR. N≥3 ± SD. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, student's t-test. (Baker and Khacho).
(Khacho 2018, unpublished)

Furthermore, gene expression profiling on MuSCs through RNA sequencing (Khacho 2018, unpublished) revealed a severe downregulation of many genes involved in MuSC self-renewal and quiescence (Figure 8A-C), specifically the MuSC quiescence markers Pax7 and CD34, which was further validated by qPCR (Figure 8D). Together, these data demonstrate that loss of Opa1 in the MuSC pool has a significant impact on the gene expression profile of MuSCs and severely impairs their ability to regenerate muscle tissue. Importantly, these data suggest that disruption of mitochondrial dynamics may play a significant role in muscle stem cell function. However, how the MuSC pool itself is affected during muscle regeneration, and how loss of Opa1 affects the function of MuSCs needs to be resolved.

2.2 Hypothesis

Impaired mitochondrial dynamics, by loss of the mitochondrial fusion protein Opa1 will disrupt muscle stem cell identity, fate and function, leading to impaired self-renewal and loss of the MuSC pool. Defects in MuSC fate and function in Opa1-knockouts may be mediated by changes in nuclear gene expression, reactive oxygen species production and stem cell metabolism.

2.3 Aims

To understand how mitochondrial dynamics can impact muscle stem cell fate and function, there are three experimental aims:

1. Identify the role of Opa1 and mitochondrial dynamics in muscle stem cells during quiescence, activation and stem cell fate.
2. Determine mechanistically how mitochondrial dynamics and loss of Opa1 affects muscle stem cell metabolism, nuclear gene expression and signalling pathways.
3. Understand the physiological role of Opa1 in muscle stem cells during aging.

Chapter 3: Materials and Methods

3.1 Mouse Model

Opa1-Pax7 CreER^{T2} mice, previously developed in the lab, were used to study the effect of loss of Opa1 in Pax7+ adult muscle stem cells. Opa1$^{Flox/Flox}$ mice with LoxP sites next to exons 10 and 13 that were previously developed[198] were crossed with Pax7 CreER^{T2} mice[199]. Progeny of the OPA1-Pax7 CreER^{T2} mice were first genotyped using Opa1-flox primers, and Control-Cre primers to determine which mice to select for experimentation (Table 1). Mice that were Opa1-floxed and expressed the Cre-recombinase were used as Opa1 MuSC knockout (Opa1-KO) mice, and mice that were Opa1-floxed that did not possess the Cre-recombinase were used as wild-type littermate controls (Opa1-WT). At 7-9 weeks of age, all (wild-type and Opa1-KO) mice received tamoxifen (200mg/kg) (Sigma T5648, 50mg/mL dissolved in corn oil) administration via gavage for five consecutive days, followed by three days' rest before any subsequent procedures were carried out. A mixture of both male and female mice were used for all experimental procedures. Confirmation of Opa1 knockout in MuSCs was performed by RT-qPCR using primers specific to Opa1, and GAPDH, B-Tubulin and B-Actin as housekeeping control genes (Table 1).

3.2 Cardiotoxin Preparation and Injections

Cardiotoxin (Latoxan L8102) was prepared by dissolving in saline to a final stock concentration of 100 μM and frozen in 100 μL aliquots at -20^{o}C[200]. At time of cardiotoxin injection, mice were injected subcutaneously with Buprenorphine and anesthetized by gas inhalation 30 minutes prior to cardiotoxin injection. 50 μL of 10 μM cardiotoxin was injected into the desired tibialis anterior (TA) muscle using an insulin syringe. All procedures were approved by the ACVS animal care committee at the University of Ottawa and complied with the guidelines

of the Canadian Council on Animal Care and the Animals for Research Act. Following cardiotoxin injection, mice were left to recover in a cage with a heating pad and were observed 1 day following cardiotoxin injection. The mice were left for the desired number of days for regeneration to occur before harvesting.

3.3 Muscle Tissue Harvest for Histology

At the time of harvest, the tibialis anterior (TA) muscle was dissected following cervical dislocation and immediately dropped in freshy prepared cold 2% (w/v) PFA (Sigma P6148) and fixed for 30 minutes, shaking on ice. Following fixation, TA muscles were washed with PBS and glycine twice for 10 minutes, then treated with 5% (w/v) sucrose for 2 hours, followed by 20% sucrose (w/v) for 2-3 days to allow for cryopreservation of the tissue. TA tissue was frozen in isopentane cooled using liquid nitrogen for 30 seconds and immediately stored at -80^{o}C for sectioning and subsequent staining procedures.

3.4 Muscle Stem Cell Isolation

Magnetic Activated Cell Sorting (MACS)

Muscle stem cells isolated using MACS isolation was carried out using an isolation kit purchased from Milteny Biotech (Porpiglia, E. & Blau, H.M. 2016). Following cervical dislocation, the tibialis anterior, extensor digitorum longus, gastrocnemius, soleus and quadriceps from the mouse hindlimb was harvested, weighed and minced, and 5mL of digestion enzyme containing 1% (w/v) collagenase B (Roche 11088831001) and 0.4% (w/v) dispase II (Roche 04942078001) was added. The muscle tissue and enzyme mixture was placed in a C-Tube and subjected to the SLICE_FACS dissociation program on the Milteny MACS Octo-Dissociator for 27 minutes. The muscle slurry was then run through a 70 μm filter (Milteny 130-110-916) and

cells were spun down at 600g for 10minutes. The resulting pellet was then treated with 400 μL of red blood cell lysis buffer (Sigma R7757) for 30 seconds, followed by addition of 10mL PBS and centrifugation at 600g for 5 minutes. Muscle stem cells were negatively selected for using the Satellite Stem Cell Isolation Kit (Milteny 130-104-268), followed by an Anti-Alpha Integrin-7 muscle stem cell selection (Milteny 130-104-261) to obtain a population of pure muscle stem cells.

Fluorescence Activated Cell Sorting (FACS)

Muscle stem cells were isolated by FACS isolation using a published protocol[201]. Briefly, subsequent to cervical dislocation, muscle tissue from the hindlimb was harvested and digested in 1% (w/v) Collagenase-B (Roche 11088831001) and 0.4% (w/v) Dispase II (Roche 04942078001) using the Milteny MACS Octo-dissociators SLICE_FACS program for 27 minutes. Following digestion muscle slurry was filtered and spun to obtain a cell pellet, which was then subjected to red blood cell lysis using 400 μL of red blood cell lysis buffer (Sigma R7757) for 30 seconds, followed by a wash with 10 mL of PBS and subsequent spin at 600g for 5 minutes. The cell pellet was then resuspended in 1 mL of FACS buffer (3mM EDTA, 10% (v/v) FBS in 1xPBS) and PE-conjugated antibodies were added, including the Lin- antibodies: Sca-1 (BD Pharmingen, 553108), CD45 (BD Pharmingen, 553081), CD31 (BD Pharmingen, 553373) and CD11b (eBiosciences, 12-0112-82) to remove non-muscle stem cells present in the population, and α-integrin-7 (Ablab, 67-0010-05) and VCAM (Biolegend, 105720) antibodies to positively select for muscle stem cells (Table 2). Samples were taken to the FACS sorter at the Flow Cytometry facility at the Ottawa Hospital Research Institute.

3.5 Single Myofiber Isolation

To isolate single myofibers, the extensor digitorum longus (EDL) muscle of each leg was harvested immediately following cervical dislocation and digested in 0.5% (w/v) collagenase B (Roche 11088815001) for 40 minutes at 37°C. The EDL muscle was then triturated in wash media (Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose and 1% (v/v) penicillin-streptomycin) to release single myofibers. Single myofibers were then washed repeatedly in wash media to remove excess debris attached to the myofibers. Myofibers were allowed to recover for 45 minutes before either being fixed immediately in 2% (w/v) PFA, warmed to 37°C for 10 minutes or placed in single myofiber culture media (DMEM 4.5 g/L glucose, 20% (v/v) FBS, 1% (v/v) chicken embryo extract, 1% (v/v) penicillin-streptomycin, 7.5ng/mL bFGF). Cultured fibers were incubated in a culture incubator at 37°C, 5% CO_2 from four hours to 96 hours, depending on the experimental design[202,203].

Treatment of single myofibers in culture

Single myofibers were treated with drugs or the required vehicle control for various timepoints ranging from four hours to 96 hours. Drug concentrations and information are indicated in Table 3.

3.6 Histology

Tissue Sectioning

Tissue was sectioned in a cross-sectional orientation at a thickness of 14 μm using the HM525NX Cryostat (University of Ottawa Histology Core) at -28°C and placed on a charged slide (Fisher Scientific 12-550-15). Slides were stored at -80°C until further processing.

Hematoxylin & Eosin Staining

Previously fixed and frozen muscle sections were stained for hematoxylin and eosin (H&E) by the Histology Core at the University of Ottawa. Stained tissue was imaged on the EVOS FLAuto2 at the Cell Biology Image Acquisition Core at the University of Ottawa.

Immunofluorescence Staining

Tissue Sections

Tissue was subjected to antigen retrieval in citrate buffer at pH=6.0 by the University of Ottawa Histology Core, followed by 1 hour blocking in 3% (w/v) BSA and primary antibody incubation overnight at 4^{o}C (Table 2). Secondary antibody staining was performed using a Biotin-Strepdavidin interaction (Biotin: Jackson Immunoresearch, 115-065-205, Strepdavidin: Jackson Immunoresearch, 016-160-084) and/or fluorescence-conjugated secondary antibody incubation (See Table 2 for antibody information). Slides were mounted using Immumount (ThermoFisher 9990402). Stained tissue sections were imaged on an Epifluorescent microscope (Zeiss AxioObserver.Z1) at the Cell Biology Image Acquisition Core at the University of Ottawa.

Single Myofibers

Single myofibers were permeabilized in 0.1% (v/v) triton and 100 mM glycine, followed by blocking in 5% (v/v) horse serum, 2% (w/v) BSA and 0.1% (v/v) triton in PBS for 5 hours at room temperature. Primary antibody was then added to myofibers and incubated overnight at 4^{o}C (Antibody concentration indicated in Table 2). Secondary antibody was added for 1 hour at room temperature, and then fibers were mounted using Immumount (ThermoFisher, 9990402) on a charged glass slide (Fisher Scientific, 12-550-15). Myofibers stained for Tom20 (mitochondrial length measurements) were imaged on the LSM880 Airyscan Confocal Microscope, and all other staining was imaged on the Zeiss AxioObserver.Z1 at the Cell Biology Image Acquisition Core at the University of Ottawa.

3.7 Gene Expression Analyses

RNA Isolation

Total RNA was isolated from muscle stem cell pellets using the PicoPure RNA column-based isolation kit (ThermoFisher, KIT0214). Isolated RNA was immediately quantified using the nano-drop 2000 spectrophotometer, aliquoted and frozen at -80^{o}C for subsequent analyses.

Quantitative Real-Time PCR (RT-qPCR)

Muscle stem cell RNA was diluted to a concentration of 5 ng/μL and prepared for RT-qPCR using the Rotor-Gene SYBR RT-qPCR kit (Qiagen, 204174). Each sample was run in triplicate using the following thermal cycler programming: 55^{o}C for 10 minutes, 95^{o}C for 5 minutes, cycling of 95^{o}C for 5 seconds and 60^{o}C for 10 seconds for 40 repeat cycles, melting from 60-95^{o}C with a 1^{o}C rise each step with 90 seconds of pre-melt conditioning on the first step and 5 seconds for each subsequent step. Every sample was run with a housekeeping gene (GAPDH, B-Actin or B-Tubulin) each run for normalization purposes (Table 1). Each gene was fitted to the appropriate standard curve and subsequently analysed.

3.8 ATP Assays

Muscle stem cells were seeded in a 96 well plate and treated with or without 20 μM oligomycin for 30 minutes to block the activity of ATP synthase and thus mitochondrial oxidative phosphorylation (to observe ATP production from glycolysis only). ATP levels were then detected using the CellTiterGlo cell viability assay (Promega G9241), a luciferase-based assay to measure the number of ATP molecules in each well. ATP concentrations were retrieved based on a standard

curve analysis. Cell counts and viability were performed to allow for normalization of data using a hematocytometer and Trypan Blue to identify any dead cells.

3.9 EdU Preparation and Injections

Edu (Baseclick EdU, BCN-001-500) was prepared at a concentration of 5 mg/mL and dissolved in saline with a combination of agitation and heating at 37^{o}C. EdU was then frozen at -80^{o}C in aliquots. EdU was injected through intraperitoneal injection at a concentration of 50 mg/kg 12 hours prior to harvesting muscle tissue.

3.10 Statistical Analyses

Statistical tests were determined and computed with Excel (Student's paired and unpaired t-tests) or Prism GraphPad (one-way, two-way or repeated measure ANOVAs with respective Post-HOC analyses). All statistics are presented with the following significance: $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***.

Primer Name	Sequence (5'-3')		Method
	Forward	Reverse	
OPA1-Flox	TTAAGACACCCCAAGAGCTTGC	CCAGCTTAGATCCCATTTGTTGACAG	Genotyping
Control	TTACGTCCATCGTGGACAGC	TGGGCTGGGTGTTAGCCTTA	Genotyping
Cre	GAACCTGATGGACATGTTCAGG	AGTGCGTTCGAACGCTAGAGCCTGT	Genotyping
Pax7GFP R10	GAATTCCCCGGGGAGTCGCATCCTGCGG		Genotyping
Sv40pA F3	CCACACCTCCCCCTGAACCTGAAACATAAA		Genotyping
Opa1	CGACTTTGCCGAGGATAGCTT	CGTTGTGAACACACTGCTCTTG	RT-qPCR
Mfn1	CCTACTGCTCCTTCTAACCCA	AGGGACGCCAATCCTGTGA	RT-qPCR
Mfn2	CTGGGGACCGGATCTTCTTC	CTGCCTCTCGAAATTCTGAAACT	RT-qPCR
Drp1	TTACGGTTCCCTAAACTTCACG	GTCACGGGCAACCTTTTACGA	RT-qPCR
Pax7	GACGACGAGGAAGGAGACAA	ACATCTGAGCCCTCATCCAG	RT-qPCR
CD34	CGCAGTTGGAGCCCTACAG	CCTCCACCATTCTCCGTGTAAT	RT-qPCR
Hes1	GCCAATTTCTCATC	GAGAGGTGGGCTGGGACTT	RT-qPCR
Myf5	CCTGTCTGGTCCCGAAAGAAC	GACGTGATCCGATCCACAATG	RT-qPCR
MyoD	TACAGTGGCGACTCAGATGC	CTGGGTTCCCTGTTCTGTGT	RT-qPCR
MyoG	CAGTGAATGCAACTCCCACA	ACCCAGCCTGACAGACAATC	RT-qPCR
SLC7A11	GTCTGCCTGTGGAGTACTGT	ATTACGAGCAGTTCCACCCA	RT-qPCR
GSS	CAAAGCAGGCCATAGACAGGG	AAAAGCGTGAATGGGGCATAC	RT-qPCR
GCLC	GGGGTGACGAGGTGGAGTA	GTTGGGGTTTGTCCTCTCCC	RT-qPCR
PCNA	TTTGAGGCACGCCTGATCC	GGAGACGTGAGACGAGTCCAT	RT-qPCR
GAPDH	TCGGTGTGAACGGATTTG	GGTCTCGCTCCTGGAAGA	RT-qPCR
B-Tubulin	GCACAATGGACTCAGTCAGG	CCCTTTGCCCAGTTATTTCC	RT-qPCR
B-Actin	AAATCGTGCGTGACATCAAA	AAGGAAGGCTGGAAAAGAGC	RT-qPCR

Table 1. Primers and sequences for genotyping and RT-qPCR.

Antibody Name	Company/Cat no.	Dilution	Assay
PE rat anti-mouse CD31	BD Pharmingen 553373	2 μL/mouse	FACS
PE rat anti-mouse CD45	BD Pharmingen 553081	2 μL/mouse	FACS
PE rat anti-mouse CD11b	eBiosciences 12-0112-82	2 μL/mouse	FACS
PE rat anti-mouse Sca1	BD Pharmingen 553108	2 μL/mouse	FACS
PE-Cy7 conjugated CD106 (VCAM)	Biolegend 105720	10 μL/mouse	FACS
647 conjugated alpha integrin-7 (rat)	Ablab 67-0010-05	10 μL/mouse	FACS
Pax7 (mouse)	DHSB PAX7-S	1:13	IF Primary
Pax7 (rabbit)	Invitrogen PA1-117	1:50	IF Primary
Tom20 (rabbit)	ProteinTech 11802-1-AP	1:500	IF Primary
MyoD (mouse)	SantaCruz sc-32758	1:100	IF Primary
MyoG (mouse)	DHSB FD5	1:100	IF Primary
Laminin (rabbit)	Abcam AB-11575	1:1000	IF Primary
Ki67 (rabbit)	Abcam AB-15580	1:1000	IF Primary
eMHC (mouse)	DHSB F1.652-s	1:15	IF Primary
GFP (chicken)	Abcam AB-13970	1:1000	IF Primary
Alexa-Fluoro 488 Rabbit	Invitrogen A11008	*1:500-1:1000	IF Secondary
Alexa-Fluoro 488 Mouse	Invitrogen A11001	*1:500-1:1000	IF Secondary
Alexa-Fluoro 488 Chicken	Jackson 703-545-155	*1:500-1:1000	IF Secondary
Alexa-Fluoro 647 Rabbit	Abcam AB-150075	*1:500-1:1000	IF Secondary
Alexa-Fluoro 647 Mouse	Abcam AB-150107	*1:500-1:1000	IF Secondary
Alexa-Fluoro 594 (Rabbit or Mouse)	Invitrogen A11005	*1:500-1:1000	IF Secondary
Cy3 Rabbit	Jackson 711-165-152	1:1000	IF Secondary
Cy3 Mouse	Jackson 715-165-150	1:1000	IF Secondary
Biotin	Jackson 115-065-205	1:250	IF Secondary
Cy3-Conjugated Strepdavidin	Jackson 016-160-084	1:250	IF Secondary

Table 2. Antibodies used for fluorescence activated cell sorting (FACS) and Immunofluorescence (IF).

***** 1:500 concentration is used for tissue immunofluorescence staining and 1:1000 is used for single myofiber immunofluorescent staining.

Drug	Company/Cat no.	Concentration
Mdivi-1	Sigma M0199	2 μM
MitoTEMPO	Sigma SML0737	10 μM
L-Buthionine Sulphoximine (BSO)	Sigma B2515	10 μM- 50 μM
Rotenone	Sigma R8875	10 nM-1 μM
L-Glutathione (GSH)	Sigma G4251	1 mM
L-Cysteine	Sigma 5360	5 mM
Glycine	Bio-Rad 1610717	5 mM
Antimycin A	Sigma A8674-50MG	50 μM
Oligomycin	Sigma O4876-25MG	5 μM
Rapamycin	Invivogen (tlrl-rap)	20 nM
Torin-1	Invivogen (inh-tor1)	10 nM
Recombinant Mouse HGF-A	R&D Systems 1200-SE	40 ng/mL
Recombinant Human HGF Protein (Active form)	Abcam Ab632	10 ng/mL

Table 3. Drugs and concentrations used for *in vitro* single myofiber cultures.

Chapter 4: Results

4.1 Characterizing the Phenotype of Opa1-KO Muscle Stem Cells

As previously demonstrated in our lab, loss of Opa1 critically impairs muscle regenerative potential, and alters gene expression of muscle stem cells. To further understand how Opa1 impacts muscle tissue and MuSC function, we first set out to establish characteristics of Opa1-KO MuSCs and muscle tissue under basal (uninjured) conditions. To further validate Opa1-KO, we performed immunostaining for the OMM protein TOM20. Analysis of mitochondrial length in Opa1-KO MuSCs revealed highly fragmented mitochondria compared with the mitochondria of wild-type littermates (Figure 8A-B). We then measured ATP levels using a luciferase-based ATP assay and observed no detectable reduction in total ATP levels, or any difference in the source of ATP from glycolysis or OXPHOS, establishing that acute (short-term) loss of Opa1 disrupts mitochondrial fusion, but does not show a detectable decline in mitochondrial ATP generation (Figure 8C-D). Given that Opa1-KO mice cannot undergo muscle regeneration, leading to a decline in muscle fiber size and number, we looked at the number of fibers and fiber size under basal Opa1-KO conditions and observed no detectable difference in muscle fiber numbers or cross-sectional area (CSA) between wild-type and Opa1-KO mice (Figure 8E-F). Furthermore, the number of Pax7+ MuSCs under basal conditions in Opa1-KO mice were consistent with wild-type counterparts (Figure 8G-H), thus confirming that the defects observed in Opa1-KO muscle tissue only occur upon muscle injury.

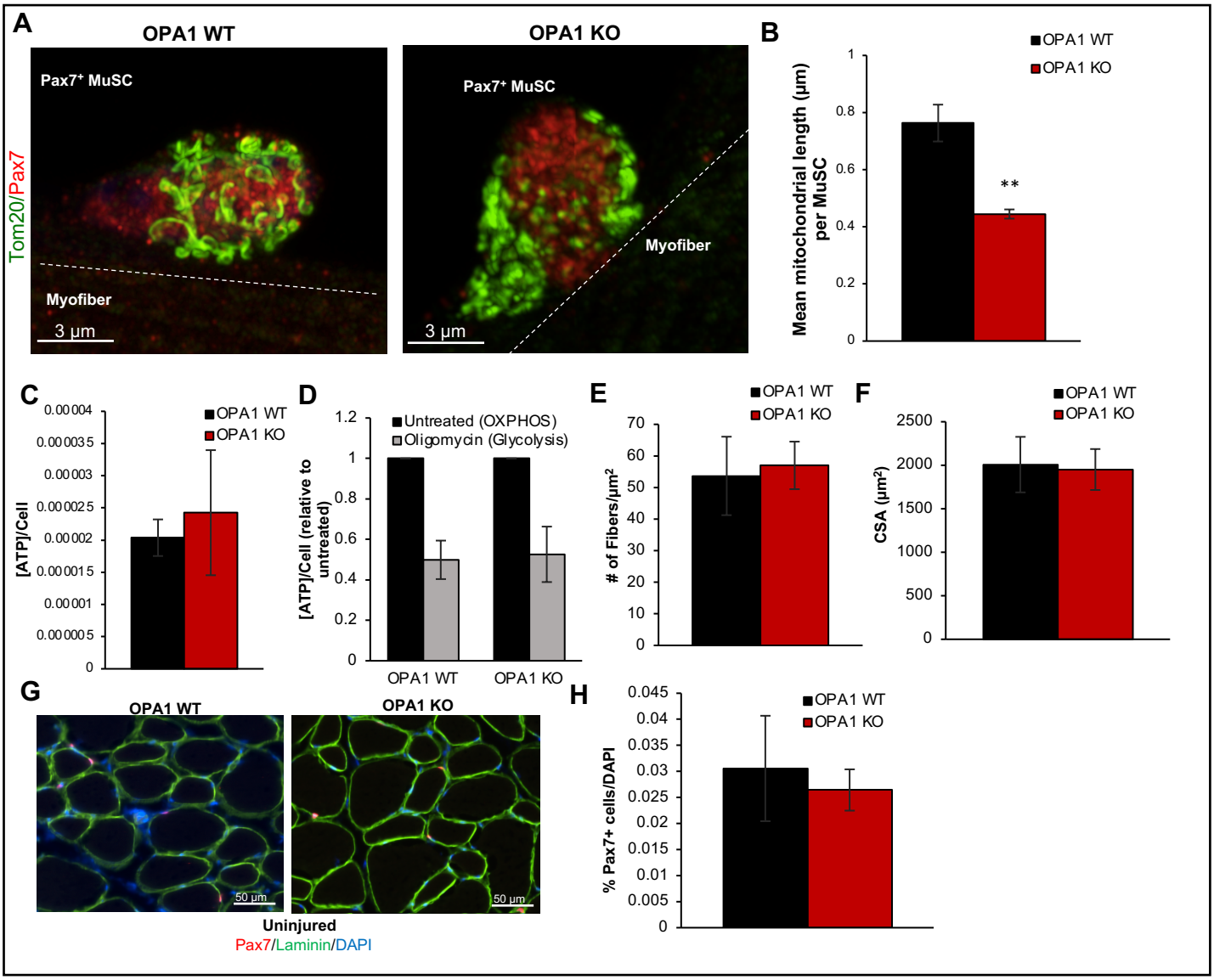

Figure 9. Characteristics of OPA1-KO MuSCs.

(A) Representative Images of OPA1-KO mitochondria (stained with Tom20) in MuSCs (stained with Pax7).

(B) Quantification of mitochondrial length in OPA1-KO muscle stem cells and wild-type counterparts. N=10 ± SE $p<0.01$ ** students unpaired t-test.

(C) Total ATP/cell in acute basal OPA1-KO MuSCs. N=3

(D) ATP source in acute basal OPA1-KO MuSCs. N=3

(E) Quantification of the number of pre-existing fibers in OPA1-KO mice compared to wildtype littermates. N=4

(F) Measurement of the cross-sectional area (CSA) of pre-existing fibers in OPA1-KO mice compared to wildtype littermates. N=4.

(G) Representative cross-sectional images of muscle stem cells (stained with Pax7) in OPA1-KO mice without injury.

(H) Quantification of Pax7+ MuSCs in OPA1-KO mice under basal conditions. N=4 ± SD.

4.2 Loss of Opa1 impairs Muscle Stem Cell Fate Decisions

Next, we examined the effect of Opa1 ablation on MuSC fate decisions. Once activated, MuSCs must then decide whether to self-renew and contribute to the stem cell pool or commit to a myogenic lineage and continue to repair injured tissue[1,2,4]. To evaluate whether disruption of dynamics affects MuSC fate decisions, we used the *in vivo* injury model previously described (Figure 7C). Immunofluorescent staining for the MuSC marker Pax7 revealed that Opa1-KO mice have a significant and consistent reduction in MuSCs as early as 4DPI, ultimately leading to an almost complete depletion in the MuSC pool by 21DPI, thus indicating a defect in the self-renewal capacity of Opa1-KO MuSCs (Figure 10A-B). Given that we observed MuSC depletion in Opa1-KO mice by 4DPI, we then performed immunofluorescent staining for MyoD and MyoG, which are commitment and differentiation markers, respectively. This quantification revealed an increase in both MyoD+ and MyoG+ cells in Opa1-KO mice at 4DPI, indicating a premature drive towards commitment (Figure 10D, E, G). One possibility for variation in the number of muscle stem cells and their committed progeny in Opa1-KO mice is a difference in proliferation. To exclude a discrepancy in proliferation as a possibility for differences in cell populations in Opa1-KO mice, we examined proliferation in Pax7, MyoD or MyoG expressing cells by staining for the proliferation marker Ki67. In all three cell populations, no difference in proliferation was observed between Opa1-WT and Opa1-KO mice, indicating that the shift in cell population is most likely a result of altered cell fate decisions *in vivo* (Figure 10C, F, H).

We next sought to examine stem cell fate using our *in vitro* system, where extensor digitorum longus (EDL) muscle fibers are isolated and cultured. Muscle stem cells, which reside on the periphery of the myofibers are activated upon isolation and can be cultured for various times

to examine activation, proliferation, stem cell fate, and early differentiation[203]. To observe stem cell fate and early differentiation in Opa1-KO MuSCs, we cultured single myofibers for 72 hours. Immunofluorescent staining for Pax7, MyoD and MyoG revealed that in clusters of Opa1-KO MuSCs, there was a decrease in Pax7+cells with a concomitant increase in MyoD+ and MyoG+ cells when compared to MuSCs on single myofibers from wild-type littermates (Figure 10I,J,K). We next treated Opa1-KO MuSCs on single myofibers with low-dose (2 μM) Mdivi-1, a putative DRP1 inhibitor, to determine if prohibiting further fragmentation could rescue self-renewal. Interestingly, Mdivi-1 was able to reduce the number of MyoD+ cells and increase the number of Pax7+ cells to comparable levels with Opa1-WT MuSCs (Figure 10L,M). Thus far, our data has demonstrated that acute loss of Opa1 skews MuSC fate decisions, favouring myogenic commitment over self-renewal while inhibition of further mitochondrial fragmentation with Mdivi-1 is able to rescue self-renewal. However, mechanistically how disruption of mitochondrial dynamics and loss of Opa1 can manipulate MuSC fate remains to be resolved.

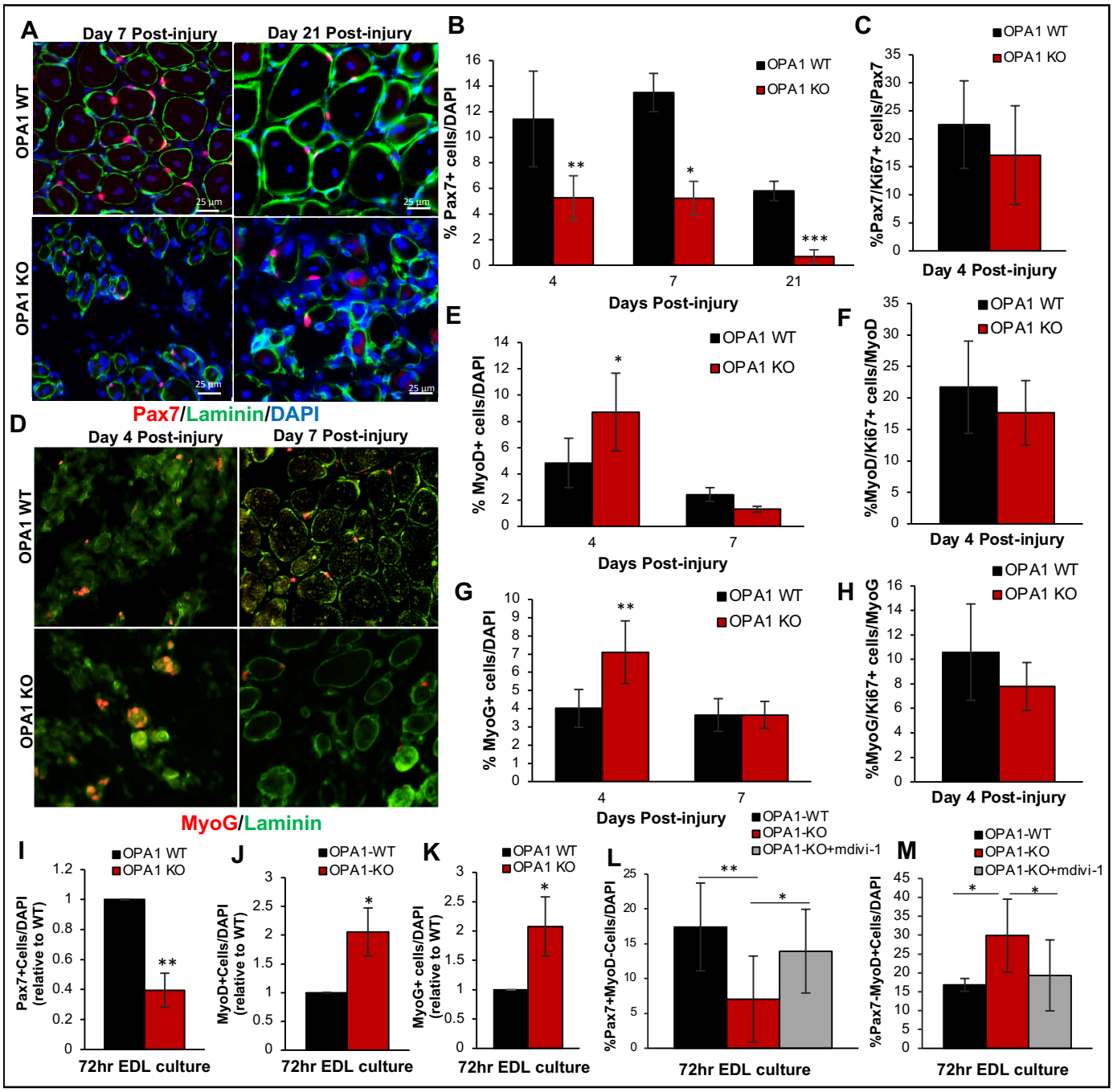
A
Day 7 Post-injury
Day 21 Post-injury
OPA1 WT
OPA1 KO
25 µm
Pax7/Laminin/DAPI
B
% Pax7+ cells/DAPI
OPA1 WT
OPA1 KO
Days Post-injury
C
%Pax7/Ki67+ cells/Pax7
Day 4 Post-injury
D
Day 4 Post-injury
Day 7 Post-injury
MyoG/Laminin
E
% MyoD+ cells/DAPI
F
%MyoD/Ki67+ cells/MyoD
G
% MyoG+ cells/DAPI
H
%MyoG/Ki67+ cells/MyoG
I
Pax7+Cells/DAPI (relative to WT)
72hr EDL culture
J
MyoD+Cells/DAPI (relative to WT)
K
MyoG+ cells/DAPI (relative to WT)
L
OPA1-WT
OPA1-KO
OPA1-KO+mdivi-1
%Pax7+MyoD-Cells/DAPI
M
%Pax7-MyoD+Cells/DAPI

Figure 10. Loss of Opa1 impairs MuSC fate decisions.

(A) Representative images of regenerating muscle at 7 and 21DPI showing Pax7+ muscle stem cells.

(B) Quantification of Pax7+ muscle stem cells at 4,7 and 21DPI. N=3 ± SD, $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** students unpaired t-test.

(C) Quantification of Pax7+Ki67+ muscle stem cells at 4DPI. N=3 ± SD.

(D) Representative images of regenerating muscle at 4 and 7DPI showing MyoG+ committed cells.

(E) Quantification of MyoD+ committing cells at 4 and 7DPI normalized to total DAPI+ cells. N=3 ± SD $p<0.05$ *, student's unpaired t-test.

(F) Total number of MyoD+Ki67+ cells in Opa1-WT and -KO muscle at 4DPI. N=3 ± SD.

(G) Quantification of MyoG+ committed cells at 4 and 7DPI in OPA1-KO muscle compared with wild-type counterparts. N=3 ± SD $p<0.01$ **, student's unpaired t-test.

(H) Total number of MyoG+Ki67+ cells in Opa1-KO muscle at 4DPI. N=3 ± SD.

(I) Quantification of Pax7+ muscle stem cells *in vitro* single myofibers at 72 hours in culture in Opa1-KO muscle relative to wild-type counterparts. N=3 ± SD, $p<0.01$ **, student's unpaired t-test.

(J) Quantification of MyoD+ committing cells *in vitro* single myofibers at 72 hours in culture in Opa1-KO muscle relative to wild-type counterparts. N=3 ± SD, $p<0.05$ *, student's unpaired t-test.

(K) Quantification of MyoG+ committed cells *in vitro* single myofibers at 72 hours in culture in Opa1-KO muscle relative to wild-type counterparts. N=3 ± SD, $p<0.05$ *, student's unpaired t-test.

(L) Quantification of Pax7+ muscle stem cells of Opa1-WT and Opa1-KO single myofibers treated with 2 μM mdivi-1 and cultured for 72hours. N=6 ± SD, $p<0.05$ *, $p<0.01$ **, student's unpaired t-test.

(M) Quantification of MyoD+ committed cells of OPA1-WT and Opa1-KO single myofibers treated with 2 μM Mdivi-1 and cultured for 72hours. N=6 ± SD, $p<0.05$ *, student's unpaired t-test.

4.3 Mitochondrial Structure in Muscle Stem Cells during Adult Myogenesis

To understand the potential role of mitochondrial dynamics on MuSC fate, we first wanted to examine mitochondrial structure under physiological conditions during MuSC activation and fate decisions. Thus, to observe mitochondrial length during the aforementioned stages of adult myogenesis, we cultured single myofibers for 0,4,12,24,48 and 72 hours. To visualize mitochondria, single myofibers were stained for the MuSC marker Pax7 and the OMM protein TOM20 and imaged using Airyscan Confocal microscopy. Interestingly, we observed a striking level of mitochondrial fragmentation that occurred at just 4 hours in culture at the onset of activation that persisted to 24 hours in culture. The observed mitochondrial fragmentation was coincident with a decrease in Opa1 gene expression in MuSCs activated following an injury (Figure 11A-C)[28,30]. Furthermore, fragmentation preceded the expression of Ki67, a marker of proliferation which occurs after 24 hours in culture (Figure 11E-F). It is well understood that mitochondrial fragmentation is required to segregate mitochondrial content into daughter cells during cell division[105,180,204]. However, mitochondrial fragmentation during MuSC activation largely precedes MuSC proliferation, potentially indicating a role for mitochondrial fragmentation in MuSC activation other than distribution of mitochondria during stem cell division.

Furthermore, following the observed fragmentation early in MuSCs, mitochondria began to re-elongate as MuSCs reached 48 hours in culture. Interestingly, at 48hours we noticed a difference in mitochondrial length in different cell populations. Cells with high expression of Pax7 tended to have increased mitochondrial length, while those with lower Pax7 expression had more fragmented mitochondria (Figure 11D). Finally, as MuSCs reached 72 hours in culture and began the process of early differentiation, mitochondrial length was substantially longer than

mitochondria in MuSCs at 24 or 48 hours in culture (Figure 11A-B). These results indicate a dynamic change in mitochondrial length as MuSCs undergo adult myogenesis *in vitro*. This data, taken together with our observations that loss of Opa1 impairs MuSC fate decisions demonstrate an important role for mitochondrial elongation in MuSC fate. However, as there is also a change in mitochondrial length from quiescence to activation, the significance of this remains to be resolved.

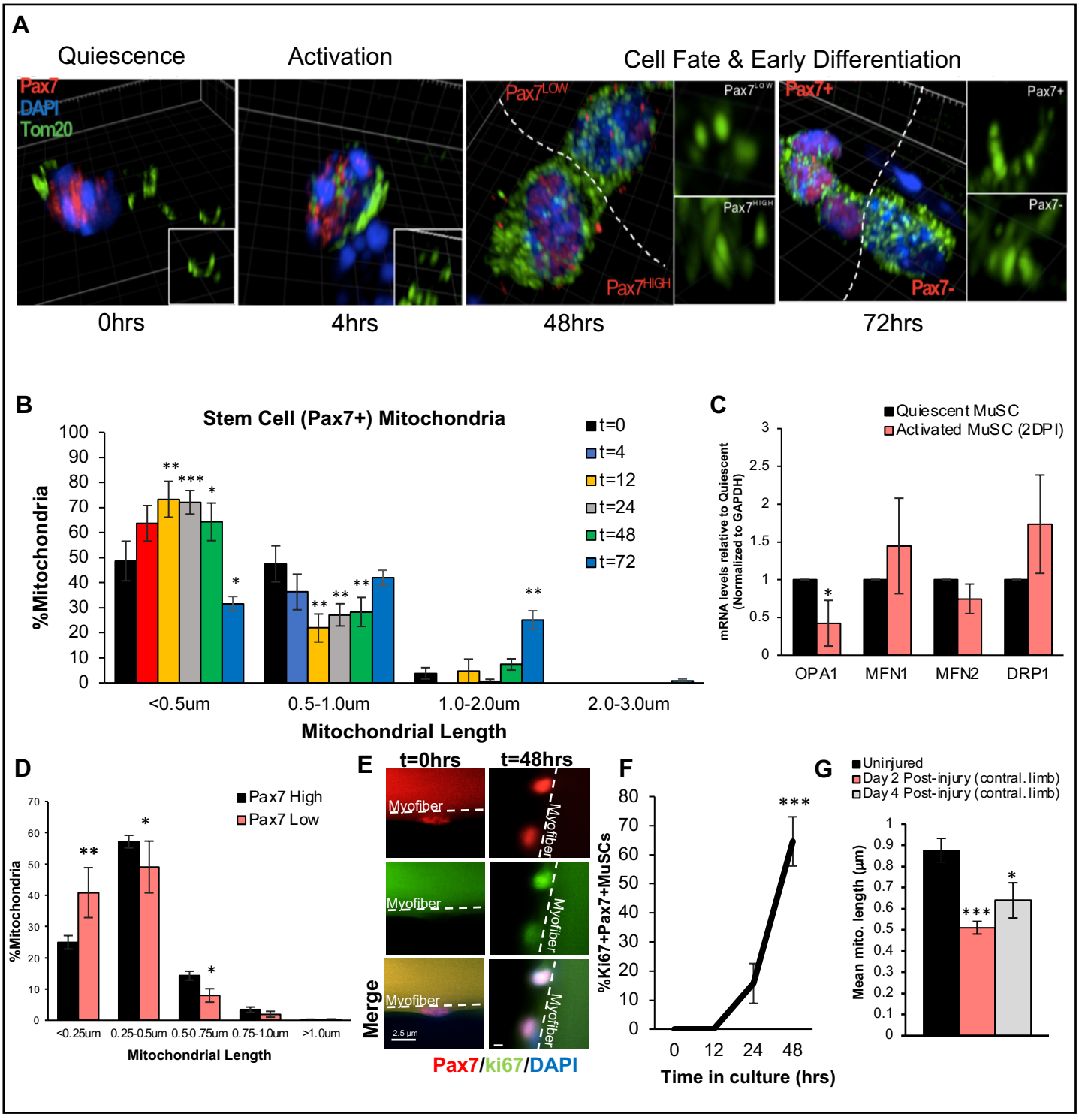
A
Quiescence
Activation
Cell Fate & Early Differentiation
Pax7
DAPI
Tom20
Pax7LOW
Pax7HIGH
Pax7+
Pax7-
0hrs
4hrs
48hrs
72hrs
B
Stem Cell (Pax7+) Mitochondria
%Mitochondria
t=0
t=4
t=12
t=24
t=48
t=72
<0.5um
0.5-1.0um
1.0-2.0um
2.0-3.0um
Mitochondrial Length
C
Quiescent MuSC
Activated MuSC (2DPI)
mRNA levels relative to Quiescent (Normalized to GAPDH)
OPA1
MFN1
MFN2
DRP1
D
Pax7 High
Pax7 Low
%Mitochondria
<0.25um
0.25-0.5um
0.5-0.75um
0.75-1.0um
>1.0um
Mitochondrial Length
E
t=0hrs
t=48hrs
Myofiber
Merge
Pax7/ki67/DAPI
F
%Ki67+Pax7+MuSCs
Time in culture (hrs)
G
Uninjured
Day 2 Post-injury (contral. limb)
Day 4 Post-injury (contral. limb)
Mean mito. length (µm)

Figure 11. Mitochondrial structure is highly dynamic in muscle stem cells during adult myogenesis.

(A) Representative images of mitochondrial structure (identified by TOM20 staining using confocal microscopy) in muscle stem cells (indicated by Pax7 staining) on single myofibers cultured for up to 72hours.

(B) Distribution of mitochondrial length manually measured in muscle stem cells at various time-points. N=7 ± SE, $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** two-way ANOVA with Neuman-Keuls post-hoc test.

(C) mRNA levels of mitochondrial dynamics genes in quiescent and activated (2DPI) muscle stem cells. N=3 ± SD, $p<0.05$, students unpaired t-test.

(D) Mitochondrial length bin in muscle stem cells cultured for 48 hours and classified as Pax7 high or low. N≥6 ± SE. Student's unpaired t-test $p<0.05$ *, $p<0.01$ **.

(E) Representative images of ki67+ muscle stem cells on single myofibers.

(F) Quantification of proliferating (Ki67+) muscle stem cells at various time-points in culture. N=3 ± SE $p<0.001$ *** Students unpaired t-test.

(G) Average mitochondrial length in muscle stem cells from completely uninjured mice, or mice with an injury in the contralateral limb at 2,4- and 21-days post-injury (DPI). N=10 ± SE, $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** one-way ANOVA with Tukey's post-hoc test.

4.4 Loss of Opa1 Reduces the Depth of Quiescence in Muscle Stem Cells

Recent hallmark studies have demonstrated that quiescence exists as a gradient, where MuSCs can remain in a deep-quiescent state, or adopt a shallow quiescence, termed "G-Alert"[28,57]. The "G-Alert" phenomenon was first described in MuSCs found in muscle that was contralateral to the muscle that received an injury and was undergoing the process of regeneration. The "G-Alert" MuSCs were characterized by increased cellular volume, increased ATP levels and a greater propensity to enter the cell cycle[57]. To determine if mitochondrial structure corresponded with the change in the depth of MuSC quiescence, we measured the length of mitochondria in MuSCs under uninjured conditions and in MuSCs contralateral to an injury at early stages of regeneration (2 and 4DPI). Remarkably, mitochondria from MuSCs contralateral to an injury early in regeneration had highly fragmented mitochondria when compared to MuSCs under basal conditions, further demonstrating a dynamic response in mitochondrial structure with a change in MuSC identity (Figure 11G). Given that loss of Opa1 leads to highly fragmented mitochondria, similar to that observed in wildtype MuSCs in a "G-Alert" state, and alters the expression of genes involved in quiescence, most notably Pax7, CD34 and Hes1, a downstream target of the Notch pathway, (Figure 8C-D) we hypothesized that loss of Opa1 causes defects in MuSC quiescence.

To evaluate the impact of Opa1 loss on MuSC quiescence and maintenance, we analysed the number of Pax7+ MuSCs in the contralateral limb at 4,7 and 21DPI and noticed a gradual decline in the number of MuSCs in Opa1-KO mice early in regeneration, leading to a depletion in the MuSC pool by the time regeneration was completed (Figure 12A). Furthermore, *in vitro* single myofiber cultures of MuSCs in the contralateral muscle at 21DPI revealed a decline in the number of Pax7+ cells, and an increase in MyoG+ committed cells (Figure 12B-C). However, as previously

established, acute loss of Opa1 in the absence of a stimulus does not lead to MuSC depletion (Figure 9G-H), which lead us to hypothesize that Opa1-KO MuSCs exist in a primed state of quiescence, but require a minor stimulus to drive them out of quiescence and cause MuSC depletion.

To investigate this idea, we then evaluated the incorporation of EdU into the contralateral limb of Opa1-KO mice at 2 and 4DPI. At both 2 and 4DPI, the number of Opa1-KO Pax7+ MuSCs that incorporated EdU were higher than wild-type counterparts (Figure 12D-F). Moreover, at 4DPI we observed an increase in EdU+ cells that were not Pax7+, inspiring us to examine the number of MyoD+EdU+ MuSCs, which were also elevated in the contralateral limb of Opa1-KO mice (Figure 12G-H). To further evaluate the quiescent state of Opa1-KO MuSCs, we examined early stages of activation by analysing *in vitro* cell cycle entry using single myofiber cultures. Interestingly, Opa1-KO MuSCs from contralateral fibers had a much greater quantity of Ki67+ cells early on compared to wild-type counterparts (Figure 12I). Finally, while analysing *in vitro* single myofibers, we noticed an interesting phenomenon in nuclear architecture. A study recently conducted demonstrated that the shape of the nucleus could give indications of the status of MuSC activation[34]. Thus, we classified nuclear morphology and observed that Opa1-KO MuSCs freshly isolated (t=0hrs in culture) from the contralateral limb had higher proportions of "small round" nuclei, which can be associated with early activation (Figure 12J-K), while Opa1-KO MuSCs cultured for 24 hours tended to have higher proportions of larger nuclei, an indicator of cell growth[205-208] (Figure 12J,L-N). These data demonstrate that loss of Opa1 reduces MuSC quiescence and leads to premature activation upon a stimulus.

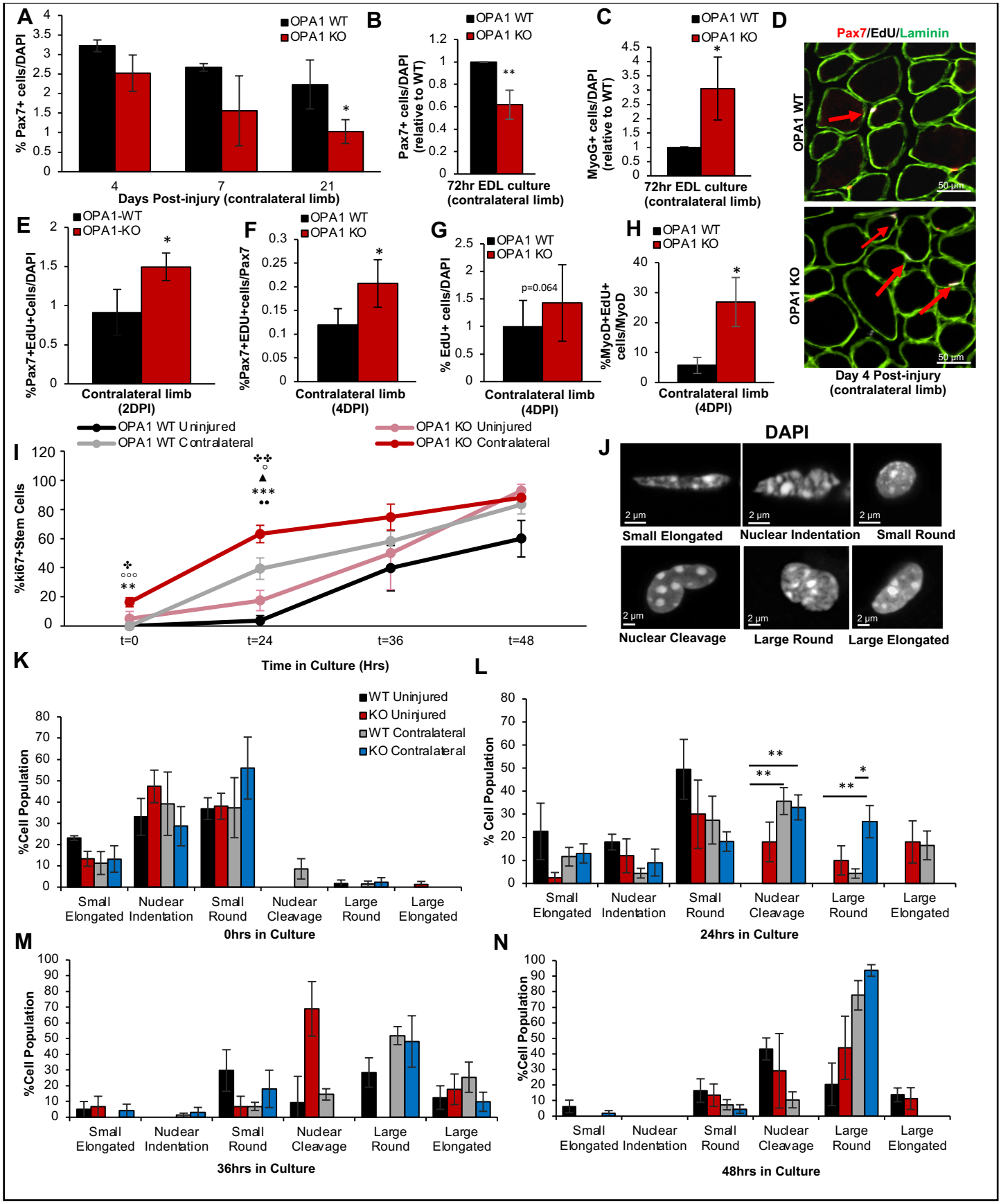
A
% Pax7+ cells/DAPI
OPA1 WT
OPA1 KO
Days Post-injury (contralateral limb)
B
Pax7+ cells/DAPI (relative to WT)
72hr EDL culture (contralateral limb)
C
MyoG+ cells/DAPI (relative to WT)
72hr EDL culture (contralateral limb)
D
Pax7/EdU/Laminin
OPA1 WT
OPA1 KO
50 µm
Day 4 Post-injury (contralateral limb)
E
%Pax7+EdU+Cells/DAPI
OPA1-WT
OPA1-KO
Contralateral limb (2DPI)
F
%Pax7+EDU+cells/Pax7
Contralateral limb (4DPI)
G
% EdU+ cells/DAPI
p=0.064
Contralateral limb (4DPI)
H
%MyoD+EdU+ cells/MyoD
Contralateral limb (4DPI)
I
OPA1 WT Uninjured
OPA1 WT Contralateral
OPA1 KO Uninjured
OPA1 KO Contralateral
%ki67+Stem Cells
t=0
t=24
t=36
t=48
Time in Culture (Hrs)
J
DAPI
2 µm
Small Elongated
Nuclear Indentation
Small Round
Nuclear Cleavage
Large Round
Large Elongated
K
WT Uninjured
KO Uninjured
WT Contralateral
KO Contralateral
%Cell Population
Small Elongated
Nuclear Indentation
Small Round
Nuclear Cleavage
Large Round
Large Elongated
0hrs in Culture
L
% Cell Population
24hrs in Culture
M
%Cell Population
36hrs in Culture
N
%Cell Population
48hrs in Culture

Figure 12. Loss of Opa1 reduces muscle stem cell quiescence.

(A) Quantification of Pax7+ cells in the contralateral limb at 4,7 and 21DPI. N=3 ± SD, $p<0.05$ students unpaired t-test.

(B) Quantification of Pax7+ muscle stem cells within a cluster on single myofibers in the contralateral limb 21DPI. N=3 ± SD, $p<0.01$ ** students unpaired t-test.

(C) Quantification of MyoG+ committed cells within a cluster on single myofibers in the contralateral limb 21DPI. N=3 ± SD, $p<0.01$ ** students unpaired t-test.

(D) Representative images of the contralateral limb immunofluorescent stained with Pax7 (red), laminin (green) and stained with EdU (white).

(E) Number of Pax7+&EdU+ Cells per total cells (DAPI) in the contralateral limb 2 days post-injury. $p<0.05$ *, student's unpaired t-test.

(F) Quantification of Pax7+&EdU+ MuSCs in the contralateral limb of Opa1-KO mice compared to wild-type littermates. N=6 ± SD, $p<0.05$ *, student's unpaired t-test.

(G) Quantification of total EdU+ cells in the contralateral limb of Opa1-KO mice compared with wild-type littermates. N=6 ± SD, $p<0.05$ *, student's unpaired t-test

(H) MyoD+&EdU+ activated MuSCs in the contralateral limb 4DPI. N=6 ± SD, $p<0.05$ * students unpaired t-test.

(I) Quantification of proliferating muscle stem cells on myofiber cultures at various-timepoints in uninjured and contralateral limbs of Opa1-KO mice and their wild-type littermates. N=7 ± SE. ● WT Uninjured vs. WT contralateral, * WT uninjured vs. KO Contralateral, ▲ WT Contralateral vs. KO uninjured, ◦ WT Contralateral vs. KO Contralateral, ✤ KO Uninjured vs. KO Contralateral. $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** two-way ANOVA with Neuman-Keuls post-hoc test.

(J) Representative images of muscle stem cell nuclear morphology.

(K-N) Quantification of the number of muscle stem cells exhibiting various nuclear morphologies at 0 (K), 24 (L), 36 (M), and 48 (N) hours in culture. N=7 ± SE, $p<0.05$ *, $p<0.01$ ** two-way ANOVA with Holm-Sidak post-hoc analysis.

4.5 Mitochondrial Structure is Altered by Systemic Factors and mTORC1

We were intrigued that as a distant injury can transition MuSCs into an alert state, this coincides with fragmentation of the mitochondria. Follow-up studies that demonstrated MuSCs could transition into a state of G-Alert also identified a systemic pathway that was responsible for the altered quiescent state of MuSCs distant to an injury[58,59,203]. It was discovered that systemic hepatocyte growth factor activator (HGFA), which is released upon injury could circulate and induce proteolytic cleavage of HGF to an active form, which would bind to the c-Met receptor on the surface of MuSCs and activate mTORC1 to allow the G-Alert transition[59]. Given that mitochondria are often sensors of external cues, we then hypothesized that mitochondria may sense systemic signals and undergo fragmentation to allow for the alteration of quiescence. To dissect out this pathway and understand if and where mitochondrial fragmentation is important, we treated MuSCs on single myofibers with either recombinant 40 ng/mL HGF-A, recombinant 10 ng/mL HGF in its active form, or mTOR inhibitors Rapamycin at 20 nM and Torin-1 at 10 nM. We first measured mitochondrial length in treated MuSCs that were cultured for four, 12 and 24 hours. Interestingly, treatment with HGF and HGFA induced a modest increase in fragmented mitochondria, while inhibition of mTOR prevented this fragmentation (Figure 13A-B). To validate if treatment affected proliferation, an indicator the “alert” state, we quantified the number of MuSCs that were Ki67+ at 24 and 36 hours in culture and observed that treatment with HGF and HGFA appeared to promote proliferation, while inhibition of mTOR with Rapamycin or Torin-1 curtailed proliferation (Figure 13C). Therefore, treatment with the systemic factor HGFA promotes mitochondrial fragmentation and enhances activation, while inhibition of mTOR, a key pathway for the “G-Alert” transition, stops mitochondrial fragmentation and reduces cell cycle entry.

Given that inhibition of mTOR abates both fragmentation and proliferation, we then set out to see if treatment with Rapamycin or Torin could reduce the rapid activation observed in Opa1-KO MuSCs. Both Rapamycin and Torin minimized fragmentation in Opa1-KO MuSCs at 4 hours in culture, and effectively reduced the number of Opa1-KO MuSCs that entered the cell cycle at 24 hours in culture (Figure 13D-E). These data demonstrate that systemic signals and activation of mTOR can alter mitochondrial dynamics to transition MuSCs into $G_{Alert,}$ though how mitochondrial fragmentation can convert MuSCs to a state of shallow quiescence still remains unknown.

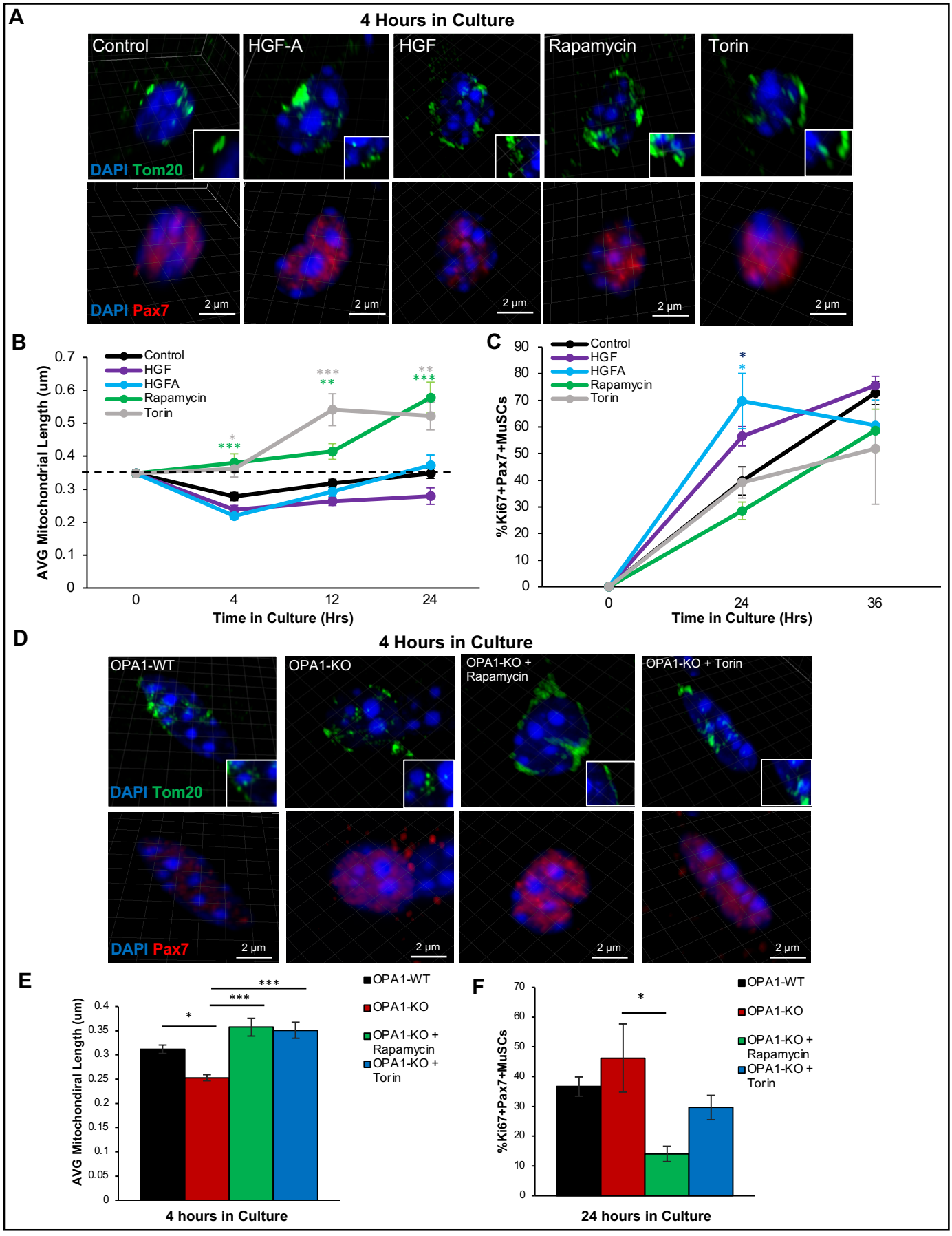
A
4 Hours in Culture
Control
HGF-A
HGF
Rapamycin
Torin
DAPI Tom20
DAPI Pax7
2 µm
B
AVG Mitochondrial Length (um)
Control
HGF
HGFA
Rapamycin
Torin
Time in Culture (Hrs)
C
%Ki67+Pax7+MuSCs
Time in Culture (Hrs)
D
4 Hours in Culture
OPA1-WT
OPA1-KO
OPA1-KO + Rapamycin
OPA1-KO + Torin
E
AVG Mitochondiral Length (um)
4 hours in Culture
F
%Ki67+Pax7+MuSCs
24 hours in Culture

Figure 13. Systemic HGF and mTOR alter mitochondrial length and muscle stem cell activation.

(A) Representative 3D images of mitochondrial structure in muscle stem cells on single myofibers at four hours in culture treated with 40 ng/mL HGF-A, 10 ng/mL HGF, or 20 nM Rapamycin and 10 nM Torin-1.

(B) Average mitochondrial length in muscle stem cells on single myofibers treated with respective drugs and cultured from 0-24 hours. Dotted line represents average mitochondrial length at 0 hours (quiescent with no drug treatment). N=5-20 ± SE. One-way ANOVA with Dunnett's Post-HOC test. $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***.

(C) Quantification of Pax7+Ki67+ muscle stem cells on single myofibers treated with respective drugs at 24 and 36 hours in culture. N=3 ± SE. One-way ANOVA with Dunnett's Post-HOC test. $p<0.05$*.

(D) Representative 3D images of mitochondrial structure of Opa1-WT and Opa1-KO muscle stem cells treated with mTOR inhibitors for 4 hours.

(E) Average mitochondrial length of Opa1-KO muscle stem cells treated with mTOR inhibitors for 4 hours in culture. N≥ 5 ± SE. One-way ANOVA with Tukey's Post-HOC test, $p<0.05$ *, $p<0.001$ ***.

(F) Quantification of Ki67+Pax7+ muscle stem cells in Opa1-KOs treated with mTOR inhibitors for 24 hours in culture. N=3 ± SE. One-way ANOVA with Tukey's Post-HOC test, $p<0.05$ *.

4.6 Redox Signalling Promotes Muscle Stem Cell Activation and Cell Cycle Entry

We first wanted to confirm that direct manipulation of mitochondrial dynamics could alter muscle stem cell activation, by treating MuSCs on single myofibers with Mdivi-1 (2 μM). Mitochondrial length was significantly increased at four, 12 and 24 hours in culture with mdivi-1 treatment, while stem cell activation and entry into the cell cycle, marked by Ki67, was curtailed by Mdivi-1 at 24 and 36 hours in culture, comparable with the results observed by inhibition of mTOR (Figure 14A,B,D). Furthermore, there was a shift in nuclear morphology that is more consistent with quiescence and early activation, indicated by an increase in the number of small elongated nuclei (Figure 14C).

We next investigated mechanisms to activate MuSCs downstream of mitochondrial dynamics. Studies have demonstrated that ROS and redox signalling can drive stem cell commitment, while glutathione has been linked to cell cycle progression[99,132,137,146,176-178]. Furthermore, a shift in metabolism and increase in OXPHOS genes are among the first transcriptional changes identified upon early activation[30,31]. Thus, we hypothesized that redox signalling may be important for activation of MuSCs and transition into the cell cycle. To test this hypothesis, we treated wild-type MuSCs on single myofibers with 1 mM GSH, 5 mM cysteine or glycine to increase intracellular levels of glutathione, 10 μM BSO to inhibit glutathione synthesis, or 10 nM rotenone to increase CI-derived ROS. Evaluation of the number of Ki67+ MuSCs at 24 hours in culture revealed that treatment with GSH, cysteine and rotenone significantly increased the number of cells that had entered the cell cycle, while no change in the number of Ki67+ MuSCs was observed upon treatment with glycine or BSO (Figure 14E). By 36 hours in culture, BSO had significantly reduced the number of Ki67+ MuSCs, while the remaining treatments showed no

change in the number of Ki67+ MuSCs (Figure 14F). As we did not observe an increase in Ki67+ cells in the majority of treatments at 36 hours in culture, we hypothesized that they may be progressing through the cell cycle, with no change in the total number of cells that have entered the cell cycle. To confirm this, we then looked at cluster size at 36 hours in culture and observed that there was a modest increase in the number of dividing MuSCs treated with glutathione and cysteine (Figure 14G). Furthermore, nuclear size and morphology also corroborated these results, as there was a shift towards increased nuclear size (Figure 14H-I). Overall, these data indicate that redox signalling is required for MuSC activation and entry into the cell cycle.

Since we observe this increase in activation and cell cycle entry in acute Opa1-KO MuSCs, we reasoned that impairing redox signalling by diminishing intracellular levels of ROS or GSH should rescue the early entry into an activated state. To validate this hypothesis, we treated Opa1-KO MuSCs on single myofibers with either BSO (10 μM) to deplete GSH levels, Mdivi-1 (2 μM) to inhibit mitochondrial fragmentation, or MitoTEMPO (10 μM) to reduce ROS levels. Treatment with redox signalling inhibitors effectively reduced the number of OPA1-KO MuSCs that had entered the cell cycle at 24 and 36 hours in culture (Figure 14J-K), indicating that changes in mitochondrial dynamics elicit redox signalling to activate MuSCs and allow their entry into the cell cycle.

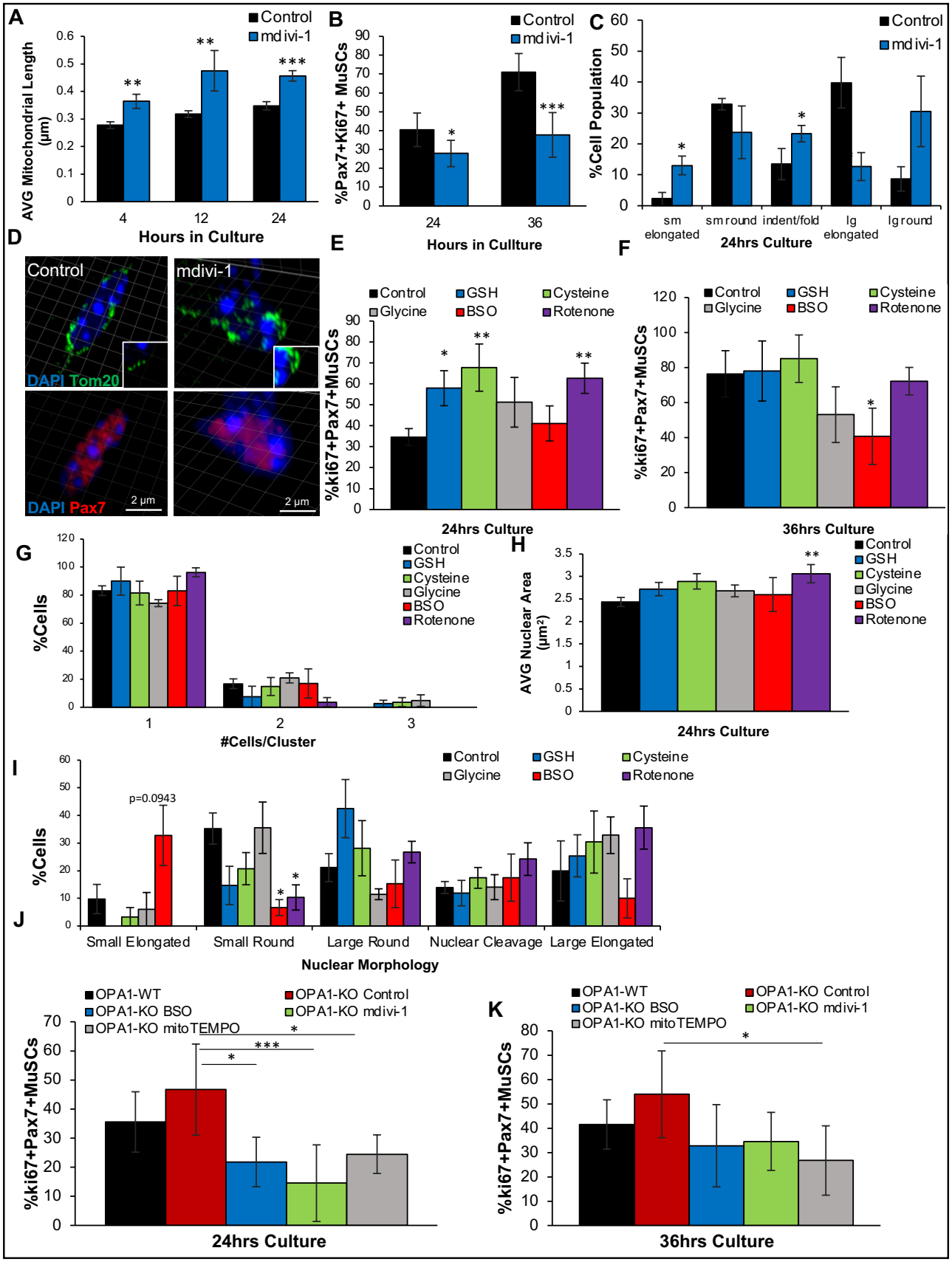
A
AVG Mitochondrial Length (µm)
Control
mdivi-1
Hours in Culture
B
%Pax7+Ki67+ MuSCs
Hours in Cullture
C
%Cell Population
sm elongated
sm round
indent/fold
lg elongated
lg round
24hrs Culture
D
Control
mdivi-1
DAPI Tom20
DAPI Pax7
2 µm
E
%ki67+Pax7+MuSCs
GSH
Cysteine
Glycine
BSO
Rotenone
24hrs Culture
F
36hrs Culture
G
%Cells
#Cells/Cluster
H
AVG Nuclear Area (µm²)
I
p=0.0943
Small Elongated
Small Round
Large Round
Nuclear Cleavage
Large Elongated
Nuclear Morphology
J
OPA1-WT
OPA1-KO Control
OPA1-KO BSO
OPA1-KO mdivi-1
OPA1-KO mitoTEMPO
K

Figure 14. Redox-signaling promotes muscle stem cell activation and cell-cycle entry.

(A) Average mitochondrial length in wild-type muscle stem cells on single myofibers cultured for four-24hours and treated with 2 μM Mdivi-1. N=5-10 ± SE. Student's unpaired t-test, $p<0.01$ **, $p<0.001$ ***.

(B) Quantification of Pax7+&Ki67+ muscle stem cells on single myofibers treated with 2 μM Mdivi-1 compared to control at 24 and 36 hours in culture. N=4 ± SD. Student's unpaired t-test, $p<0.05$ *, $p<0.001$ ***.

(C) Nuclear morphology classification in wild-type muscle stem cells on single myofibers cultured for 24 hours and treated with 2 μM Mdivi-1, sm = small, lg = large. N=4 ± SD. Student's unpaired t-test $p<0.05$ *.

(D) Representative 3D images of mitochondria in muscle stem cells on single myofibers treated with 2 μM Mdivi-1 and cultured for 4 hours.

(E) Quantification of Pax7+&Ki67+ muscle stem cells on single myofibers treated with 1 mM GSH, 5 mM cysteine or glycine, 10 μM BSO, or 10 nM rotenone for 24 hours in culture. N=3 ± SD. One-way ANOVA with Dunnett's Post-HOC test, $p<0.05$ *, $p<0.01$ **.

(F) Quantification of Pax7+&Ki67+ muscle stem cells on single myofibers treated with 1 mM GSH, 5 mM cysteine or glycine, 10 μM BSO, or 10 nM rotenone for 36 hours in culture. N=3 ± SD. One-way ANOVA with Dunnett's Post-HOC test, $p<0.05$ *.

(G) Quantification of cluster size in Pax7+ muscle stem cells on single myofibers with respective treatments (E) at 36 hours in culture. N=3 ± SD.

(H) Nuclear area measurements in wild-type muscle stem cells on single myofibers with respective treatments (E) at 36 hours in culture. One-way ANOVA with Dunnett's Post-HOC test, $p<0.01$ **.

(I) Classification of nuclear morphology in wild-type muscle stem cells on single myofibers with respective treatments (E) at 36 hours in culture. One-way ANOVA with Dunnett's Post-HOC test, $p<0.05$*.

(J) Quantification of Pax7+&Ki67+ muscle stem cells on single myofibers in Opa1-WT and Opa1-KO muscle stem cells treated with 10 μM BSO, 2 μM Mdivi-1, and 10 μM MitoTEMPO for 24 hours in culture. One-Way ANOVA w Tukey's Post-HOC test, $p<0.05$ *, $p<0.001$ ***.

(K) Quantification of Pax7+&Ki67+ muscle stem cells on single myofibers in Opa1-WT and Opa1-KO muscle stem cells treated with 10 μM BSO, 2 μM Mdivi-1, and 10 μM MitoTEMPO for 36 hours in culture. One-Way ANOVA w Tukey's Post-HOC test, $p<0.05$ *.

4.7 Chronic Loss of Opa1 Impairs Muscle Stem Cell Fate and Function

The importance of Opa1 and mitochondrial dynamics on the MuSC pool has been thus far established by these results, however how expression of Opa1 can affect MuSCs in a more physiological context has yet to be examined. Depletion and dysfunction of the MuSC pool is hallmark in a variety of degenerative diseases and during aging[77,209,210]. Moreover, a decline in the expression of Opa1 has also been observed in aging and can affect skeletal muscle tissue[194]. To understand the relevance of Opa1 loss in MuSCs throughout aging, we developed a model of chronic Opa1 loss in which we knocked out OPA1 using the same tamoxifen inducible model as previously described and aged the Opa1 KO mice for 3-months. Initially, we quantified the number of Pax7+ MuSCs *in vivo*, under basal conditions and observed no difference in the number of MuSCs between wild-type and Opa1-KO mice 3-months after tamoxifen injection (Figure 15A-B). Next, we induced a CTX-injury in these mice, harvested the tissue at 7DPI, and quantified the number of MuSCs in the injured and contralateral limbs. Strikingly, Opa1-KO mice are completely depleted of MuSCs at 7DPI in the injured limb and show a major decline of MuSCs in the contralateral limb (Figure 15A,C-D). Moreover, H&E staining revealed that there are no detectable newly forming, centrally nucleated fibers in 3-month Opa1-KO mice (Figure 15E-F). Next, using *in vitro* single myofiber cultures, we examined fate decisions in chronically ablated mice. Interestingly, after being cultured for 72 hours, the number of cells within an Opa1-KO cell cluster was severely diminished, although myogenic regulatory factors MyoD and MyoG were still being expressed (Figure 15G-K). This indicated to us that chronic loss of Opa1 may affect the ability of MuSCs to proliferate and divide. Thus, to confirm this hypothesis we cultured chronic Opa1-KO MuSCs on single myofibers from 0-72hours and stained for Ki67. Strikingly, there was a severely impaired ability of Opa1-KO MuSCs to express Ki67 as early as 48 hours in culture (Figure 15L).

To gain insight into what causes MuSC dysfunction in the absence of Opa1 after 3-months, we performed gene expression analysis and noticed a unique gene expression profile upon chronic loss of Opa1 (Figure 15M). Among the changes in gene expression, we observed that Gclc, the rate-limiting enzyme of glutathione synthesis, which is important for cell cycle progression[176,177,211], had decreased expression in Opa1-KO MuSCs compared to wild-type littermates. Given that GSH treatment in young wild-type MuSCs enhanced entry into the cell cycle, we reasoned that supplementation with GSH may be able to rescue proliferation in chronic Opa1-KO MuSCs. As such, we treated chronic Opa1-KO MuSCs on single myofibers with 1 mM GSH or 5 mM cysteine and glycine and quantified the number of Ki67+ cells and the number of cells within a cluster after 72 hours in culture. Remarkably, supplementation with GSH or cysteine and glycine was able to partially rescue the number of Ki67+ cells, and the size of clusters (Figure 15N-O). These data demonstrate that, in contrast to what we observe upon acute loss of Opa1, prolonged knockout of Opa1 causes major MuSC dysfunction, which can be ameliorated by supplementing with GSH.

As previously demonstrated, acute loss of Opa1 does not impair mitochondrial ATP generation, as indicated by no detectable change in total ATP or the source of ATP (Figure 9C-D). Given that acute Opa1-KO MuSCs do not exhibit impairments in proliferation or other dysfunction, we hypothesized that chronic loss of Opa1 would induce mitochondrial dysfunction. Therefore, we measured levels of ATP and the source of ATP in Opa1-KO mice, one and three months after receiving tamoxifen injection. In contrast to acute Opa1-KO mice, chronic ablation of Opa1 does reduce total levels of ATP, and slightly shifted the source of ATP towards glycolysis

(Figure 15R-U). To solidify that mitochondrial metabolic impairment is the cause of the observed MuSC dysfunction, we induced mitochondrial dysfunction pharmacologically by treating young wild-type MuSCs with high doses of the metabolic inhibitors Rotenone (1 μM), Antimycin-A (50 μM), Oligomycin (5 μM), and BSO (50μM) and assessed proliferative ability and cluster formation after 72 hours in culture. Interestingly, we observed a significant decline in both the number of Ki67+ cells, and the total number of cells within a cluster at 72 hours in culture with all treatments, which was consistent with results upon chronic loss of Opa1 (Figure 15P-Q). These results demonstrate that chronically diminishing the mitochondrial fusion protein Opa1 in MuSCs impairs mitochondrial function, alters MuSC gene expression and causes severe MuSC dysfunction.

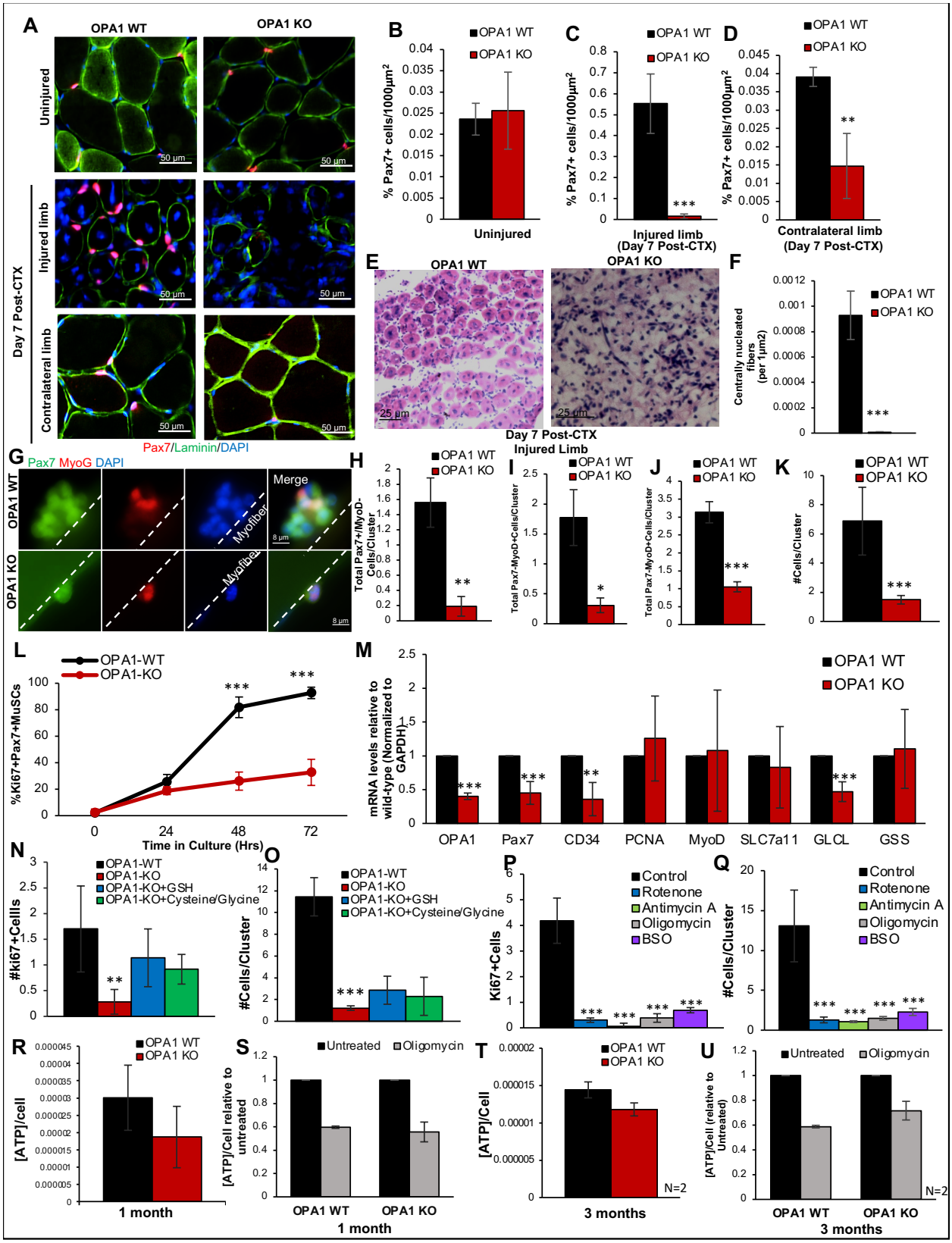
A
OPA1 WT
OPA1 KO
Uninjured
Injured limb
Contralateral limb
Day 7 Post-CTX
50 µm
Pax7/Laminin/DAPI
B
OPA1 WT
OPA1 KO
% Pax7+ cells/1000µm²
Uninjured
C
Injured limb
(Day 7 Post-CTX)
D
Contralateral limb
(Day 7 Post-CTX)
E
25 µm
F
Centrally nucleated fibers (per 1µm2)
G
Pax7 MyoG DAPI
Merge
Myofiber
8 µm
H
Total Pax7+/MyoD-Cells/Cluster
Day 7 Post-CTX
Injured Limb
I
Total Pax7-MyoD+Cells/Cluster
J
Total Pax7-MyoD+Cells/Cluster
K
#Cells/Cluster
L
OPA1-WT
OPA1-KO
%Ki67+Pax7+MuSCs
Time in Culture (Hrs)
M
mRNA levels relative to wild-type (Normalized to GAPDH)
OPA1
Pax7
CD34
PCNA
MyoD
SLC7a11
GLCL
GSS
N
OPA1-KO+GSH
OPA1-KO+Cysteine/Glycine
#ki67+Cells
O
#Cells/Cluster
P
Control
Rotenone
Antimycin A
Oligomycin
BSO
Ki67+Cells
Q
#Cells/Cluster
R
[ATP]/cell
1 month
S
Untreated
Oligomycin
[ATP]/Cell relative to untreated
T
[ATP]/Cell
N=2
3 months
U
[ATP]/Cell (relative to Untreated)

Figure 15. Chronic loss of Opa1 severely impairs muscle stem cell fate and function.
(A) Representative images of Pax7+ muscle stem cells in chronically ablated Opa1-KO mice completely uninjured, or 7DPI injured and contralateral limbs.
(B) Quantification of Pax7+ muscle stem cells in chronically ablated Opa1-KO mice completely uninjured, or Opa1-KO mice 7DPI injured (C) and contralateral (D) limbs. N=3 ± SD, $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***. Student's unpaired t-test.
(E) Representative H&E images of muscle fibers in chronically ablated Opa1 mice 7DPI.
(F) Quantification of total newly formed, centrally nucleated fibers in chronically ablated Opa1 mice compared to wild-type littermates. N=3 ± SD, $p<0.001$ *** students unpaired t-test.
(G) Representative images of muscle stem cells on single EDL myofibers at 72 hours in culture, stained with Pax7 and MyoG.
(H,I,J) Quantification of the number of cells in each cluster on single myofibers at 72hours in culture. N=6 ± SD, $p<0.05$ *, $p<0.01$ **, $p<0.001$ *** Student's unpaired t-test.
(K) Quantification of muscle stem cell populations within each cluster on single myofibers cultured for 72 hours. N=6 ± SD, $p<0.01$ ***, student's unpaired t-test.
(L) Quantification of Pax7+&Ki67+ muscle stem cells on single myofibers from 0-72hrs in culture in chronic OPA1-KO mice compared to wild-type counterparts. N=6 ± SD, $p<0.001$ ***, student's unpaired t-test.
(M) Gene expression array of genes involved in muscle stem cell quiescence, activation and oxidative stress sensing in 3-month-old Opa1-KO mice compared to age-matched wild-type littermates. N=3 ± SD, student's unpaired t-test, <0.05*, $p<0.01$ **, $p<0.001$ *** .
(N) Quantification of Ki67+ cells in chronic Opa1-KO muscle stem cells on single myofibers treated with GSH 1 mM, or 5 mM Cysteine+Glycine cultured for 72hours. N=6 ± SD. One-Way ANOVA w Tukey's Post-HOC test, $p<0.01$ **.
(O) Quantification of cluster size in chronic Opa1-KO muscle stem cells on single myofibers treated with GSH 1 mM, or 5 mM Cysteine+Glycine cultured for 72hours. N=6 ± SD. One-Way ANOVA w Tukey's Post-HOC test, $p<0.001$ ***.
(P) Quantification of Ki67+ muscle stem cells on single myofibers treated with 1 µM Rotenone, 50 µM Antimycin-A, 5 µM Oligomycin, or 50 µM BSO for 72hours. N=3 ± SD. One-Way ANOVA w Dunnett's Post-HOC test, $p<0.001$ ***.
(Q) Quantification of cluster size of muscle stem cells on single myofibers treated with 1 µM Rotenone, 50 µM Antimycin-A, 5 µM Oligomycin, or 50 µM BSO for 72hours. N=3 ± SD. One-Way ANOVA w Dunnett's Post-HOC test, $p<0.001$ ***.
(R) Total ATP levels in Opa1-KO MuSCs 1-month post-tamoxifen treatment compared to wild-type littermates. N=3.
(S) ATP levels from Opa1-KO MuSCs 1-month post-tamoxifen treatment relative to untreated (indicating ATP produced from OXPHOS) or in the presence of oligomycin (indicating ATP produced by Glycolysis). N=3.
(T) Total ATP levels in Opa1-KO MuSCs 3-months post-tamoxifen treatment compared to wild-type littermates. N=2.
(U) ATP levels from Opa1-KO MuSCs 3-months post-tamoxifen treatment relative to untreated, with or without oligomycin treatment. N=2.

4.8 Characteristics of Aging Muscle Stem Cell Function and Mitochondrial Structure

Finally, we looked at characteristics of MuSC mitochondria and function in wild-type aging mice. First, gene expression analysis revealed a significant decline in both Opa1 and Pax7 expression in aged mice (Figure 16A). Furthermore, the genes involved in GSH synthesis were also decreased, similarly to what is observed in chronically ablated Opa1 MuSCs (Figure 16B). To see if the decline in Opa1 gene expression coincided with a decrease in mitochondrial length, we measured mitochondria from MuSCs at the onset of aging (16 months) and observed a slight but significant reduction in mitochondrial length compared to young mice of the same background (10 weeks old) (Figure 16B). Next, we examined MuSC function and maintenance during aging. Using mice that were 16 months of age, we quantified the total number of Pax7+ MuSCs under basal conditions and upon injury (7 days post CTX injury) and observed a small, but significant decline in the number of MuSCs of aged mice compared to young counterparts (Figure 16D-E). Furthermore, analysis of MuSC fate decisions *in vitro* revealed a reduction in the number of Pax7+ cells and an increase in the number of MyoD+ cells, indicating fate decisions skewed towards commitment (Figure 16F-G). Thus, at the onset of aging, mitochondrial fragmentation is observed along with a simultaneous decrease in MuSC maintenance ability upon activation.

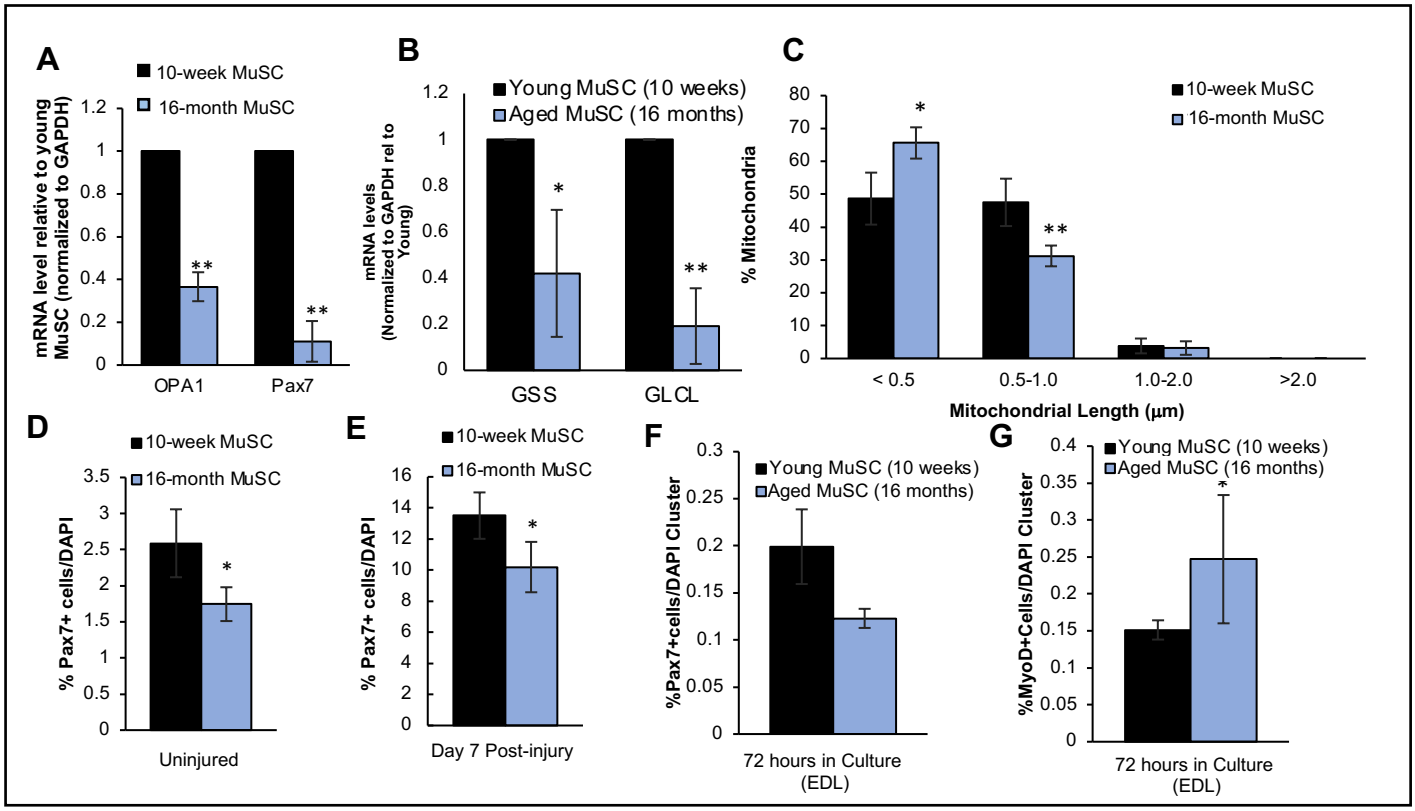

Figure 16. Aging mice have defects in mitochondrial structure and altered gene expression.

(A) mRNA levels of Opa1 and Pax7 in aged muscle stem cells compared to young wild-type counterparts. N=3 ± SD, p<0.01 **, student's paired t-test.

(B) mRNA levels of glutathione synthesis genes Gss and Glcl in aged muscle stem cells compared to young wild-type counterparts. N=4 ± SD, p<0.05 *, p<0.01 **, student's paired t-test

(C) Distribution of muscle stem cell mitochondrial length in aged muscle stem cells compared to young mice. N=7 ± SE. p<0.05 *, p<0.01 ** student's paired t-test.

(D) Quantification of basal levels of muscle stem cells in 16-month-old mice compared to 10-week-old mice. N=3 ± SD, p<0.05 *, student's paired t-test.

(E) Quantification of Pax7+ muscle stem cells in 16-month-old mice at 7DPI compared to 10-week-old mice. N=3 ± SD, p<0.05 *, student's paired t-test.

(F) Quantification of Pax7+ cells within a cluster of cells on single myofibers cultured for 72hrs in aged mice. N=3 ± SE.

(G) Quantification of MyoD+ cells within a cluster of cells on single myofibers cultured for 72hrs in aged mice. N=3 ± SE, p<0.05 *, student's paired t-test.

Chapter 5: Discussion

Muscle stem cells reside primarily in a quiescent state until a sufficient stimulus promotes activation and muscle reparation[1,2]. The ability of MuSCs to exit quiescence and undergo a decision in fate between self-renewal and commitment is essential for muscle tissue regeneration and MuSC maintenance[77,212]. In this work, we place mitochondrial dynamics and specifically Opa1 as a critical regulator of the identity of MuSCs from quiescence to self-renewal and commitment (Figure 17). Acute loss of Opa1 in Pax7+ MuSCs, disrupting mitochondrial fusion leads to substantial defects in muscle regeneration. Upon closer look, loss of Opa1 impairs the ability of MuSCs to undergo self-renewal, shifting to commitment and leading to depletion of the MuSC pool following regeneration. The notion that impairing mitochondrial dynamics can alter stem cell fate decisions has also been demonstrated in many other systems, including NSCs, HSCs, T-cells, ESCs, and cancer stem cells[132,158,160,175,180,213], however our data is the first to demonstrate that dysregulated mitochondrial dynamics affects fate decisions in the muscle stem cell system.

This work primarily focused on the loss of mitochondrial fusion protein Opa1 as an indicator of impaired mitochondrial dynamics. Therefore, it is possible that the effect on MuSC fate decisions is specifically due to the loss of Opa1 function, which doubles as a cristae structure modulator[107,214]. To address this issue, we also treated MuSCs with low concentrations of the putative DRP1 inhibitor, Mdivi-1. The choice to treat MuSCs with 2 μM Mdivi-1 was based on a recent study that demonstrated treating with high doses of Mdivi-1 increased ROS levels, whereas low dose Mdivi-1 functions as a DRP1 inhibitor[215]. Our data shows that OPA1-KO MuSCs treated with Mdivi-1 undergo similar fate decisions as wild-type MuSCs, indicating a manipulation of mitochondrial structure either favouring fission or fusion is sufficient to alter fate decisions.

Furthermore, the effect of Mdivi-1 on the commitment and differentiation of stem cells has been noted in NSCs, epithelial stem cells, and human iPSCs, among many others[159,180,216]. Thus, our data demonstrating that loss of OPA1 and mitochondrial plasticity in MuSCs dysregulates fate decisions supplements the current literature and further denotes the importance of mitochondrial structure in stem cell function, though this does not delineate the exact role of mitochondrial dynamics in adult myogenesis.

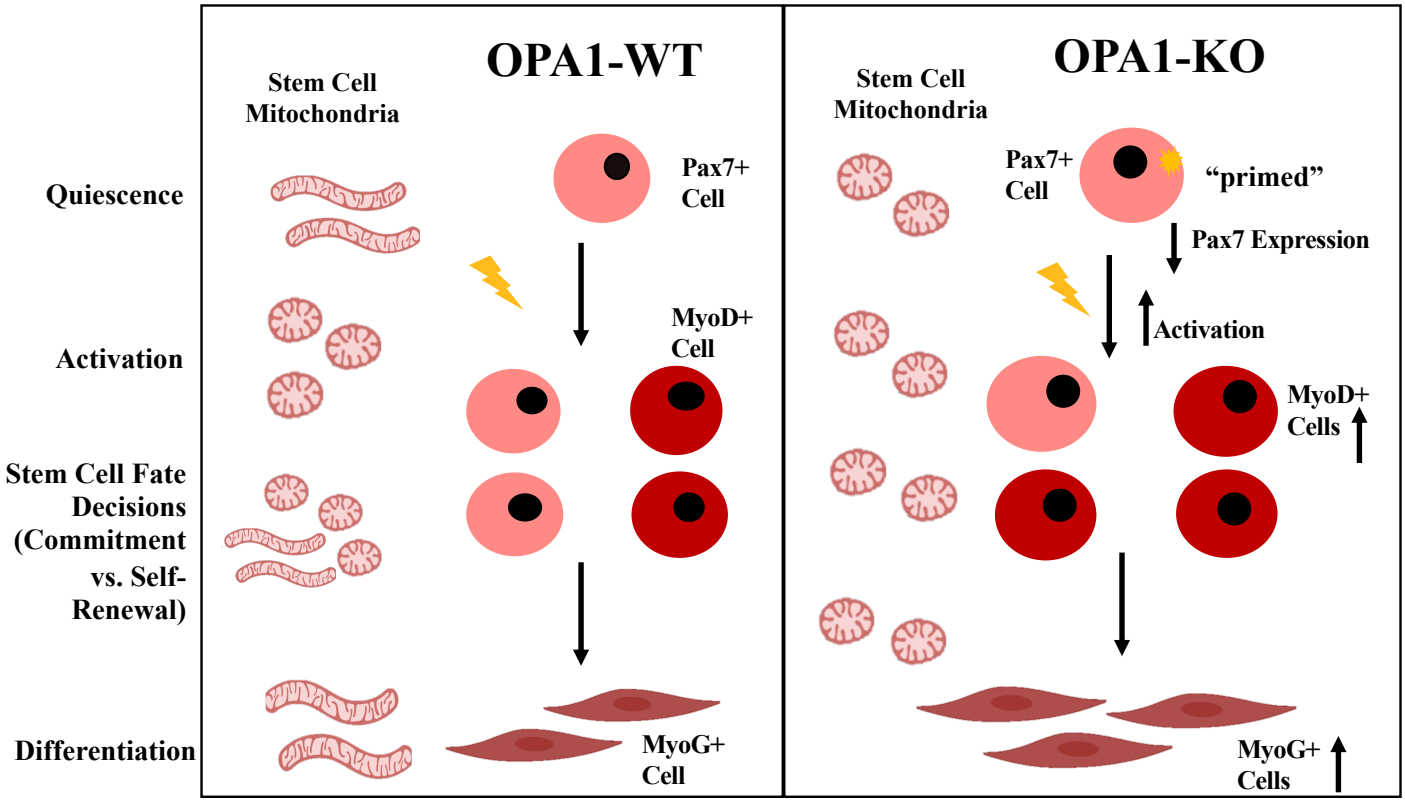

Figure 17. Acute mitochondrial fragmentation drives muscle stem cell activation and myogenic commitment. Mitochondrial structure is dynamic during adult myogenesis, allowing for muscle stem cells to progress through differentiation. However, acute loss of Opa1 in MuSCs, causing fragmented mitochondria "primes" MuSCs for activation, leading to an increase in MyoD+ and MyoG+ committed cells at the expense of Pax7+ self-renewing cells.

To better understand the relevance of mitochondrial dynamics in adult myogenesis, we performed in-depth examination of mitochondrial length. We observed a striking fragmentation at just 4 hours in culture that persisted as MuSCs reached 48 hours in culture. Contemporary studies have identified that MuSCs in the early stages of activation, approximately 3-5 hours after dissection, have massive changes in gene expression, many of which genes are involved in mitochondrial metabolism[30,31]. Therefore, in addition to early alteration in mitochondrial metabolism, for the first time we have identified alterations in mitochondrial structure at the cusp of MuSC activation. Furthermore, studies conducted in our lab show that loss of Opa1 in MuSCs reduces the quiescence genes Pax7, CD34, and Hes1 suggesting a role for Opa1 and mitochondrial fragmentation in MuSC quiescence and activation (Figure 8).

In addition to a dynamic change in mitochondrial length upon activation, we also identified differences in mitochondrial length in cell populations expressing varying levels of Pax7 at 48 hours in culture. As MuSCs undergo mitosis, they can divide asymmetrically or symmetrically. Cells dividing asymmetrically give rise to one daughter cell destined to commit, and one daughter cell designated for self-renewal[1,4,24]. Furthermore, studies have demonstrated that daughter cells expressing high levels of Pax7 retain template DNA and are destined for self-renewal[65]. Our results further establish that Pax7-high cells, which may be destined for self-renewal, inherit longer mitochondria. Furthermore, mitochondria in cells cultured for 72 hours are elongated when compared with those at earlier timepoints. Previous investigations have noted that as stem cells undergo differentiation, mitochondria begin to elongate and mature when compared to stem cell mitochondria, which is accompanied by an increase in mitochondrial metabolism and extensive mitochondrial biogenesis[103,217]. Thus, our data demonstrating mitochondrial elongation upon early

differentiation corroborates the current literature, potentially underlying a role for mitochondrial elongation in differentiation.

Muscle stem cell quiescence, classically defined as a state of dormancy has recently been described as a gradient, whereby MuSCs can exist in a deep state of quiescent meeting the criteria of dormancy, or they can adopt a state of shallow quiescence termed an alert state[28]. Interestingly, the idea that Opa1 could regulate quiescence was further recognized when we examined the MuSC pool *in vivo*. Opa1-KO muscle that has received no injury does not exhibit any muscle stem cell depletion when compared to wild-type counterparts. However, when an injury is received, MuSCs in the limb contralateral to the injury were depleted. This therefore presented us with a model where loss of Opa1 under acute basal conditions alters gene expression, reducing MuSC quiescence, although is insufficient alone to force them into activation. However, upon a stimulus, even that of a distant injury is adequate to allow exit from quiescence and into premature activation. In fact, studies have identified that MuSCs in the limb contralateral to an injury do adopt a state of G_{Alert}[57]. To further understand if this was the case of Opa1-KO MuSCs, we evaluated EdU incorporation into the contralateral limb, and entry into the cell cycle *in vitro*. Both data promoted the idea that Opa1 could regulate MuSC quiescence and activation. Furthermore, we noticed a change in the nuclear morphology of Opa1-KO MuSCs upon activation. Although this has not been previously studied, some studies have suggested that nuclear folding is an early marker of differentiation, while other studies have observed a relationship between round nuclei and the presence of MyoD[34,35,218-221]. Despite the current challenge in determining the significance of altered nuclear morphology, this presents a potentially interesting marker of early activation in MuSCs and warrants further studies and validations. Altogether, our data is the first to demonstrate

a critical role for Opa1 in the maintenance of MuSC quiescence and given the implications of a distant injury to the contralateral muscle stem pool, may provide an interesting avenue of research into stem cell depletion.

With the knowledge a distant injury can affect the contralateral stem cell pool we reasoned there may be a systemic factor that modifies the state of quiescence in the MuSCs. To our knowledge, there has been two systemic pathways identified that can transition MuSCs into an alert state[58,59]. Most notably, it was identified that systemic circulating HGF activator (HGF-A) could covert HGF into its active form, thus allowing the binding of HGF to the cMET receptor on the surface of muscle stem cells and activating mTORC1[59]. Our data demonstrate that mitochondrial fragmentation is an important aspect of the shift from quiescence to activation. Furthermore, we've shown that in the contralateral limb, MuSC mitochondria exhibit more fragmentation that mitochondria of deeply quiescent MuSCs. We therefore further analysed how mitochondrial fragmentation could further respond to systemic signals and modify quiescence. Our data reveal that mitochondria are able to respond to treatment with both human recombinant active HGF, and the HGF activator protein (HGF-A) and increase fragmentation and entry into the cell cycle, while treatment with mTOR inhibitors is able to subdue mitochondrial fragmentation and reduce the level of activation. These data are the first to place mitochondrial fragmentation in the systemic pathway that transitions MuSCs to G_{Alert} (Figure 18). As previously mentioned, another systemic pathway involving the fully reduced form of HMGB1 was also identified. Future studies following this project could look at factors within this systemic pathway to determine if mitochondria sense systemic from alternative sources.

Finally, our data show that treatment of OPA1-KO MuSCs with mTOR inhibitors is capable of subduing activation, as indicated by reduced cell-cycle entry. One interesting study has demonstrated that mTOR can control mitochondrial dynamics, by stimulating translation of mitochondrial fission process 1 (MTFP1), which allows for phosphorylation of DRP1 to initiate mitochondrial fission[222]. Interestingly, the results of mTOR inhibition have similar effects to Mdivi-1 treatment, another inhibitor of DRP1. This may indicate that in addition to the importance of OPA1 in maintaining quiescence, DRP1 may also be essential in transition MuSCs into activation. Interestingly, Rapamycin and rapalogs that are clinically utilized as cancer treatment drugs have also demonstrated anti-aging remedies in mice[223-225]. Overall, these data provide a novel role for mitochondrial fragmentation as sensors of external systemic cues through mTOR activation, as well as potentially provides an interesting opportunity for therapeutic intervention with DRP1 inhibitors in circumstances where loss of quiescence causes stem cell depletion.

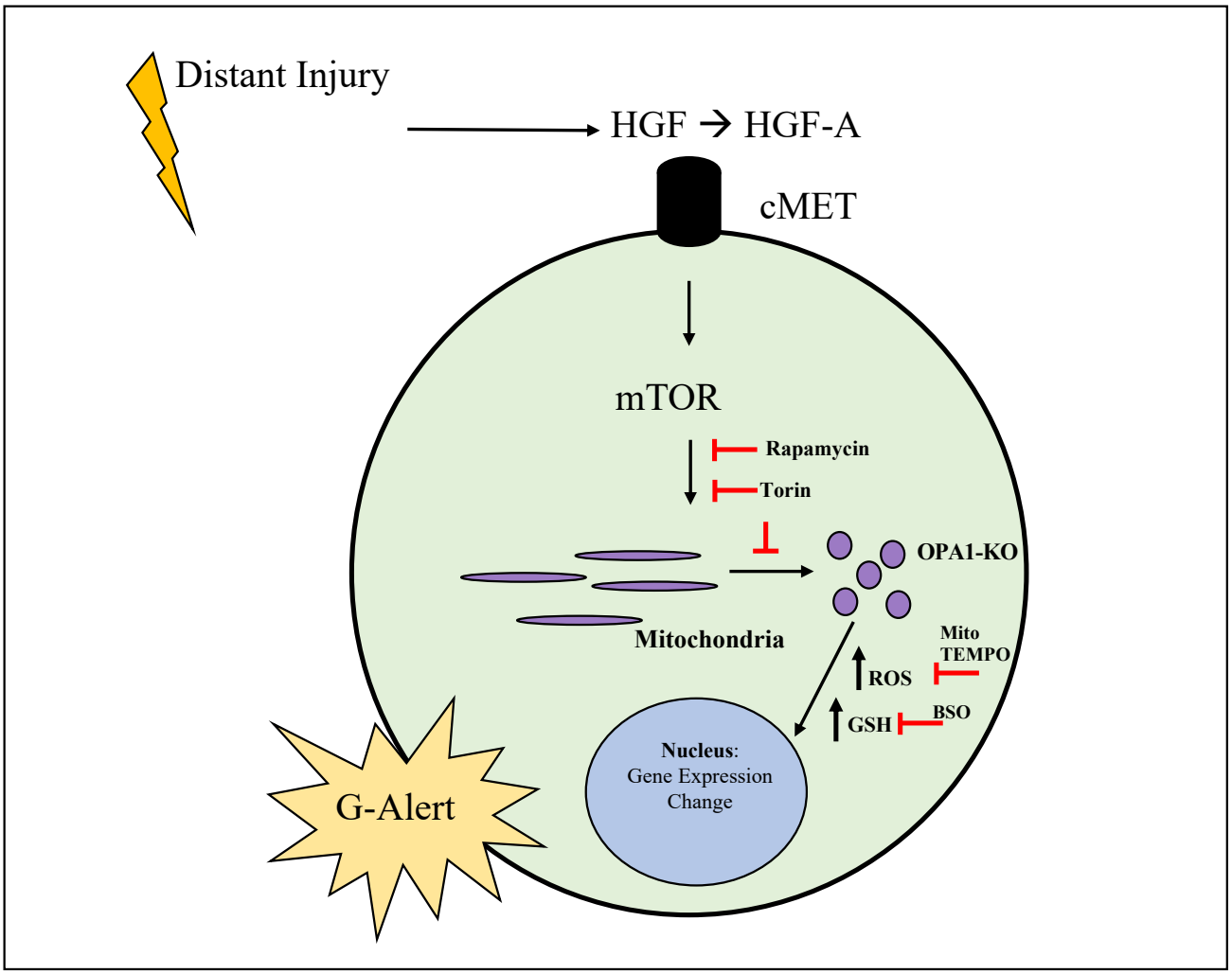

Figure 18. Mechanism of mitochondrial fragmentation to activate muscle stem cells. Mitochondria act as sensors of external systemic signals through mTOR activation, subsequently undergoing fragmentation and eliciting redox signaling to alter expression of MuSC quiescence genes to reduce the depth of muscle stem cell quiescence.

In addition to identifying the systemic mechanism by which mitochondrial fragmentation elicits changes in quiescence, we have also established redox signalling as a downstream mechanism for stem cell activation (Figure 18). Our data show that in the presence of GSH, or the GSH precursor cysteine entry into the cell cycle is increased, while treatment with BSO, an inhibitor of GSH synthesis halted cell cycle entry. Furthermore, rotenone, which at low doses causes electron leak and increased ROS through complex I[226,227] was also sufficient to increase the number of MuSCs that had entered the cell cycle. The relationship between mitochondrial dynamics and ROS signalling to promote stem cell commitment has been demonstrated, however few studies have actually shown the importance of redox signalling during stem cell activation[99,132,137,146]. Moreover, it is known that glutathione is required for progression through the cell cycle, specifically during S-phase[174,176,228-230]. Finally, the parallel relationship between ROS and GSH levels has been thoroughly characterized, where increasing levels of ROS promote synthesis of GSH as an antioxidant defense[107,166-168]. Thus, the relationship established here between ROS and glutathione for the regulation of stem cell quiescence and activation provides new insight into the mechanisms by which ROS signalling can elicit the progression of stem cells towards commitment and differentiation. Although the work done here does establish a link between ROS signalling, GSH and cell cycle entry the exact mechanisms by which redox signalling can elicit changes in activation and cell cycle progression in stem cells remains to be resolved.

Thus far, we have established that acute loss of Opa1, leading to rapid fragmentation without generating dysfunctional mitochondria is a necessary requirement to transition MuSCs out of quiescence and permit activation of the myogenic program while promoting MuSC

commitment. However, long-term mitochondrial fragmentation leads to pathogenesis and impaired mitochondrial function[107,231-235]. Mitochondrial dysfunction is a prominent pathogenic factor in muscle wasting diseases and age-induced sarcopenia[77,209,210,236]. Our data demonstrates that just three months after loss of Opa1, prominent mitochondrial dysfunction leads to impairment in stem cell function. Upon acute loss of Opa1 in MuSCs, ATP production is not affected. However, just three months post-Opa1-KO, MuSC ATP production is impaired, leading to mitochondrial dysfunction. Furthermore, acute loss of Opa1 leads to a lack of muscle regeneration by 21 days post-injury, whereas with chronic loss of Opa1 muscle regeneration fails by just 7 days post-injury, indicating severe defects in the muscle regenerative process upon prolonged loss of Opa1.

Further investigations identified that an inability to properly proliferate lead to stem cell depletion in chronic Opa1-KO muscle stem cells. Notably, failure to proliferate did not stem from an inability to activate, as indicated by the expression of the myogenic activation marker MyoD and early expression of the cell cycle marker Ki67[64]. Interestingly, the inability of chronic Opa1-KO MuSCs to divide was accompanied by a decline in the expression of glutathione synthesis genes, which, as previously discussed is indispensable for cell cycle progression[176,228-230]. Biological aging is often associated with an increase in oxidative stress, and studies have demonstrated that depletion of glutathione is common during aging[237-239], and in fact was observed in wild-type aged MuSCs in this study as well. Therefore, the ability to supplement with glutathione or antioxidants is a vital opportunity to ameliorate stem cell dysfunction with underlying mitochondrial deficiencies, which is demonstrated in our chronic Opa1 model, where addition of GSH is able to partially rescue proliferation. However, the inability of GSH to fully

rescue proliferation defects signifies the involvement of another mechanism. Given that GSH and redox reactions have been reported to be necessary for transition through S-phase, it is possible another metabolic checkpoint may be required later in the cell cycle, such as M-phase[240].

Mitochondrial dysfunction, in addition to its association with aging, has also been shown to be a prominent pathogenic factor in early stem cell exhaustion. A recent paper identified impaired mitochondrial OXPHOS as the leading cause of active T-cell exhaustion[175]. Similarly, we observe in young wild-type MuSCs treated with high doses of oxidative inhibitors that proliferation is severely impaired. Notably, the phenotype observed upon induction of oxidative impairment is strikingly similar to that observed in 3-month Opa1-KO mice, solidifying the role of mitochondrial dysfunction in the chronic Opa1 phenotype. Therefore, our data gathered from our chronic Opa1-KO model demonstrates an accelerated aging phenotype, where mitochondrial dysfunction is prominent and severely impairs the ability of MuSCs to progress through myogenic differentiation, diminishing their repair capacity. This is in striking contrast to our acute Opa1-KO model, whereby rapid mitochondrial fragmentation acts as a physiological mechanism to drive MuSCs into the myogenic program and through differentiation.

Finally, this study examined some aspects of metabolism and muscle stem cell function in aging mice. For this work, we used 16-month-old mice, which can be considered aged mice but not necessarily geriatric[241]. None-the-less, our work has demonstrated that even in mice 16 months of age have a reduction in mitochondrial fusion, glutathione synthesis genes, and impaired MuSC fate decisions. Furthermore, even at 16 months of age, some minor stem cell depletion can be observed. This data corroborates the current literature, as a reduction in many genes involved in

mitochondrial dynamics, redox homeostasis and MuSC maintenance[33,80,184,194,210,242]. Ultimately, this work puts mitochondrial fragmentation as an aspect of mitochondrial dysfunction at the onset of MuSC aging. As changes in mitochondrial structure is an adaptive and transient process, the ability to target mitochondrial dynamics before the onset of MuSC senescence and muscle dysfunction in aging is an attractive therapeutic strategy to combat muscle wasting under disease and pathophysiological conditions.

Chapter 6: Conclusion

In conclusion, mitochondrial dynamics is an essential process that regulates many aspects of metabolic function, and ultimately cellular function. In this work we've demonstrated that modifications to mitochondrial structure are required to drive adult MuSCs out of quiescence and allow activation of the myogenic program to drive myoblast commitment and differentiation. Our intricate studies place mitochondrial dynamics at the heart of sensing external cues to drive a redox signalling program to promote MuSC activation. Thus, acute mitochondrial fragmentation is a critical physiological mechanism for adult stem cells, while chronic mitochondrial fragmentation leads to pathogenesis and mitochondrial dysfunction, as is often observed in MuSCs of aged individuals and patients with muscle wasting conditions (Figure 19). This work provides novel insights into the role of mitochondrial dynamics in adult stem cell function, as well as provides innovative avenues of treatment to ameliorate conditions where dysfunctional mitochondrial fission and fusion leads to stem cell dysfunction.

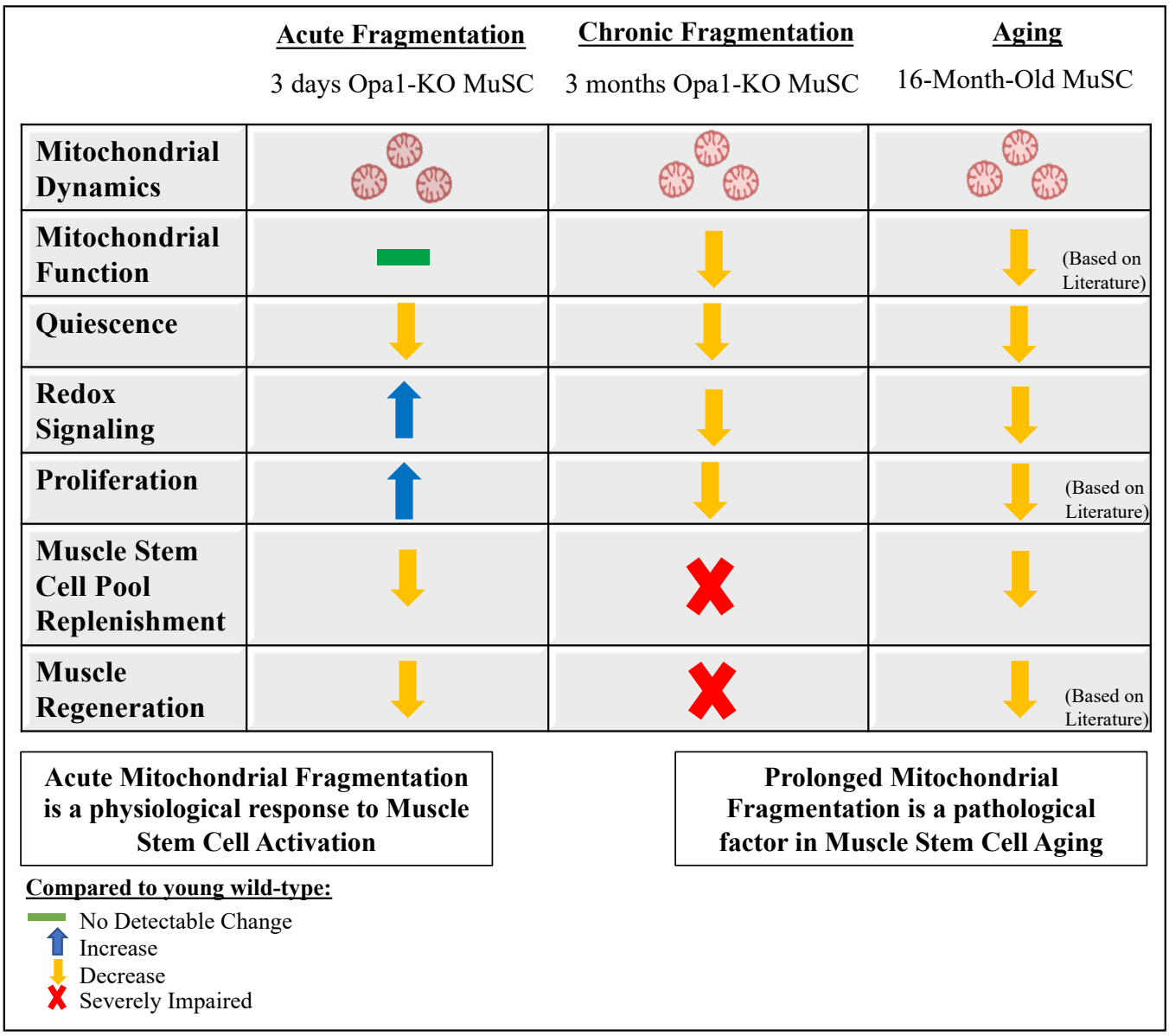

Figure 19. Comparative model of acute and chronic loss of Opa1 with physiological aging. Acute fragmentation acts as a physiological cue to progress muscle stem cells through the myogenic program, whereas chronic fragmentation is pathological, leading to mitochondrial and muscle stem cell dysfunction. Physiological aging exhibits similar characteristics as muscle stem cells with chronic fragmentation. Green bars represent no change, yellow arrows indicate a decline, blue arrows indicate and increase, and red X's represents a complete absence of the action. A decline in mitochondrial function in aging mice has been demonstrated by many studies[243-246]. Furthermore, physiological aging leads to senescence and a lack of proliferation[83,247-249] and muscle regeneration[43,78,81,82].

Chapter 7: Limitations

Although this work presents novel insights into the role of mitochondrial dynamics in muscle stem cell fate and function, there are some limitations. First, this study focuses on the quiescent state of MuSCs. One inherent characteristic of MuSCs is their rapid activation upon removal from their origin[28,30,31]. Therefore, studying true quiescence of the MuSC population poses a challenge. However, since our studies primarily focus on the change in the quiescent state from a wild-type, to Opa1-KO mice we are still able to observe a notable shift in quiescence. Future studies conducted on quiescence could utilize novel techniques, such as *in situ* fixation, where muscle tissue is fixed immediately following dissection before any subsequent isolation procedures are carried out, thus preserving quiescence[31].

Furthermore, another challenged posed when utilizing adult MuSCs is the rarity of the population. Therefore, when considering experimental techniques, the amount of sample possible to obtain must be taken into account, thus limiting the feasible techniques. For example, techniques such as the Seahorse mitochondrial respiration assay, and various assays requiring protein are a challenge with the sample amount we are able to obtain. Methods to overcome these limitations including increasing the number of animals used to obtain the desired amount of sample or working in a primary myoblast culture system where the stem cells can multiply, and thus more cells would be available for downstream applications.

Finally, the techniques used to induce regeneration of adult muscle tissue in this study are extensive, involving severe myonecrosis. While useful in understanding the full extent of muscle regeneration, more physiological methods of inducing MuSC activation may present us with

results that are clinically translatable and allow us to really understand the extent of the quiescence defect upon loss of Opa1. To achieve these results, high-intensity exercise models, which have demonstrated the ability to activate muscle stem cells, could be utilized[11,13,200,250].

Chapter 8: Future Directions

Results obtained from this study have opened avenues for many future studies. First, this study briefly identified both ROS and GSH as metabolites required for MuSC activation and entry into the cell cycle. However, this study does not directly measure ROS or GSH levels, nor does it fully decipher how exactly ROS and GSH work to transition MuSCs into activation and through commitment. Thus, future studies can focus on measuring ROS levels through mitochondrial superoxide (MitoSOX) staining, and GSH levels through various assays, metabolomics or high-pressure liquid chromatography (HPLC). Investigations into the exact mechanism by which ROS and GSH can activate MuSCs could focus on different redox reactions, one of which could potentially be protein glutathionylation[167].

Furthermore, this study examines some aspects of MuSC mitochondrial function, including ATP measurements and mitochondrial length, but a more comprehensive look into metabolic function in MuSCs at various stages of myogenic progression, and in the absence of Opa1 would advance the current discoveries. First, in addition to Opa1 being an IMM fusion protein, it is also responsible for cristae remodelling and supercomplex assembly[120,123,124,251]. Thus, future studies could examine cristae morphology through electron microscopy, and supercomplex assembly through blue-native gel electrophoresis under the aforementioned conditions. This would provide greater insight into the metabolic mechanisms that regulate MuSC activation.

Finally, future studies could further investigate the physiological relevance of loss of Opa1 on the MuSC pool, or conditions that affect mitochondrial dynamics and metabolic function. Examples of this include using disease models to study the impact of disease on metabolism and mitochondrial dynamics. Furthermore, using aging or disease models, one could investigate the effect of prolonged supplementation with glutathione on the maintenance and function of the MuSC pool. Lastly, to gain a better understanding of the physiological relevance of loss of Opa1 on the quiescent MuSC pool, studies could focus on minor stem cell activation mechanisms, such as exercise. These studies would provide us with insight on the clinical relevance of impaired mitochondrial dynamics in the muscle stem cell pool.

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