THE IMACT OF PARKIN DEPENDENT MITOPHAGY ON MUSCLE STEM CELL FATE DECISION, LINEAGE PROGRESSION, AND MUSCLE REGENERATION

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ABSTRACT

Skeletal muscles are considered one of the most dynamic tissues due to their capabilities of remodelling, repair, and regeneration attributed to their unique population of stem cells known as muscle stem cells (MuSCs). Mitochondrial function, maintenance, and remodelling has been linked with the ability of the MuSCs to maintain quiescence, undergo self-renewal, and differentiate in order to regenerate muscle. Subsequently, mechanisms affecting mitochondrial properties in MuSCs, including mitophagy, are suggested to play an important role. To determine the role of mitophagy in MuSC behavior, we developed a mouse model harboring a conditional MuSC specific inactivation of the key mitophagy gene Parkin. *In vitro* single myofiber experiments showed that Parkin deficiency triggered premature expression of activation and differentiation MuSC markers and lowered the proportion of quiescent cells indicating premature activation/commitment at the expense of the quiescent/self-renewing MuSC pool. Furthermore, cell cycle re-entry and proliferation were impaired in absence of Parkin, as evidenced by a reduction of MuSC cluster size, and the number of Ki67 (proliferation marker) expressing cells. Moreover, immunofluorescent experiments in the Tibialis Anterior (TA) muscle form cardiotoxin injured mice showed reduction in the Pax7+ and Ki67+ MuSCs at 7- and 14- days post injury indicating defects in the MuSC self-renewal and proliferation during muscle regeneration. The injured muscle also had a significant reduction in fiber size post injury indicating improper muscle regeneration and a defect in the regenerative potential. Furthermore, post-injury the Parkin deficient muscle also had a significant decrease in the number of Pax7+ self-renewing MuSCs signifying depletion of the MuSC pool. Together, our data suggests that manipulation of Parkin-mediated mitophagy alters muscle stem cell fate and lineage progression causing an imbalance in MuSC self-renewal and proliferation. Alterations in Parkin mediated mitophagy also impairs muscle regeneration and promotes the activation and commitment of MuSCs at the expense of MuSC self-renewal.

Keywords: MuSC, mitophagy, Parkin-dependent mitophagy, muscle regeneration, stem cells.

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LIST OF ABBREVIATIONS :

ACVS	Animal Care and Veterinary Service
AMBRA1	Autophagy and beclin 1 regulator 1
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATG7	Autophagy related 7
ATP	Adenosine triphosphate
BAK	BCL2 antagonist killer 1
BCL2	B-cell leukemia/lymphoma 2
bFGF	Basic fibroblast growth factor
BNIP3	BCL2 interacting protein 3
BNIP3L	BCL2 interacting protein 3 Like
BRCA1	Breast cancer gene 1
BSA	Bovine serum albumin
C/EBPb	CCAAT/enhancer binding proteins
CALCR	Calcitonin receptor
Ccnd	Cyclin D1
CD11b	Cluster of differentiation 11b
CD31	Cluster of differentiation 31
CD34	Cluster of differentiation 34
CD45	Cluster of differentiation 45
Cdc25	Cell division cycle 25 a
Cdkn1b	Cyclin-dependent kinase inhibitor 1B
Cdkn1c	Cyclin-dependent kinase inhibitor 1C

cDNA	copy DNA
CO2	Carbon dioxide
CSA	Cross-sectional area
CTX	Cardiotoxin
CXCR4	C-X-C chemokine receptor type 4
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNM1L	Dynamin-1 like
DPI	Days Post Injury
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2′-deoxyuridine
EGF	Epidermal growth factor
eIF2a	Eukaryotic translation initiation factor 2A
eMyHC	Embryonic myosin heavy chain
ERK	Extracellular signal-regulated kinase
ERK1/2	Extracellular signal-regulated kinase 1/2
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FAO	Fatty acid oxidation
FBS	Fetal bovine serum

FGF-2	Fibroblast growth factor 2
FOXO	Forkhead box O
FOXO3	Forkhead box O3
FUNDC1	FUN14 domain containing 1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
Gas1	Growth arrest specific 1
H&E	Hematoxylin and eosin
Hes1	Hes family BHLH transcription factor 1
Hey1	Hes related family BHLH transcription factor with YRPW Motif 1
HeyL	Hes related family BHLH transcription factor with YRPW Motif Like
HGF	Hepatocyte growth factor
HIF2A	Hypoxia-inducible factor-2alpha
HSC	Hematopoietic stem cell
IF1	Inhibitory factor 1
IGF-1	Insulin-like growth factor-1
IL12	Interleukin 12
IL13	Interleukin 13
IL1a	Interleukin 1a
IL1b	Interleukin 1b
IL6	Interleukin 6
IL6-JAK STAT3	Interleukin 6 Janus kinase (JAK)/signal transducer and activator of transcription
IMM	Inner mitochondrial membrane
INF-y	Interferon-gamma
JNK	Janus kinase

KO	Knockout
LAMP1	Lysosomal associated membrane protein 1
LC3	Light chain 3
LIR	Light chain 3 interacting region
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MFN1	Mitofusin 1
MFN2	Mitofusin 2
miRNA	Micro-RNA
MMP	Mitochondrial membrane potential
MPC	Myogenic precursor cell
MRF4	Myogenic regulatory factor 4
MRFs	Myogenic regulatory factors
MSC	Mesenchymal stem cell
MTCH2	Mitochondrial carrier homolog 2
mTOR	Mammalian target of rapamycin
MuSCs	Muscle stem cells
Myf5	Myogenic factor 5
MyoD	Myoblast determination protein
MyoG	Myogenin
NBR1	Neighbor of BRCA1 gene 1
NDP52	Nuclear dot protein 52
NICD	Notch intracellular domain
NOTCH3	Neurogenic locus notch homolog protein 3

NSC	Neural stem cells
OCT	Optimal cutting temperature compound
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OPTN	Optineurin
OXPHOS	Oxidative phosphorylation
Pax3	Paired homeobox transcription factor 3
Pax7	Paired homeobox transcription factor 7
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3K-AKT-mTORc1	Phosphoinositide-3-kinase-mammalian target of rapamycin complex 1
PINK1	Phosphatase and tensin homolog (PTEN)-induced putative kinase 1
PKB	Protein kinase B
Pmp22	Peripheral myelin protein-22
PRKN	Parkin
qPCR	Quantitative polymerase chain reaction
Rb1	Retinoblastoma protein
RBP-jk	Recombination signal binding protein for immunoglobulin kappa J region
Rgs2	Regulator of G protein signaling 2
Rgs5	Regulator of G protein signaling 5
RHOT	Ras homolog family member T

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative polymerase chain reaction
SEM	Standard error of the mean
SIX1	Sine oculis homeobox homolog 1
Spry1	Sprouty1
TA	Tibialis Anterior
TNF-a	Tumor necrosis factor alpha
NF kB	Nuclear factor kappa light chain enhancer of activated B cells
TOM20	Translocase of outer mitochondrial membrane 20
TTP	Tristetrapolin
UCP2	Uncoupling protein 2
USP15	Ubiquitin-specific peptidase 15
USP30	Ubiquitin-specific peptidase 30
USP35	Ubiquitin-specific peptidase 35
VCAM1	Vascular adhesion molecule-1
VDAC	Voltage-dependent ion channel
WT	Wildtype

1 INTRODUCTION

1.1 Skeletal Muscles

Due to their capability of remodeling, repair, and regeneration, adult skeletal muscles are considered one of the most dynamic and plastic tissues representing approximately 40% of the human body weight[1,2]. Adult skeletal muscles are postmitotic comprised of bundles of multinucleated, striated contractile myofibers with an intricate network of blood vessels, nerves, and the extracellular matrix[1,3]. Each myofiber has numerous myofibrils along the length of each fiber and holds many sarcomeres, which are the subunits of the functional unit of contraction[3,4]. The interaction between actin and myosin filaments occurs at the sarcomere, which translate energy into force and motion[3]. Skeletal muscles are provided with nourishment and the transmission of force due to the interactions between muscle fibers and the connective tissue surrounding them, called fascia[4]. Skeletal muscle fibers are formed through a regulated process called myogenesis, whereby the fusion of myogenic progenitors, myoblasts forms myotubes, eventually leading to the formation of muscles[4].

1.2 Muscle injury and repair

In response to injury, skeletal muscles undergo regeneration and maintain their function and size through regeneration [2,3]. Even after successive and severe muscle injuries, proper regeneration of muscle size and function can occur[2]. Skeletal muscle can become injured from different events including diseases, exposure to myotoxic agents, ischemia, muscle lacerations, and rare genetic myopathies.[5,6]. Any imbalance between injury and regeneration can lead to the deterioration of muscle function, causing multiple diseases[2]. The disruption of muscle tissue homeostasis due to injury has three main phases: degeneration/inflammation, regeneration, and remodeling phases[6]. Post injury, muscle degeneration and inflammation occur with necrosis of the muscle fiber due to the unregulated influx of calcium through the sarcolemma lesions[5,6]. This causes the activation of enzymes that contribute to muscle damage and

produce mitogenic substances in muscle and immune cells[6]. After muscle degeneration, inflammatory cells including neutrophils and macrophages are recruited to contribute to cell lysis, remove cellular debris, stimulate myoblast proliferation, and promote of myotube formation[5,6]. Muscle regeneration begins 4-5 days post injury, peaks at 14 days, and then gradually reduces 3 to 4 weeks later[6]. Muscle regeneration depends on the balance between pro-inflammatory and anti-inflammatory factors that determines whether muscle fiber replacement and reconstitution of functional muscle, or scar formation will occur[5]. During muscle regeneration, connective tissue remodelling occurs with gap formation between the damaged muscle fibers, which are filled with hematoma[5]. The size, location, and nature of the hematoma are used to clinically classify muscle injury and late removal of the hematoma delays skeletal muscle regeneration, promotes fibrosis, and causes a reduction in the biomechanical properties of the healing muscle[6,7]. As muscle regeneration progresses, revascularization of the injured skeletal muscle occurs with the innervation of regenerated myofibers[6]. As muscle fibers are terminally post-mitotic, they cannot divide to contribute to muscle growth and repair [3,6]. Thus, following injury muscle repair is accomplished by a unique population of muscle stem cells (MuSCs), satellite cells[3,6].

1.3 Muscle stem cells

1.3.1 ***Overview***

Muscle stem cells are located between the basal lamina and the plasmalemma of the muscle fibers and account for 2%-10% of the total myonuclei[8,9]. They have a high nucleus-to-cytoplasm ratio with increased heterochromatin amount and a reduced organelle content[5,8]. More MuSCs are found on the slow oxidative muscles compare to the fast glycolytic muscles with a higher number of MuSCs at neuromuscular junctions[5,8]. During development MuSCs are derived from the myogenic progenitors originating from the dermomyotome which are characterized by the expression of paired box proteins Pax3 and Pax7[10]. During embryonic myogenesis, the muscle precursor cells activate and differentiate,

expressing myogenic regulatory factors (MRFs) including Myf5, MyoD, MyoG and MRF4, forming embryonic muscle compartments however, a subset of the myogenic precursor cells don't express MRFs, retaining their Pax3/Pax7 expression that are hypothesized to give rise to MuSCs found in the adult skeletal muscle[11](**Fig. 1)**. Quiescent MuSCs can also be identified by a number of specific markers of attachment and adhesion molecules and cell surface receptors that distinguish them from there differentiated counterparts, including integrin alpha-7, M-cadherin, CD34, Calcitonin-Receptor (CALCR), C-X-C Chemokine Receptor type-4 (CXCR4), Vascular Cell Adhesion Molecule 1 (VCAM1), heparin sulfate proteoglycans syndecan-3 and syndecan-4 etc.[3,12–15]. Studies have demonstrated that satellite cells are a heterogenous population to maintain cellular homeostasis and the distinct subpopulations can be recognized based on their gene expression, epigenetic markers, cell surface markers, and functionality [16,17]. A large-scale single-cell RNA sequencing analysis depicted two distinct subpopulations of satellite cells with a close-to-quiescence cluster expressing genes encoding growth arrest-specific protein 1 (Gas1), Hes1, Pax7, and Calcr, and an early activation cluster expressing ribosomal genes Myf5 and Myod. Moreover, the distinct satellite cell subpopulations also exhibit differences in cell surface markers with $CD34^{high}$ expression in the quiescent naïve state and the $CD34^{low}$ expression in the committed, primed state. Quiescent MuSCs also express negative cell cycle regulators including cyclin-dependent kinase inhibitor 1B and 1C (Cdkn1b, Cdkn1c), the retinoblastoma tumor suppressor protein (Rb1), regulator of G-protein signalling 2 and 5 (Rgs2, Rgs5), peripheral myelin protein 22 (Pmp22), and the negative regulator of fibroblast growth factor signalling Sprouty 1 (Spry1). These genes act collectively to prevent activation of quiescent satellite cells and impairments in their ability to maintain quiescence is shown to reduce self-renewal capacity and muscle regeneration[10].

1.3.2 Regulation of MuSC states

Under resting conditions, MuSCs are in a transient cell cycle inhibition state known as the quiescent state or a reversible G0 state[10,16] . The ability of MuSCs to remain in this state is essential for the long-term

conservation of the satellite cell pool[10]. MuSC quiescence dysregulation results in imbalances in their fate decisions leading to stem cell depletion and altering tissue homeostasis and regeneration[16]. MuSCs also exist in a primed quiescent state, ready for activation, known as the G_{Alert} state[10]. MuSCs in the G_{Alert} state are observed to be larger, have higher metabolic activity, have higher intracellular ATP, and can perform their first division faster than the G_0 MuSCs[16]. The different MuSC states are regulated through a network of transcriptional, post-transcriptional, epigenetic, and metabolic regulation[16].

1.3.3 Maintenance of a quiescent state:

Quiescent MuSCs exhibit several general features distinguishing them from activated cells. They are small, have reduced levels of transcription and translation and favor catabolism with functional importance of autophagy and fatty acid oxidation. They also have low levels of oxidative phosphorylation in mitochondria which contribute to the quiescent properties by limiting production of ROS and cellular damage[17]. MuSC quiescence is highly controlled and different signalling pathways, transcriptional and post-transcriptional regulation, and epigenetic regulation help maintain this state.

1.3.3.1 Signalling Pathways

Notch signalling is known to be highly active in the quiescent MuSCs and is essential for the maintenance of the quiescent state[17]. In MuSCs, the disruption of Notch signalling by the inducible deletion of RBP-jk leads to their spontaneous activation and differentiation[18]. Moreover, inhibition of downstream targets of Notch signaling including Hey1 and HeyL lead to defects in MuSC quiescence maintenance[17]. Contrastingly, activation of Notch signalling by overexpression of Notch intracellular domain (NICD) has shown to favor self-renewal of MuSCs at the expense of their activation, proliferation, and differentiation[19]. It also directly induces Pax7 transcription, activates CALCR, and suppresses cell migration contributing to the quiescent state of MuSCs[17]. Furthermore, the calcitonin receptor signalling

pathway (CALCR) is also shown to help maintain the quiescent state of the MuSC and its loss in adult MuSCs is shown to cause MuSC apoptosis[20]. Moreover, pathway analysis of the quiescent state indicated over-representation of components of the tumour necrosis factor (TNF)-NF kB, IL-6-Janus kinase (JAK)-STAT3, and AMP-activated protein kinase (AMPK) pathways [16].

1.3.3.2 Transcriptional and post-transcriptional regulation

Transcriptional factors, including CCAAT/enhancer-binding protein beta (C/EBPβ), Forkhead box O (FOXO) and hypoxia-inducible factor 2A (HIF2A) are known to promote stem cell quiescence[16]. C/EBPβ is expressed in quiescent satellite cells and its loss promotes their premature exit from quiescence causing spontaneous activation and differentiation[21]. Moreover, its forced expression also upregulates 28 quiescence activated genes[21]. The FOXO family of forkhead transcription factors also regulate the quiescent state of MuSCs. It is shown that FOXO3, through activation of Notch signalling promotes the quiescent state of stem cells and its conditional deletion impairs MuSC self-renewal, promoting their differentiation[22]. Furthermore, FOXO deficient satellite cells highly express MyoG and myogenesis-related genes while downregulating quiescent genes including Notch and CALCR[23]. Thus, FOXO genes positively effect quiescence by upregulating quiescent genes and repressing activation-induced transcriptional programmes[16]. Furthermore, it has been shown that satellite cells are in an intrinsic hypoxic state and express HIF2A which helps maintain their quiescence, increases their self-renewal, and block their myogenic differentiation[24]. It binds to the promoter of the Sprouty1 (Spry1) gene, which regulates quiescence, and activates its expression[24,25] . The retinoblastoma (Rb) protein is also responsible for promoting MuSC quiescence as knocking down Rb in MuSC promotes their premature differentiation with a reduced capacity for their self-renewal[26]. Additionally, several non-coding RNAs such as microRNAs (miRNAs) also have a critical role in the regulation of stem cell quiescence. MiR-489 is highly expressed in quiescent MuSCs and is quickly downregulated during activation and it actively maintains MuSC quiescence by suppressing DEK, an oncogene which promotes

proliferative expansion of myogenic progenitors[27]. miR-195 and miR-497 are also known induce cell cycle arrest, promoting quiescence by targeting cell cycle genes *Cdc25* and *Ccnd*[28]. Notch signalling induces miR-708 which also regulates quiescence by antagonizing cell migration through targeting the transcripts of the focal-adhesion-associated protein Tensin 3 inhibiting focal adhesion kinases (FAK)[29]. Other post-transcriptional regulators which promote quiescence include Tristetrapolin (TTP), Staufen 1, and eIF2a[16]. TTP binds to MyoD mRNA and decays it inhibiting satellite cell activation keeping them in a quiescent state[30]. Quiescence is also regulated by Staufen 1 which blocks the translation of MyoD in the quiescent MuSCs preventing their activation[31]. Finally, phosphorylation of translation initiation factor eIF2a is another post-translational modification required to maintain MuSC quiescence. Phosphorylated eIF2a silences the translation of accumulating mRNAs, maintaining low protein synthesis, preventing the activation of MuSCs[32] .

1.3.4 Transition to an activated state

MuSC quiescence exit is the transition from a quiescent G0 state to the G1 phase of the cell cycle[33]. Upon stimulation, such as muscle damage, exercise, or pathogenic conditions, satellite cells activate, re-entering the cell cycle, proliferate, and give rise to myogenic precursor cells called myoblasts[34]. The basal lamina guides the MuSCs committed to myogenesis to migrate and proliferate[35]. MuSCs undergo dramatic epigenetic and transcriptomic changes with different signalling pathways in the transition state[17]. Furthermore, stem cell activation and proliferation are also regulated by the release of different cytokines and growth factors by the extracellular matrix including fibroblast growth factor 2 (FGF-2), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF-1), tumor necrosis factor (TNF-a), and epidermal growth factor (EGF) etc.[3,36].

1.3.4.1 Signalling Pathways

One of the main signalling pathways during the activated state of MuSCs is the p38a/b mitogen-activated protein kinase (MAPK) pathway[16,34,37]. Once activated, p38a/b MAPK quickly translocates to the nucleus and inactivates TTP to stabilize MyoD mRNA promoting MuSC activation[10,16,30,37]. P38a is also shown to turn off proliferation promoting JNK pathway, downregulating cyclin D1 expression causing cell cycle exit and initiating muscle differentiation[37]. Moreover, p38 MAPK signalling pathways is also a critical regulator of skeletal muscle differentiation and fusion as Lluis *et al.* showed that treatment with p38a/b inhibitors prevented the fusion of myoblasts into myotubes and the induction of muscle specific genes[38]. The canonical Wnt signalling pathway is also involved in the regulation of satellite cell activation, proliferation, and differentiation and the Wnt signals are known to control the expression of myogenic regulatory [34,39]. Moreover, the PI3K-AKT-mTORC1 signalling pathway is also rapidly induced upon activation and the AKT- mediated activation of mTOR signalling and repression of FOXO transcription factors are associated with MuSC activation[16,17]. Finally, even though Notch signalling is crucial for maintaining MuSC quiescence, some Notch activity is required for maintaining the proliferative state of MuSCs but not essential for cells post activation[40]. The adaptor protein, Numb, which prevents Notch translocation to the nucleus, also plays a key role in the commitment of progenitor cells[12,41].

1.3.4.2 Transcriptional and post-transcriptional regulation

Gene expression and large-scale transcriptional changes also occur as MuSCs activate and proliferate[36,42,43]. Thus, the switch from a quiescent to an activated MuSC state is accomplished through a complex interplay between signalling pathways, metabolic profiles, gene expression, and extracellular signals. During MuSC activation, the myogenic regulatory factors MyoD and Myf5 become highly upregulated alongside Pax7 expression. MyoD regulates the expression of Cell division cycle 6 (Cdc6) which renders the chromatin accessible for DNA replication, allowing cell cycle re-entry [34]. It also withdraws the cells from the cell cycle, enhancing myogenin and p21 expression[35]. Once MuSCs become

activated, MyoD expression persists in proliferating MuSCs and differentiated myotubes[35]. Increased expression of MyoD, MRF4, and myogenin expression stimulates the differentiation of the cells[35]. Furthermore, the post-transcriptional regulator DEK mediates intron removal normally retained in quiescent MuSCs, expanding their proteome by promoting the expression of genes involved in cell cycle regulation and lineage progression[44]. Paxpb1, a nuclear protein, is also shown to regulate a late cell-growth checkpoint, essential for the quiescent MuSCs to re-enter the cell cycle upon activation[45]. As MuSC commit and progress along the myogenic path, they transiently express the transcription factor Myogenin (MyoG) which drives the expression of several skeletal muscle genes including embryonic form of myosin heavy chain (eMyHC) [15,36,46]. As myoblasts fuse into myotubes and differentiate in mature fibers, embryonic MyHCs will be progressively replaced by adult isoforms to complete the regeneration process [15,46]

1.3.5 MuSC fate decisions during regeneration

As MuSCs activate and proliferate, they undergo great energetic shifts due to the increased biosynthetic activity and cell proliferation to support regeneration[2,5,12,13,16] . MuSC also possess the ability to commit to the myogenic lineage, to aid in muscle regeneration, or to self-renew, to maintain the stem cell pool[2,36,47,48]. Both the processes, differentiation into myotubes and the return to quiescence, requires the MuSCs to exit the cell cycle and re-enter the G0 arrest state[16]. Regulation of these highly controlled fate decisions is necessary to ensure a balance amongst regeneration post injury and the maintenance of a MuSC pool [1,2,36,47,48].

1.3.6 MuSC proliferation

Once activated, MuSCs begin proliferating, expressing the proliferation marker Ki67 and proliferating cell nuclear agent (PCNA)[15]. Ki67 is present during all active phases of the cell cycle including the G1,

S, G2 and the mitosis state[15]. Due to the increased cellular demands during activation, mTORC1 senses the energetic and nutritional status within the cells and directs them towards growth and proliferation[17]. Moreover, the WNT1, WNT3a and WNT5a ligands are also shown to promote MuSC proliferation[34]. Furthermore, the microenvironment of the cells also plays an important role in their proliferation and the secreted TNF-α, IFNγ, IL1α, IL1β, IL6, IL12, and IL13 secreted by immune cells promote the activation and proliferation of MuSCs[49,50].

1.3.7 MuSC division

MuSC population is controlled by a balance amongst asymmetric and symmetric cell division[51–53]. Symmetric division yields either two differentiated daughter cells known as symmetric commitment which can leads to stem cell exhaustion, or it yields two daughter cells maintaining stem cell properties to expand the muscle stem cell pool at the expense of muscle regeneration[51,52]. Asymmetric division yields one stem cell and a committed progenitor cell for the maintenance of the MuSC pool and to ensure proper muscle regeneration[51,52]**(Fig. 1)**. The relative orientation of the MuSC division determines the fate of the daughter MuSC[51]. If MuSCs divide in a planar orientation along the basal membrane, two daughter cells that expand muscle stem cool are generated[51,53]. If MuSCs divide in an apical-basal orientation, then asymmetrical division occurs where the committed cell initiates contact with the plasmalemma of the myofiber whereas the self-renewing cell maintains contact with the basal lamina[51,53]. Moreover, slow-dividing activated MuSCs produce self-renewing cells whereas the fast-dividing activated MuSCs produce cells daughter cells that promote commitment[54]. Furthermore, the expression of Scrib, cell polarity protein, also determines the fate of the dividing MuSCs, with high expression in activated MuSCs and no expression in quiescent MuSCs[55].

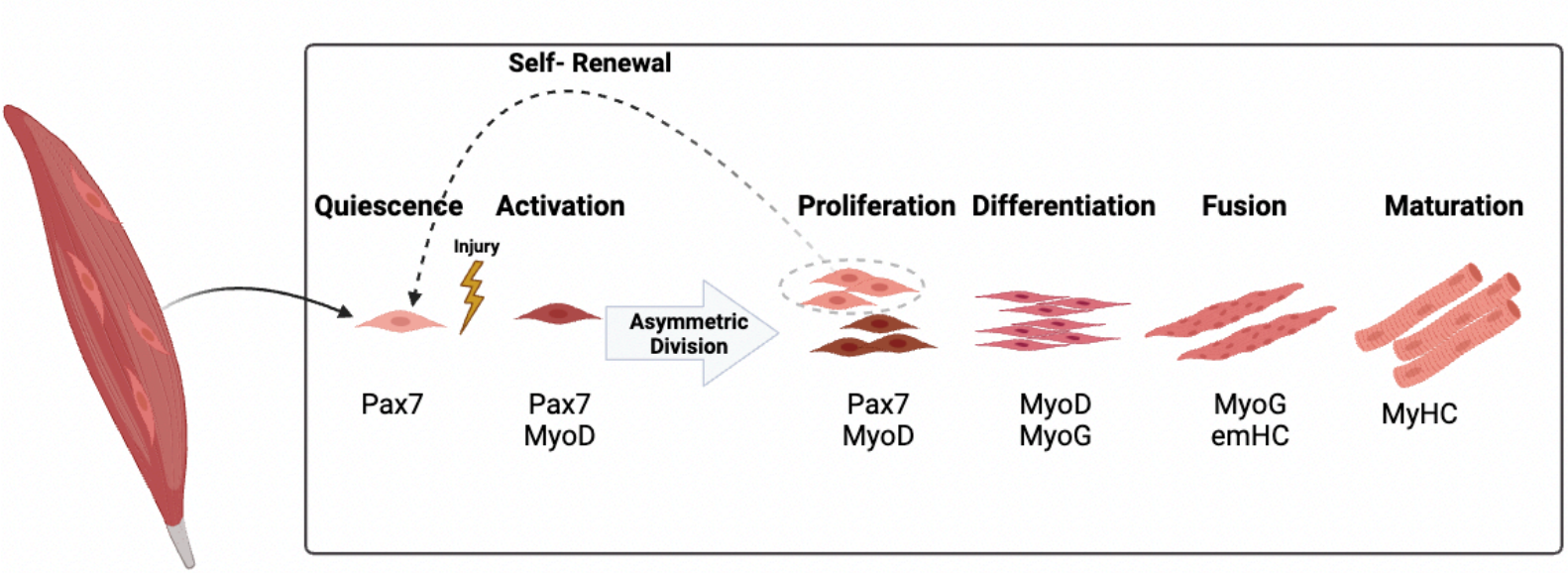

Self- Renewal
Quiescence
Activation
Injury
Proliferation
Differentiation
Fusion
Maturation
Asymmetric Division
Pax7
Pax7
MyoD
Pax7
MyoD
MyoD
MyoG
MyoG
emHC
MyHC

Figure 1. Schematic of Muscle stem cell fate decisions.

MuSCs lineage progression and the different markers they present at each stage. Created using BioRender.

1.3.8 Regulation of differentiation and commitment of MuSCs

As muscle stem cells exit their quiescent state and begin proliferating into myogenic precursor cells (MPC) or myoblasts to regenerate the injured muscle[47,49,51]. Differentiation of committed MuSCs is controlled by several myogenic regulatory factors (MRFs) including Myf5, MyoD, myogenin and Mrf4, and the Wnt, TGF-β, and p38α/β MAPK pathways[16,47,49,51,56]. p38α represses Pax7 expression at the epigenetic level and activates the MyoD transcriptional activity while also leading to cell cycle exit by inducing the expression of cell cycle inhibitors including $p21^{CIP1}$, $p19^{ARF}$ and p57[10,47,49,51,57]. MyoD also induces the expression of miR-206 and miR-133 which also promote Pax7 downregulation[58]. After multiple rounds of proliferation, many MPCs enter myogenic/terminal differentiation as the initiate the expression of myogenin (MyoG) and Mrf4[47,49,51,59] . Once they begin differentiating, myoblast exit the cell cycle, elongate, and fuse into multinucleated muscle cells forming mature muscle fibers[47,49]. Metabolically, differentiating MuSCs switch to oxidative phosphorylation, have increased mitochondrial mass, and upregulate the TCA cycle enzymes and electron transport chain proteins to sustain the increased energy demands of mature myofibers[16,60–63].

1.3.9 Regulation of the re-establishment of quiescence in MuSCs

As the muscle regenerates, a majority of the MuSCs differentiate to repair the muscle however, to ensure proper regeneration post subsequent injuries the muscle needs to replenish its satellite cell population[36]. This ability to have a constant stem cell population is due to the high self-renewal capacity of MuSCs[36]. Some MuSCs remain in an undifferentiated quiescent state even while the muscle is in a regenerative state[64]. Alongside their immediate niche, the Wnt, p38α/βMAPK (mitogen activated protein kinase), JAK-STAT3 and ERK1/2 signalling pathways, Spry1, Sine oculis homeobox homolog 1 (SIX1), Myf5, Pax3 and Pax7 are also regulators of muscle stem cell self-renewal[2,3,56,64–68]. Spry1 promotes cell cycle exit by inhibiting the ERK signalling pathway[16]. Furthermore, FOXO3 is also suspected to have a role in the suppression of myogenic differentiation and the return to quiescence through the induction of

Notch signalling[16,22]. The expression of NOTCH3 receptor also promotes the activation of Notch signalling inducing the return of MuSCs to quiescence[16]. Moreover, miRNA-195, miRNA-497, and miRNA-489 are also some of the micro-RNAs involved in the regulation of MuSC self-renewal[13,27,28,64].

1.4 Mitochondria and Stem cells

Mitochondria have been identified as key players in cell growth and senescence indicating the importance of mitochondrial fitness for cellular homeostasis[69,70]. Mitochondria are involved in stem cell fate and function in healthy tissues and during aging and diseases[71]. Accumulation of damaged mitochondria and mitochondrial dysfunction has shown to cause stem cell aging, stress induced aging, and cell senescence[72,73]. Several studies have been conducted to study the link amongst mitochondrial maintenance and stem cell function. Katajisto *et al.* showed that during asymmetric division, daughter cells prone to commitment receive aged mitochondria whereas daughter cells returning to quiescence obtain mainly young and new mitochondria emphasizing the role of mitochondrial signalling in stem cell division[74]. Several studies done on mouse models with mutations in mitochondrial DNA showed defects in self-renewal, proliferation, and differentiation of neural-, hematopoietic-, intestinal-, and induced pluripotent- stem cells[75–80]. Furthermore, stem cell identity, fate, and function are regulated by mitochondrial dynamics[81–84]. In the quiescent state, the mitochondria of most stem cells, including HSCs, MSCs and ESCs, are in an immature state being perinuclear-localized, spherical, and fragmented with few and underdeveloped cristae[85]. Recent studies have shown that quiescent MuSCs however, have elongated mitochondrial morphology which becomes smaller and fragmented during their proliferative stage[82,84]. Moreover, quiescent MuSCs rely on mitochondria to produce ATP through β-oxidation of fatty acids and OXPHOS and have low levels of ATP and ROS as their goal is to preserve the nuclear genome, epigenome, and mitochondrial genome for differentiated cells[85–87] **(Fig. 2)**. Upon differentiation, the mitochondria morphology changes becoming well-developed to produce energy more efficiently[84–87](**Fig. 2)**. Their mitochondrial content increases with enlarged, elongated, and tubular mitochondria with

complex cristae and an increase in mitochondrial ATP, OXPHOS and ROS levels also occurs[84–86,88](**Fig. 2)**. Furthermore, mitochondrial fusion proteins, mitofusin 1 (MFN1), mitofusin 2 (MFN2) and optic atrophy 1 (OPA1) are important in stem cell fate decisions suggesting that the regulation of mitochondrial dynamics is crucial for stem cell maintenance, commitment, and differentiation[75,81–83,86,88,89].

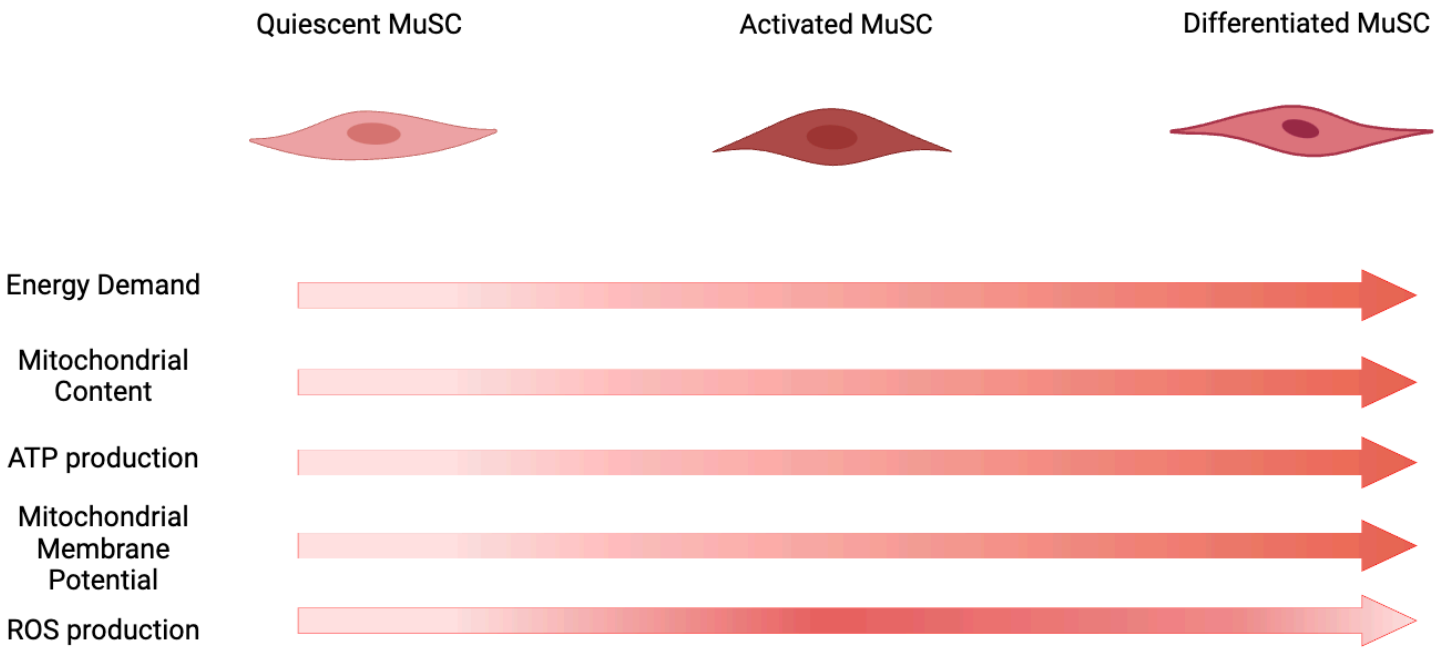

Quiescent MuSC
Activated MuSC
Differentiated MuSC
Energy Demand
Mitochondrial Content
ATP production
Mitochondrial Membrane Potential
ROS production

Figure 2. Mitochondrial Properties during MuSC lineage progression.

Changes in energy demand, mitochondrial content, ATP production, membrane potential and ROS production as a MuSC goes from quiescence to activation, to finally differentiation. Created using BioRender.

To maintain quiescence and stemness, most stem cells have low mitochondrial metabolism with inhibition of OXPHOS through different mechanisms[90–93]. Studies have shown that mitochondrial carrier homolog 2 (MTCH2) suppresses OXPHOS function within stem cells and its loss leads to a promotion of mitochondrial respiration and premature HSC differentiation[93–95]. Other proteins involved in the inhibition of OXPHOS function through suppression of glucose oxidation and ATP-synthase activity include uncoupling protein-2 (UCP2) and inhibitory factor 1 (IF1)[96–99]. Through these studies, it is indicated that oxidative phosphorylation is actively repressed in stem cells and plays a key role in stem cell differentiation[91–100]. Maintenance of quiescence and stemness has been associated with improved mitochondrial function highlighting the role of mitochondrial activity and metabolism being crucial in stem cell fate decisions[86,93,101–104]. Moreover, ROS activity has also been linked with stem cell fate where low ROS levels maintain quiescence and self-renewing stem cell capacity whereas increased levels of ROS promote stem cell differentiation and proliferation[91,100,101,105]. Suppression of ROS through different mechanisms also occurs in stem cells to maintain their quiescent state[91,100]. Furthermore, fatty acid oxidation (FAO), occurring within the mitochondria, is also known to be involved in stem cell pool maintenance and function[106]. Driven by mitochondrial bioenergetics and dynamics, fatty acid oxidation has shown to promote the self-renewal of ESCs, NSCs and HSCs[106–108]. Suppression of FAO has shown to cause commitment of HSCs leading to a decline in the stem cell pool[109]. Thus, it is imperative to regulate fatty acid oxidation through mitochondrial dynamics for the maintenance of stem cell pluripotency.

Through these studies it can be determined that stem cells have low mitochondrial abundance with underdeveloped cristae, low network complexity, low rates of OXPHOS with a reliance on FAO and low ROS emissions[69,70,75–83,85,86,88–111]. Deviating from these properties leads to stem cell activation,

commitment, proliferation, and differentiation, as mitochondria act as signalling hubs that receive and generate signals imperative in the regulation of cell fate decisions [69,70,75–83,85,86,88–111].

While several studies are focused on the role of mitochondrial structure, content, and activity in stem cell fate, the role of mitochondrial quality control in MuSC fate is not well understood.

1.5 Mitophagy

Recent studies have linked the accumulation of dysfunctional mitochondria and improper mitochondrial phenotype in each state, to stem cell aging and impaired self-renewal thus, it is imperative to prevent the accumulation of such mitochondria through different quality control mechanisms including mitophagy to maintain cellular homeostasis[69,71,112–114]. Mitophagy is a mitochondrial quality control mechanism responsible for the selective removal of damaged and dysfunctional mitochondria to regulate cellular homeostasis[115–118]. It is a mitochondrial turnover mechanism which selectively targets and degrades the damaged and dysfunctional mitochondria[119].

Mitophagy is responsible for basal mitochondrial turnover but can also be induced in pathophysiological conditions[120]. It is activated during the development and differentiation of different cell types and functions as a stress-response mechanism including depolarization, oxidative stress, hypoxia etc.[120]. The mechanism for mitophagy involve the sensing, sequestration, and trafficking mitochondria to lysosomes for degradation[120,121]. Mitophagy can be stimulated through different mechanisms and multiple signalling cascades with the regulatory pathways classified as ubiquitin-dependent or ubiquitin-independent which then interact with the light chain 3 (LC3) protein on the autophagosome membrane[120,122]. All mitophagy adaptors have an LC3-interacting region (LIR) motif which binds to the LC3 on autophagosomes which subsequently grows around the mitochondria engulfing them within the autophagosome[120]. Mitochondrial fission also occurs during mitophagy as the mitochondrion becomes altered to be engulfed

into the autophagosome[123]. Finally, fusion of the autophagosome with the lysosome results in the elimination of the targeted dysfunctional mitochondria[120].

1.5.1 Pink1/Parkin mediated mitophagy

One of the key pathways of mitophagy is mediated by the phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK1) and the E3-ubiquitin ligase Parkin (PRKN) known as the PINK1-Parkin mitophagy[122,124,125]. It regulates the ubiquitin dependent mitophagy necessary for several mitochondrial physiological processes including mitochondrial dynamics, mitochondrial biogenesis, and transport and recruitment of autophagic machinery[122,124,125]. In functional mitochondria, PINK1 is translocated to the inner mitochondrial membrane (IMM) where it is cleaved by different proteases and the truncated PINK1 is degraded by the ubiquitin-proteosome system[122,124] **(Fig. 3)**. Following a loss of MMP, uncleaved PINK1 is stabilized on the outer mitochondrial membrane (OMM) which then promotes the recruitment of the E3 ligase-PARKIN via phosphorylation of surface proteins on the mitochondrial surface[120,122,124] **(Fig. 3)**. Moreover, *PINK1* also phosphorylates ubiquitin (Ub) and poly-ubiquitin chains on dysfunctional mitochondria that bind *PARKIN* to facilitate its activation and the activated *PARKIN* further generates poly-Ub chains, amplifying the mitophagy signals[122,124]. Activated *PARKIN* drives the ubiquination of different substrates including mitofusin 1 (MFN1), mitofusin 2 (MFN2), voltage-dependent anion channel (VDAC), ras homolog family member T (RHOT/MIRO), and BAK proteins to aid in the elimination of the damaged mitochondria[124]. Different autophagy adaptors including nucleoporin 62 (p62), neighbor of breast cancer gene 1 (BRCA1) gene 1 (NBR1), nuclear domain 10 protein 52 (NDP52) and optineurin (OPTN) recognize and bind the polyubiquitinated chains to the LC3-ninteracting region (LIR) mediating the engulfment of the damaged mitochondria into autophagosomes[120,124] **(Fig. 3)**. Deubiquitinating enzymes including USP15, USP30 and USP35 eliminate PRKN-generated Ub chains, preventing mitophagy[124].

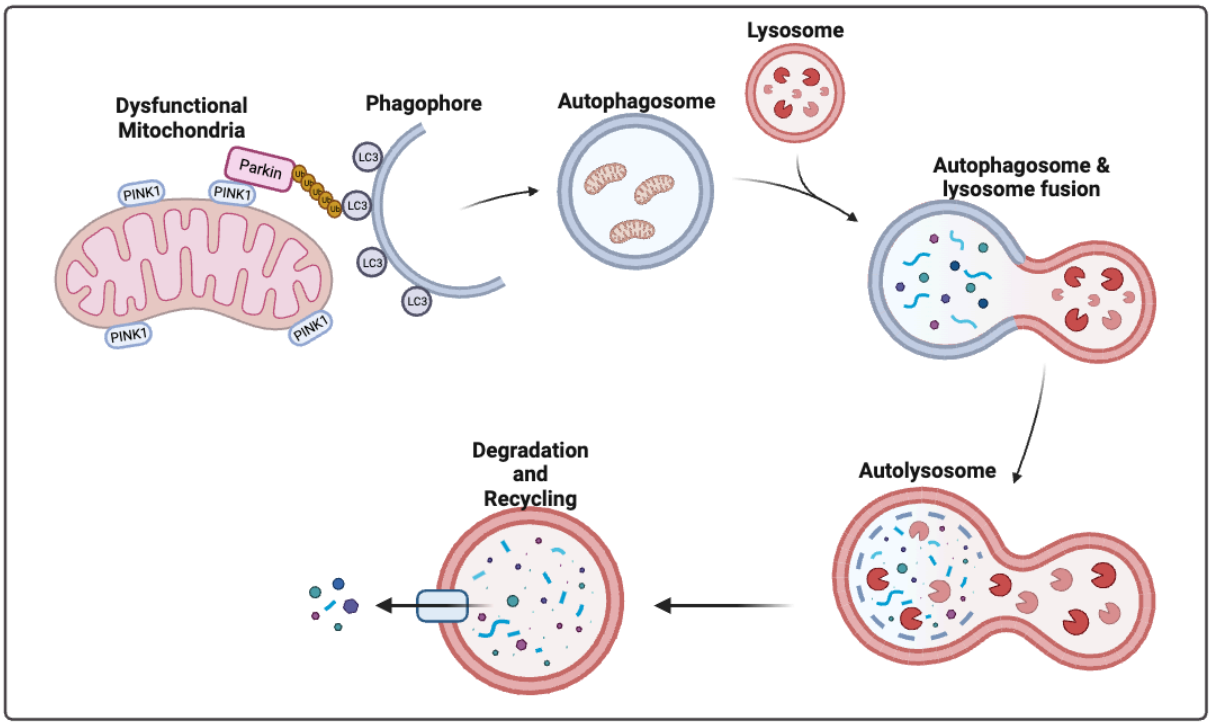

Lysosome
Dysfunctional Mitochondria
Phagophore
Autophagosome
Autophagosome & lysosome fusion
Parkin
PINK1
PINK1
PINK1
PINK1
LC3
LC3
LC3
LC3
Degradation and Recycling
Autolysosome

Figure 3. Schematic of Pink1-Parkin mediated mitophagy.

Stages of a dysfunctional mitochondria going through mitophagy, being engulfed by a phagophore and fusing with a lysosome to go towards degradation. Created using BioRender.

1.5.2 Pink1/Parkin independent mitophagy

Although Pink1/Parkin dependent mitophagy is well studied, Pink1/Parkin independent mitophagy pathways are also being looked at for cellular homeostasis. These pathways primarily rely on receptor proteins that directly interact with the LC3 or the LC3 homolog, γ-Aminobutyric acid type A (GABAA) receptor-associated protein (GABARAP) through their LIR motifs to activate mitophagy[120,122,124]. These LIR-containing proteins include B-cell leukemia/lymphoma 2 (BCL2) interacting protein 3 (BNIP3), NIX also known as BCL2 Interacting Protein 3 Like (BNIP3L), FUN-14 domain containing protein 1 (FUNDC1), autophagy and beclin 1 regulator 1 (AMBRA1) etc.[124,126,127]. BNIP3, BNIP3L and FUNDC1 all play a role in hypoxia-mediated mitophagy[124–130]. Moreover, BNIP3 promotes mitochondrial fragmentation through interactions with OPA1, inducing mitophagy, while BNIP3L is important in programmed mitophagy during cell differentiation and maturation[124,125,127,129,130]. FUNDC1 interacts with mitochondrial fission and fusion proteins DNM1L and OPA1 stimulating mitophagy[124,128,131]. Finally, AMBRA1 is involved in both Pink1/Parkin-mediated ubiquitin dependent and receptor mediated mitophagy[124,128,132,133]

A balance amongst the ubiquitination and deubiquitination of PRKN-dependent mitophagy is crucial for mitochondrial and cellular homeostasis.

1.6 Mitophagy and Stem Cells

Studies have shown that macro-autophagic activity plays a pivotal role in stem cell fate and is required for stem cell quality control and maintaining their cellular homeostasis[134–136]. A study by García-Prat, L. *et al.* reported that basal autophagic activity occurs in quiescent MuSCs, suggesting a role of mitophagy in the maintenance of a MuSC quiescence state[137]. It was also reported that a loss of autophagy through inactivation of *ATG7* in MuSCs caused increased mitochondrial dysfunction and oxidative stress leading to senescence, MuSC pool exhaustion and defects in muscle regeneration[137]. Studies have also shown that mitophagy signalling is crucial during stem cell differentiation [138]. Moreover, Mohiuddin *et al.*

demonstrated that levels of mitophagy change at different stages of muscle repair following injury, suggesting a role of mitophagy in muscle regeneration[139]. Furthermore, Liang *et al.* demonstrated an increased recruitment of Parkin and enhanced colocalization of TOM20 and LAMP1 in quiescent self-renewing HSCs suggesting a role of mitophagy in the maintenance of a quiescent HSC state[69,140]. Similarly, a study conducted by Wen Yin and colleagues demonstrated that the quiescent state of mesenchymal stem cells (MSC) is maintained and controlled by mitophagy and any dysregulation in mitophagy can cause MSC apoptosis and senescence[141]. More poignantly, PINK1-Parkin mediated mitophagy has been shown to have a protective role in transplanted bone marrow MSCs against stress induced apoptosis and senescence[72]. It has also shown to protect MSCs from hyperglycemia-induced injury due to mitochondrial dysfunction and decrease apoptosis in endothelial cells in diabetic rats[142]. Although several studies have looked at the role of mitophagy in stem cell homeostasis, the role of mitophagy as a quality control mechanism in MuSCs is unclear and needs to be examined.

1.7 Previous Work

Studying the role of mitophagy in muscle stem cells, our lab found that mitophagy is prominent in the quiescent state of MuSCs and rapidly downregulated upon activation and that PINK1 plays a role in maintaining mitophagy in the quiescent state. Moreover, to study the impact of mitophagy on MuSC fate decisions, our lab developed a germline Pink1 knockout mouse model. Using the cardiotoxin (CTX) injury model and the single myofiber culture, previous members of our lab demonstrated that impairing PINK1 promotes muscle stem cell commitment at the expense of self-renewal, indicative of altered fate decisions. Furthermore, it was also shown that PINK1 loss also leads to a progressive decline in the muscle regenerative capacity. Altogether, these datasets suggests that disruption of mitochondrial quality control mechanism, such as mitophagy, may play a significant role in MuSC function. However, as the PINK1 mouse model used was a germline full body knockout, compensatory up-regulation of PINK1 independent pathways, and problems with the immune system response may occur. Thus, to accurately

evaluate the role of Pink1-Parkin mitophagy in MuSCs, our lab developed a tamoxifen inducible MuSC specific conditional knockout of Parkin.

2 HYPOTHESIS:

Mitochondrial maintenance, and remodelling are important regulators of different types of stem cells fate and function including MuSCs. Accumulation of damaged mitochondria has shown to cause stem cell senescence, premature commitment, and impaired self-renewal. It is imperative to clear dysfunctional mitochondria through quality control mechanisms including mitophagy to ensure optimal stem cell fate and function, maintaining cellular homeostasis. Thus, we hypothesize that inhibition of Parkin-mediated mitophagy causes an imbalance in MuSC fate decision which leads to impaired muscle regeneration.

3 OBJECTIVES:

3.1 Determine the effect of Parkin ablation within MuSCs on their fate decision and lineage progression.

3.2 Determine the effect of loss of Parkin in MuSCs on muscle injury and muscle regeneration.

4 METHODS

4.1 Mouse Model

4.1.1 Parkin KO mice

Parkin$^{Flx/Flx}$ mice, with flanked exon 7, was developed and obtained from Lexicon Pharmaceuticals to study the effect of a loss of Parkin in adult muscle stem cells. The Parkin$^{Flx/Flx}$ mice were crossed with Pax7 CreER$^{T2+/-}$ mice to develop a Cre-flox conditional Parkin exon 7 KO mouse model in our lab. At 7-10 weeks of age, 200mg/kg of tamoxifen was administered to Parkin$^{WT/WT}$, Pax7 Cre$^{+/-}$ mice (Control) and Parkin$^{Flx/Flx}$, Pax7 Cre$^{+/-}$ mice ($_{MuSC}$Parkin$^{-/-}$) via gavage for five consecutive days to activate the cre-recombinase. A period of three days of rest for gene deletion was ensured before any experimental procedures were carried out. Both male and female mice were used for all experimental procedures.

4.1.2 Quantitative RT-PCR

Total RNA was isolated from muscle stem cell pellets using the PicoPure RNA isolation kit (Thermo Scientific KIT0204) according to manufacturer's recommendations. Isolated RNA was quantified using the nano-drop 2000 spectrophotometer, and reverse transcribed with the iScript Reverse Transcription Supermix for Rt-qPCR (Bio-Rad 1708840) according to manufacturer's instructions. cDNA was reverse transcribed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad 1725271) according to manufacturer's instructions and RT-qPCR was performed in a CFX96 real time PCR detection system at the University of Ottawa.

4.2 Cardiotoxin preparations and injections

Cardiotoxin (Latoxan L8102) was prepared by dissolving it in saline (0.9% NaCl) to a final concentration of 10uM. Mice were injected subcutaneously with Buprenorphine and anesthetized by gas inhalation for

30 minutes prior to the cardiotoxin injections. 50 μL of the 10 μM cardiotoxin was injected, using an insulin-type disposable syringe, 1-2mm deep into the right tibialis anterior (TA) muscle. The contralateral uninjured muscle was used as a control. Following the injections, mice were allowed to recover in a heated room and transferred to a clean cage for muscle regeneration until the time to harvest the muscle. All procedures were approved by the University of Ottawa's Animal Care and Veterinary Services (ACVS) committee and complied with the guidelines of the Canadian Council on Animal Care and the Animals for Research Act.

4.3 Muscle Tissue Harvesting

At the time of the tibialis anterior (TA) muscle harvesting, mice were sacrificed by cervical dislocation. The TA was dissected, weighed, and cut in half in a cross-sectional orientation. One half of the muscle was flash frozen by embedding it in optimal cutting temperature compound (OCT) (Fisher 23-730-571) and immediately immersing it in ice-cold isopentane using liquid nitrogen for 30 seconds and stored in -80C for sectioning and subsequent staining procedures. The other half of the muscle was immersed in freshly prepared cold 2% (w/v) paraformaldehyde (PFA) (Sigma P6148) and fixed for 30 minutes, shaking on ice. Following fixation, the muscle was washed twice with phosphate buffered saline (PBS) for 5 minutes and twice in glycine for 10 minutes. The muscle was then treated with 5% (w/v) sucrose for 2 hours, followed by 20% sucrose (w/v) for 2-3 days to cryopreserve the tissue. The TA muscle was them embedded in OCT and frozen in ice-cold isopentane using liquid nitrogen for 30 seconds and stored at -80°C for sectioning and subsequent staining procedures.

4.4 MuSC isolation

4.4.1 Magnetic Activated Cell Sorting (MACS)

MuSCs isolated through MACS was performed using an isolation kit purchased for Milteny Biotech (Milteny 130-042-501). Following cervical dislocation, hindlimb muscles of mice were harvested, minced, and digested in 1% (w/v) Collagenase-B (Roche 11088831001) and 0.4% (w/v) Dispase II (Roche 04942078001) using the Milteny MACS Octo-dissociator SLICE_FACS program for 27 minutes. Post digestion, the muscle slurry was filtered through a 70um filter (Milteny 130-110-916) and centrifuged for 10 minutes at 600g to obtain a cell pellet. The cell pellet was treated with red blood cell lysis buffer (Sigma R7757) for 30 seconds, followed by a PBS wash and a subsequent spin at 600g for 10 minutes. The cell pellet was resuspended in 1mL of FACS buffer (3mM EDTA, 10% (v/v) FBS in 1xPBS) and MuSCs were negatively selected for by incubating the solution with the Satellite Stem Cell Isolation antibody (Milteny 130-104-268) for 30 minutes on ice in a dark environment. The MuSC solution was filtered through the negative selection magnetic LS column (Miltenyi,130-042-401) capturing cells bound to the negative selection antibody. The flowthrough was then incubated for 30 minutes on ice in a dark environment with an Anti-Alpha Integrin-7 muscle stem cell selection antibody (Milteny 130-104-261) for MuSC selection. The solution was filtered through the positive selection magnetic MS column (Mitenyi 130-042-201), with the magnets capturing MuSCs. The MuSCs were stored in -80°C for subsequent experiments.

4.4.2 Fluorescence Activated Cell Sorting (FACS)

Following cervical dislocation, hindlimb muscles of mice were harvested, minced, and digested in 1% (w/v) Collagenase-B (Roche 11088831001) and 0.4% (w/v) Dispase II (Roche 04942078001) using the Milteny MACS Octo-dissociator SLICE_FACS program for 27 minutes. Post digestion, muscle slurry was filtered and spun to obtain a cell pellet which was subsequently treated with red blood cell lysis

buffer (Sigma R7757) for 30 seconds. The pellet was then washed with PBS and spun at 600g for 10 minutes. The cell pellet was resuspended in 1mL of FACS buffer (3mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) FBS in 1xPBS) with conjugated antibodies (phycoerythrin (PE)-conjugated antibodies: stem cell antigen-1 (Sca-1) (BD Pharmingen, 553108), cluster of differentiation 45 (CD45) (BD Pharmingen, 553081), cluster of differentiation 31 (CD31) (BD Pharmingen, 553373) and cluster of differentiation 11b (CD11b) (eBiosciences, 12-0112-82) to remove non-MuSCs present in the population, and a- integrin-7 (Ablab, 67-0010-05) and VCAM-1 (Biolegend, 105720) to mark MuSCs). Cell samples were sorted through FACS at the Flow Cytometry facility at the Ottawa Hospital Research Institute.

4.5 Single Myofiber Isolation

At the time of harvest, mice were sacrificed by cervical dislocation and the extensor digitorum longus (EDL) muscles were harvested for *in vitro* single myofiber isolation. The muscles were immediately digested in 0.5% (w/v) collagenase B (Roche 11088831001) for 30-45 minutes at 37C. The digested EDL muscle was then triturated using fetal bovine serum (FBS)- coated tips in wash media (DMEM containing 4.5g/L glucose and 1% (w/v) penicillin-streptomycin) to release individual myofibers. The myofibers were then washed three times in wash media to remove cellular debris attached to the myofibers. For T1 (timepoint 1 hour post isolation) myofibers were then fixed in warmed 2% (w/v) PFA for 10 minutes followed by three PBS washes and stored in PBS at 4°C until subsequent staining procedures. For timepoints 24, 48, 72 and 96 hours post isolation (T24, T48, T72, and, T96 respectively), post trituration, myofibers were cultured in single myofiber culture media (DMEM 4.5 g/L glucose, 20% (v/v) FBS, 2% (v/v0 chicken embryo extract, 1% (v/v) penicillin-streptomycin, 7.5ng/mL basic fibroblast growth factor (bFGF)) and incubated in a culture incubator at 37C, 5% CO_2 for 24, 48, 72 and, 96 hours. After desired

culture time, single myofibers were fixed in 2% (w/v) PFA for 10 minutes followed by three PBS washed and stored in PBS at 4°C until subsequent staining procedures.

4.5.1 EdU treatments of myofibers

EdU (Sigma, BCK-EDU647) was prepared at a concentration of 10mM and dissolved in DMSO (Sigma, D2438-5X10ML) through agitation. EdU was subsequently frozen at -20C in aliquots. Single myofibers were treated with EdU at different timepoints at a final concentration of 10uM for 2 hours prior to fixation before subsequent staining procedures.

Single EDL fibers were isolated as previously described and cultured in EDL culture media for 24 hours. 1 hour prior to harvest, 500 mL of culture media was removed from each well, and 500 mLof pre-warmed culture media containing 20 mM EdU dissolved in DMSO was added, at a final concentration of 10 mM EdU per well. Fibers were left to incubate with 10 mM EdU for 1 hour and the harvested and fixed in 2% PFA. Fibers were then permeabilized and blocked as previously described. Prior to primary antibody staining, the EdU detection reaction was carried out using the BaseClick EdU detection kit (Sigma, BCK647-IV-IM-M) following the manufacturer's protocol. Briefly, 500 mL of reaction cocktail was added to each well and the reaction took place on a rocker protected from darkness at room temperature for 30 minutes. Following this, fibers were washed in PBS 3x5 minutes and proceeded to primary antibody staining.

4.6 Histology

4.6.1 Tissue sectioning

TA muscles were sectioned in a cross-sectional orientation at a thickness of 14μm using HM525NX Cryostat (University of Ottawa Histology Core) at -28°C and placed on charged slides (Fisher Scientific 22-037-246). Slides were stored at -80°C until subsequent processing and staining procedures.

4.6.2 Hematoxylin and Eosin Staining

Flash frozen TA muscles sections were fixed in 2% (w/v) PFA for 30 minutes and washed three times with PBS. Fixed tissue sections were stained for hematoxylin and eosin (H&E) by the Histology Core at the University of Ottawa. The stained tissue sections were then imaged on the EVOS FLAuto2 microscope at the University of Ottawa.

4.7 Immunofluorescence staining

4.7.1 Tissue sections

Sucrose treated frozen tissue sections were sent for antigen retrieval (citrate buffer at pH of 6.0) to the Histology Core at the University of Ottawa. The sections were subsequently blocked in 3% (w/v) bovine serum album (BSA) for one hour and incubated with primary antibodies overnight at 4°C (TABLE 2). Secondary antibody staining was conducted using a Biotin-Streptavidin interaction (Biotin: Jackson Immunoresearch, 115-065-205, Strepdavidin: Jackson Immunoresearch, 016-160-084) and/or fluorescence-conjugated antibody incubations (TABLE 2). Slides were mounted using InvitrogenTM ProLongTM Gold Anrifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific P36935). Stained tissue sections were allowed to cure overnight and subsequently imaged on the EVOS FLAuto2 microscope at the University of Ottawa.

4.7.2 Single myofibers

Single myofibers were permeabilized in a solution of 0.1% (v/v) triton and 0.1M glycine in PBS for 10 minutes at room temperature followed by blocking in 5% (v/v) horse serum, 2% (w/v) BSA and 0.1% (v/v) triton in PBS for 1hour at room temperature. The myofibers were then incubated with primary antibody overnight at 4°C (TABLE 2) with secondary antibody incubation the following day for 1 hour at room temperature. Stained myofibers were mounted using InvitrogenTM ProLongTM Gold Anrifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific P36935) on charged glass slides (Fisher Scientific 22-037-246) and cured overnight. The myofibers were imaged on the EVOS FLAuto2 microscope at the University of Ottawa.

4.7.3 Primary MuSCs.

Following FACS sorting, isolated MuSCs were cytospun onto charged glass slides (Fisher Scientific 22-037-246) and fixed with 4% (w/v) PFA for 15 minutes at room temperature. The cells were then washed with PBS thrice for 5 minutes each. Fixed MuSCs were then quenched with 50mM NH_4Cl for 10 minutes at room temperature and then washed thrice with PBS for 5 minutes each. The MuSCs were then permeabilized in 0.1% (v/v) triton in PBS for 10 minutes at room temperature and then washed in PBS, thrice for 5 minutes each. The cells were then blocked in 10% (v/v) FBS in PBS in PBS for 1 hour at room temperature. Cells were then incubated with primary antibodies for 1 hour at room temperature (TABLE 2) followed by secondary antibody incubation for 1 hour at room temperature in the dark. Stained cells were mounted using InvitrogenTM ProLongTM Gold Anrifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Scientific P36935) on charged glass slides (Fisher Scientific 22-037-246) and cured overnight. MuSCs were imaged on the LSM880 Airyscan Confocal Microscope at the University of Ottawa.

4.7.4 Mitophagy Assessment Imaris 3-D reconstruction

Following FACS sorting, primary MuSCs were stained with outer mitochondrial membrane protein TOM20 and autophagosome protein LC3 and imaged using the LSM880 Airyscan Confocal Microscope at the Cell Biology and Image Acquisition Core Facility at the University of Ottawa. Airyscan images were then processed and 3D reconstruction of mitochondria, autophagosomes and their co-localization were performed on the Imaris Software.

4.8 Statistical Analysis

Statistical analyses were realized using GraphPad Prism 8. Quantitative results were presented as means ± standard error of the mean (SEM). Statistical differences were assessed using an unpaired t-test for two groups and two-way analysis of variance (ANOVA) for multiple comparisons. Statistical significance is displayed as ns: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Minimum number of replicates per experiment varied across study (See individual figure legends).

TABLE 1. PRIMERS FOR GENOTYPING AND QUANTITATIVE POLYMERASE CHAIN REACTIONS.

Primer	Sequence (5'-3')		Method
	Forward	Reverse	
Parkin WT	TTACGTCCATCGTGGACAGC	TGGGCTGGGTGTTAGCCTT	Genotyping
Parkin KO	AATGGATGAGTTCAAGGTTGC ACAG	AACTCCAGAGCTAGGATAGGGCATA	Genotyping
Cre	GAACCTGATGGACATGTTCAG G	AGTGCGTTCGAACGCTAGAGCCTGT	Genotyping
Parkin	GAGCTTCCGAATCACCTGAC	CCATCTGGGAGCTAGGAATG	qPCR
HPRT	CAAACTTTGCTTTCCCTGGT	TCTGGCCTGTATCCAACACTTC	qPCR

TABLE 2. ANTIBODIES USED FOR FACS AND IMMUNOFLUORESCENCE.

Antibody	**Company/Catalogue Number**	**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-1)	Biolegend 105720	10 μL/mouse	FACS
647 conjugated alpha integrin-7 (rat)	Ablab 67-0010-05	10 μL/mouse	FACS
Pax7 (mouse)	DSHB PAX7-S	1:14	IF Primary
Pax7 (rabbit)	Invitrogen PA1-117	1:100	IF Primary
MyoD (mouse)	SantaCruz sc-32758	1:50	IF Primary
MyoG (mouse)	DHSB F5D	1:12	IF Primary
Ki67 (rabbit)	Abcam ab15580	1:500	IF Primary
eMyHC (mouse)	DHSB F1.652-s	1:10	IF Primary
Parkin (mouse)	ab77924	1:200	IF Primary
Alexa-Fluor 488 (rabbit)	Invitrogen A11008	1:1000	IF Secondary
Alexa-Fluor 647 (mouse)	Abcam AB-150107	1:1000	IF Secondary
Alexa-Fluor 594 (mouse)	Invitrogen A11005	1:1000	IF Secondary
Biotin	Jackson 115-065-205	1:1000	IF Secondary
Cy3-Conjugated Streptavidin	Jackson 016-160-084	1:1000	IF Secondary

5 RESULTS

5.1 Confirmation of Parkin knockout in MuSC

To study the impact of Parkin deletion on adult muscle stem cells, we developed a tamoxifen-inducible muscle stem cell-specific conditional knockout of Parkin. Before beginning the experiment, we wanted to validate the deletion of Parkin in our $_{MuSC}Parkin^{-/-}$ mouse model. Thus, qPCR was performed on MuSCs isolated from control and $_{MuSC}Parkin^{-/-}$ mice using magnetic beads cell sorting (MACS). Analysis of gene expression revealed a near complete absence of Parkin transcripts in the knockout mouse model **(Fig. 4)**.

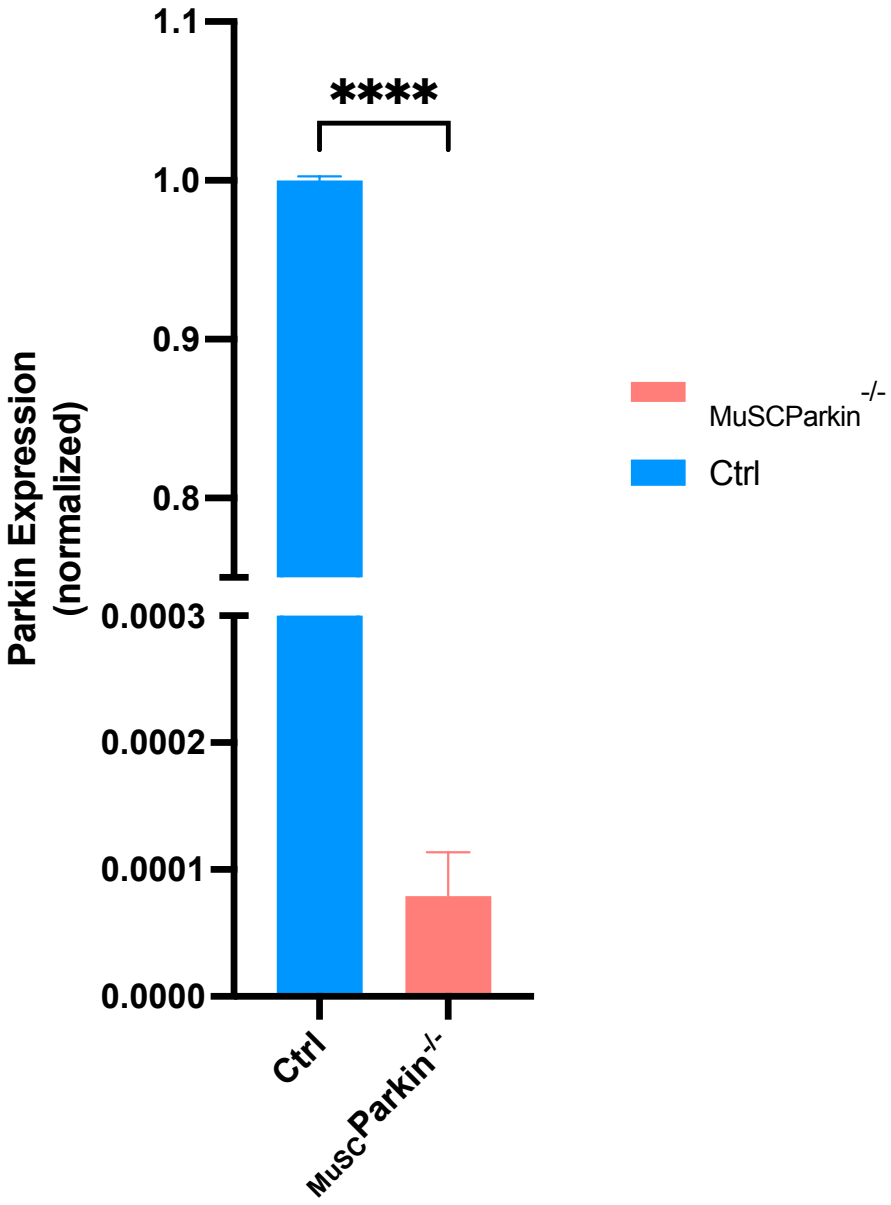

1.1
1.0
0.9
0.8
0.0003
0.0002
0.0001
0.0000
****
Parkin Expression
(normalized)
Ctrl
MuSCParkin-/-
MuSCParkin-/-
Ctrl

Figure 4. Gene expression analysis of control and $_{MuSC}$Parkin$^{-/-}$ MuSCs.

Validation of gene expression of Parkin in control and $_{MuSC}$*Parkin*$^{-/-}$ MuSCs normalized to housekeeping gene Hypoxanthine-guanine phosphoribosyltransferase (Hprt). Results represent mean ± SEM for n= 3 mice per group. Statistics were realised using an unpaired t test. Statistical significance is displayed as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

5.2 Mitophagy assessment in $_{MuSC}$Parkin$^{-/-}$ mice compared to controls.

To investigate the levels of mitophagy in $_{MuSC}$*Parkin*$^{-/-}$ mice compared to controls, MuSCs were isolated through FACS, fixed at isolation and five hours post isolation and, immunolabelled for Tom20 (outer mitochondrial membrane marker) and LC3 (autophagosome marker). The MuSC were imaged, and 3D reconstructed in Imaris for mitophagy assessment. A significant reduction in the colocalization of mitochondria and autophagosomes was observed in the $_{MuSC}$*Parkin*$^{-/-}$ cells in the quiescent and activated state **(Fig 5)**. These results are indicative of a reduction in mitophagy levels in $_{MuSC}$*Parkin*$^{-/-}$ MuSCs compared to controls.

a.
Quiescence
Activation
Control
MuSC Parkin-/-
Control
MuSC Parkin-/-

b.

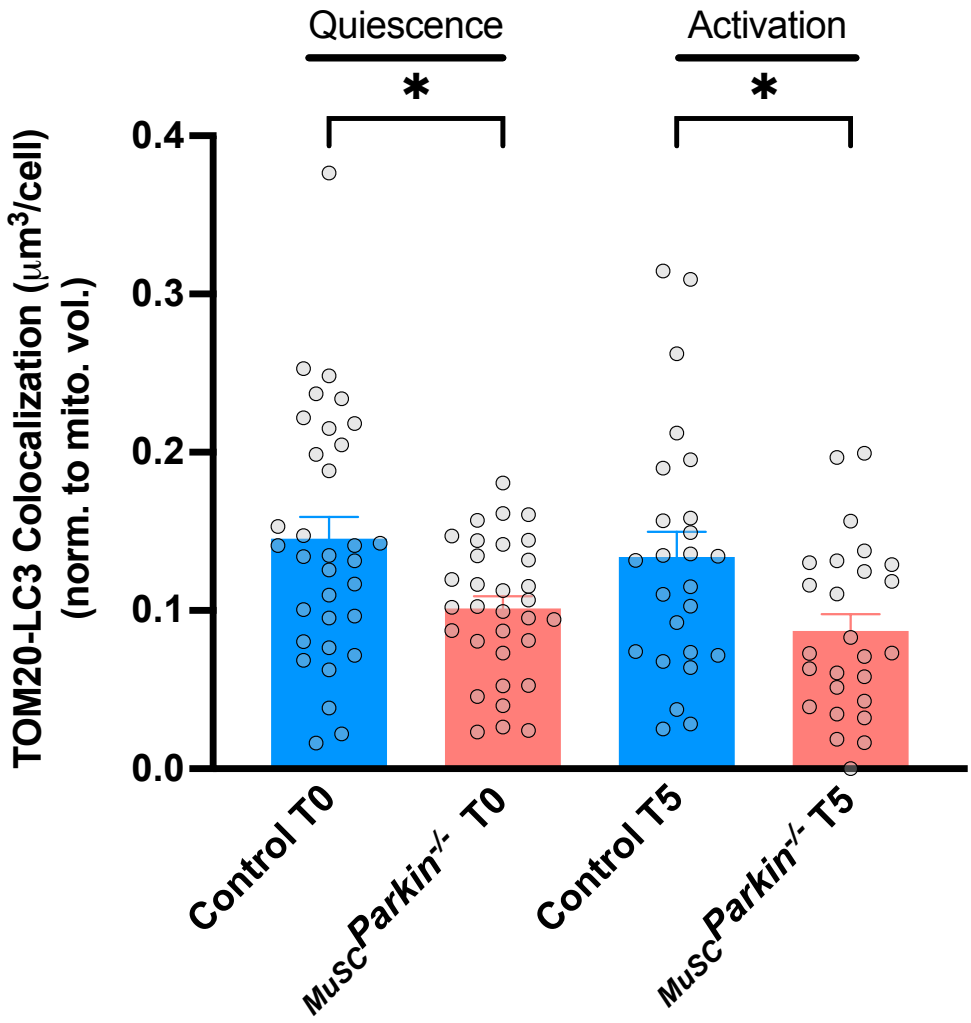

Quiescence
Activation
*
*
TOM20-LC3 Colocalization (μm³/cell)
(norm. to mito. vol.)
0.4
0.3
0.2
0.1
0.0
Control T0
MuSCParkin-/- T0
Control T5
MuSCParkin-/- T5

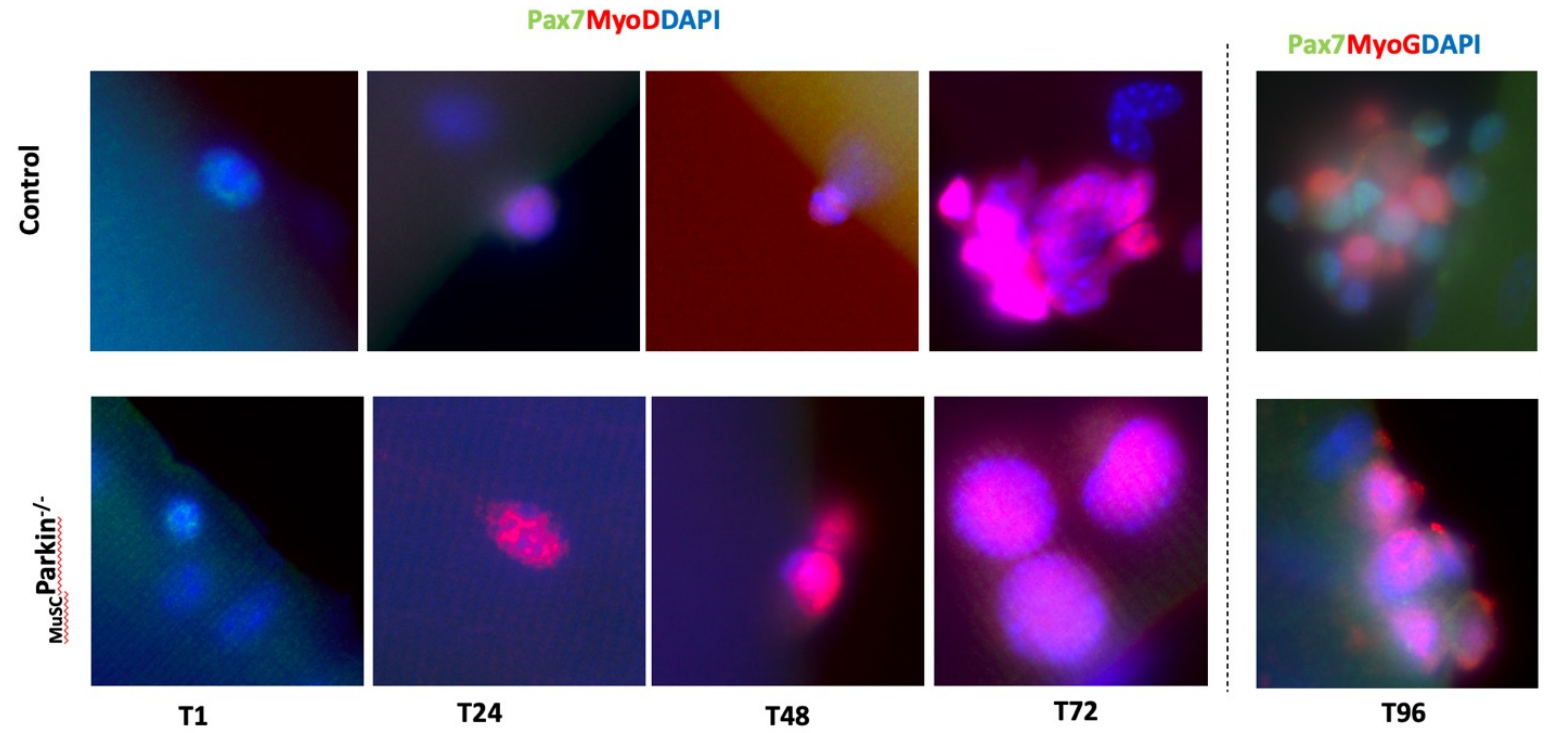
Figure 5. Parkin deficiency impairs mitophagy in $_{MuSC}$Parkin$^{-/-}$ MuSCs.

a. Imaris 3D reconstruction of quiescent and activated control and $_{MuSC}$Parkin$^{-/-}$ MuSCs stained with Tom20 (green) and LC3 (red) to evaluate colocalization (yellow) for mitophagy assessment. In the right end panels, mitochondria and autophagosomes have been removed to highlight changes in colocalization b. Colocalization of mitochondria and autophagosome in MuSCs normalized to mitochondria volume at T0 and T5 post isolation. Data is presented as mean ± SEM for n=35 cells purified from 2 mice per genotype. Statistics were realised using a one-way ANOVA test with data cleaned for outliers. Statistical significance is displayed as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

5.3 Parkin knockout leads to earlier commitment and activation of MuSCs in vitro

To determine if a Parkin deficiency in muscle stem cells affects their fate decision, EDL fibers from Control and $_{MuSC}Parkin^{-/-}$ mice were isolated, cultured, and fixed at different timepoints to track progression from quiescence to activation and early differentiation in vitro through Pax7, MyoD and MyoG immunostainings. 24 hours post isolation, a significant increase in the proportion of activated and committed cell ($Pax7^{+}/MyoD^{+}$ and $Pax7^{-}/MyoD^{+}$) along with a reduction in quiescent ($Pax7^{+}$)-cells were observed in $_{MuSC}Parkin^{-/-}$ compared to controls **(Fig. 6b)** suggesting earlier activation and commitment of MuSCs in absence of Parkin. This trend persisted at 48 hours post isolation where the proportion of cells expressing solely MyoD was increased in absence of Parkin, while the proportion of cells expressing solely Pax7 was reduced **(Fig. 6b)**. Moreover, 72 hours post isolation, no significant difference amongst the MuSC population proportions was observed however, a significant decrease in the number of MuSC populations was observed **(Fig. 6b, 6c)**. Furthermore, 96 hours post isolation, a significant increase in the proportion of cells expressing $MyoG^{+}$ was observed in $_{MuSC}Parkin^{-/-}$ along with a reduction in the proportion of Pax7 expressing cells **(Fig. 6b)**, indicative of a premature differentiation in absence of Parkin. Collectively these results suggest that a loss of Parkin in MuSCs promotes earlier activation, commitment, and differentiation at the expense of self-renewal.

a.


Pax7MyoDDAPI
Pax7MyoGDAPI
Control
MuSC Parkin-/-
T1
T24
T48
T72
T96

b.

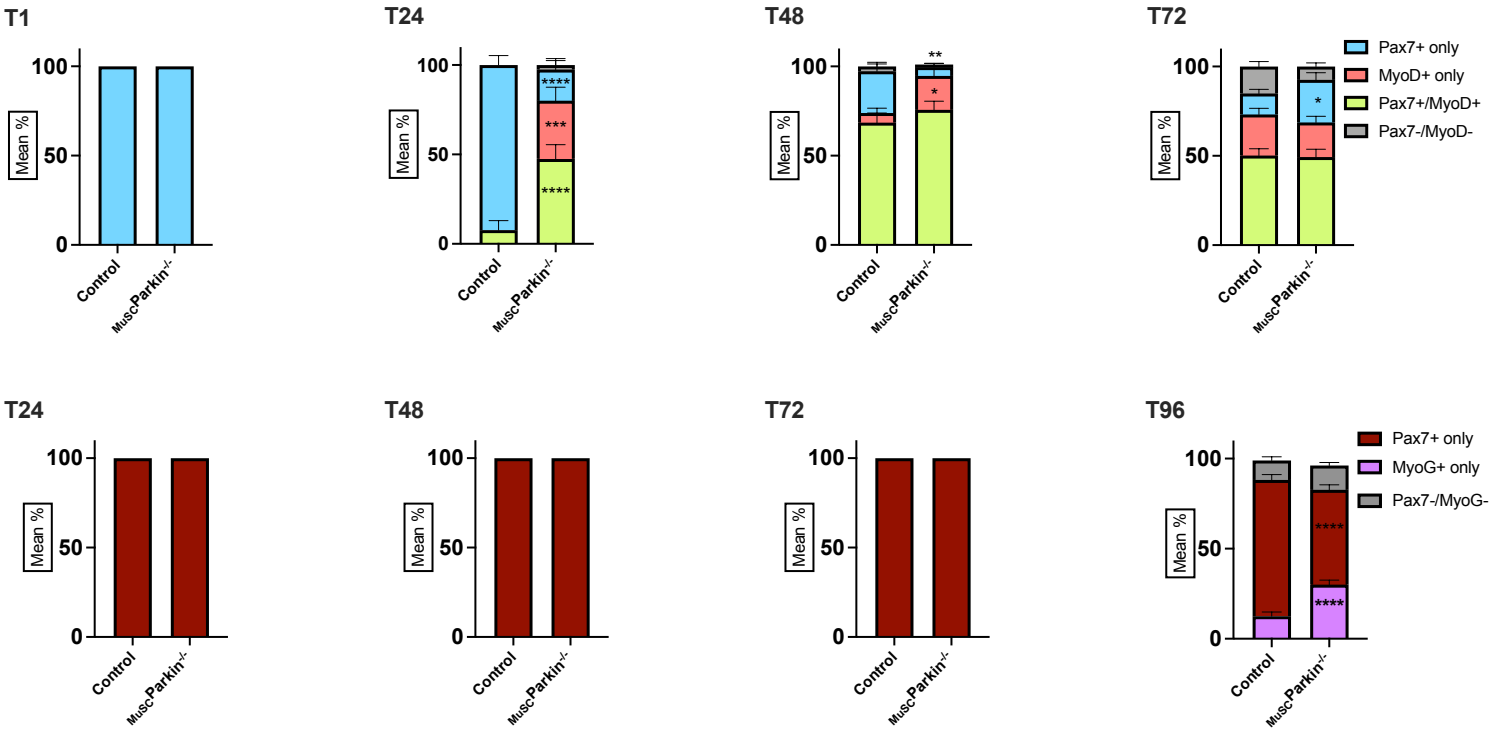
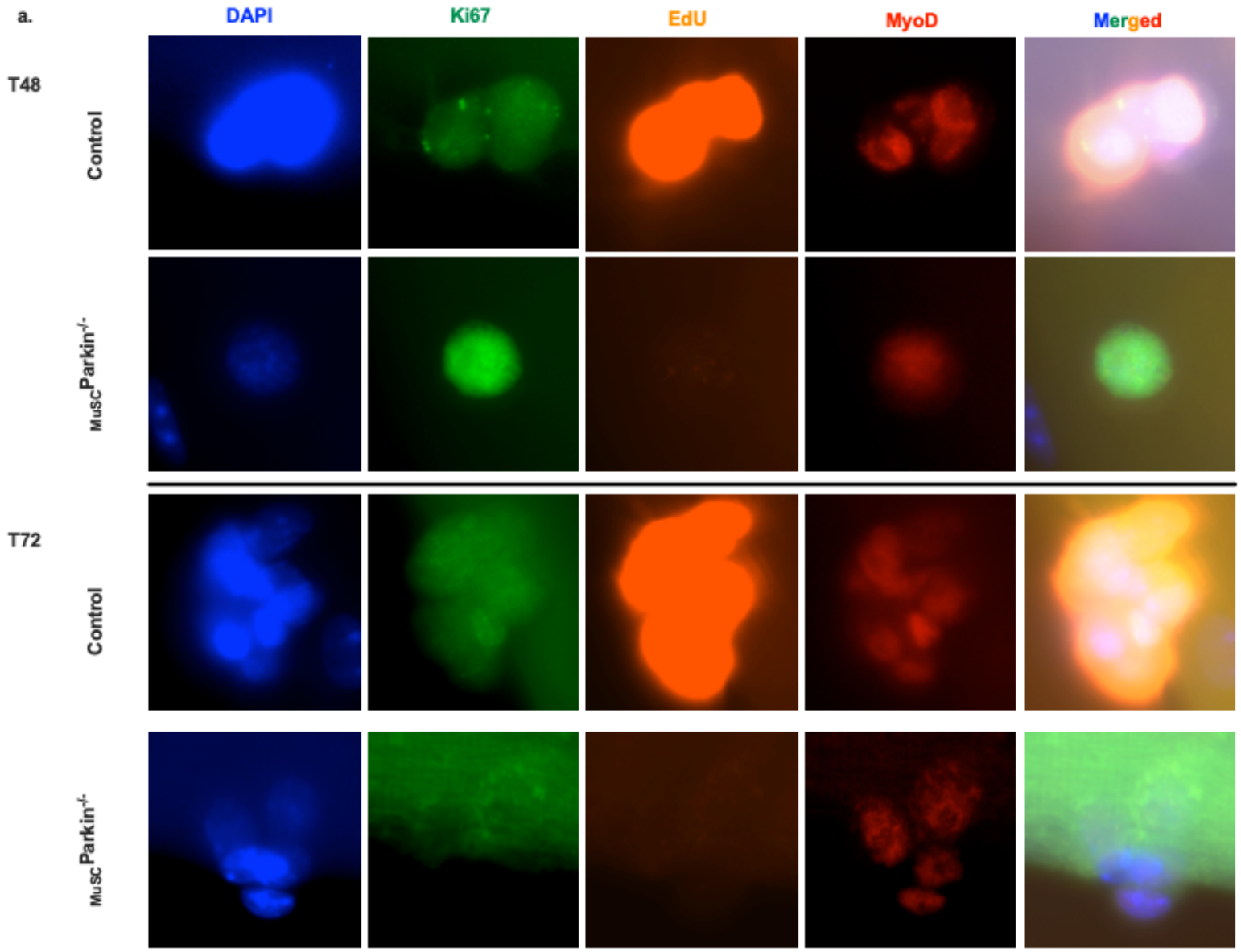

T1
T24
T48
T72
T96
Mean %
100
50
0
Control
MuSC Parkin-/-
Pax7+ only
MyoD+ only
Pax7+/MyoD+
Pax7-/MyoD-
MyoG+ only
Pax7-/MyoG-
****
***
**
*

Figure 6. Parkin deficiency in vitro promotes commitment and impairs self-renewal of MuSCs.

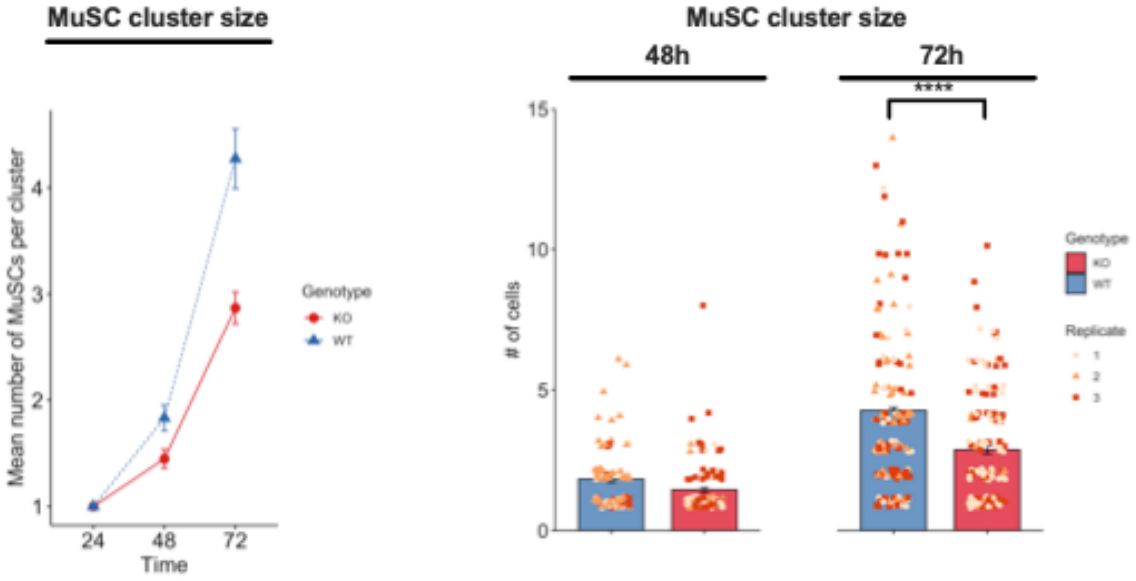
a. Representative epifluorescence images of control vs $_{MuSC}Parkin^{-/-}$ satellite cells on EDL single fibers at different isolation timepoints. Magnification: 100X. b. Percentage of cells per fiber in control vs $_{MuSC}Parkin^{-/-}$ EDL single fibers at T1, T24, T48, T72 and T24, T48, T72, T96 post isolation. Data is presented as mean ± SEM for n=4. Statistics were realised using a two-way ANOVA test. Statistical significance is displayed as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

5.4 Ablation of Parkin leads to impairments in cell cycle progression of MuSCs.

While analyzing the MuSC fate decisions data, a persistent decrease in the number of MuSCs per cluster at 72 hours post isolation was observed in the $_{MuSC}Park2^{-/-}$ mice suggesting impaired proliferation **(Fig. 7b).** Thus, to further investigate the proliferation differences and cell cycle entry in the Parkin deficient mice, EDL fibers from Control and $_{MuSC}Park2^{-/-}$ mice were isolated, cultured, and fixed at different timepoints to track the proliferation and cell cycle re-entry of the MuSCs. MuSCs were incubated with EdU 2 hours pre fixation, fixed at different timepoints, and immunolabelled with MyoD and Ki67. At 48 hours post isolation, no significant differences between genotypes were found in the MuSC cluster size, actively proliferating MuSCs ($Ki67^{+}/EdU^{+}$), non-proliferating MuSCs ($Ki67^{-}/EdU^{-}$), and MuSCs in arrest post division ($Ki67^{-}/EdU^{+}$) however, a significant increase in $Ki67^{+}/EdU^{-}$ MuSCs was observed in MuSCs from $_{MuSC}Park2^{-/-}$ mice suggesting an increased retention of MuSCs in the G1 phase **(Fig. 7b, 7c)**. Furthermore, at 72 hours post isolation, a significant decrease in the MuSC cluster size, and $Ki67^{+}/EdU^{+}$ MuSCs was observed in $_{MuSC}Park2^{-/-}$ fibers indicating impairments in actively proliferating MuSCs **(Fig. 7c)**. Moreover, a significant increase in the non-proliferating MuSCs ($Ki67^{-}/EdU^{-}$) and MuSCs in arrest post first round of division ($Ki67^{-}/EdU^{+}$ MuSCs) was also observed in the Parkin deficient mice **(Fig. 7c).** No differences in Parkin deficient $Ki67^{+}/EdU^{-}$ MuSCs was observed at 72 hours **(Fig. 7c)**. This data suggests delayed cell cycle re-entry and increased cell cycle arrest after 1st division that underlie the proliferative defect observed in the Parkin deficient MuSCs.


a.
DAPI
Ki67
EdU
MyoD
Merged
T48
Control
MuSCParkin-/-
T72
Control
MuSCParkin-/-

b.


MuSC cluster size
Mean number of MuSCs per cluster
Time
24
48
72
Genotype
KO
WT
MuSC cluster size
48h
72h
****
# of cells
Genotype
KO
WT
Replicate
1
2
3

c.

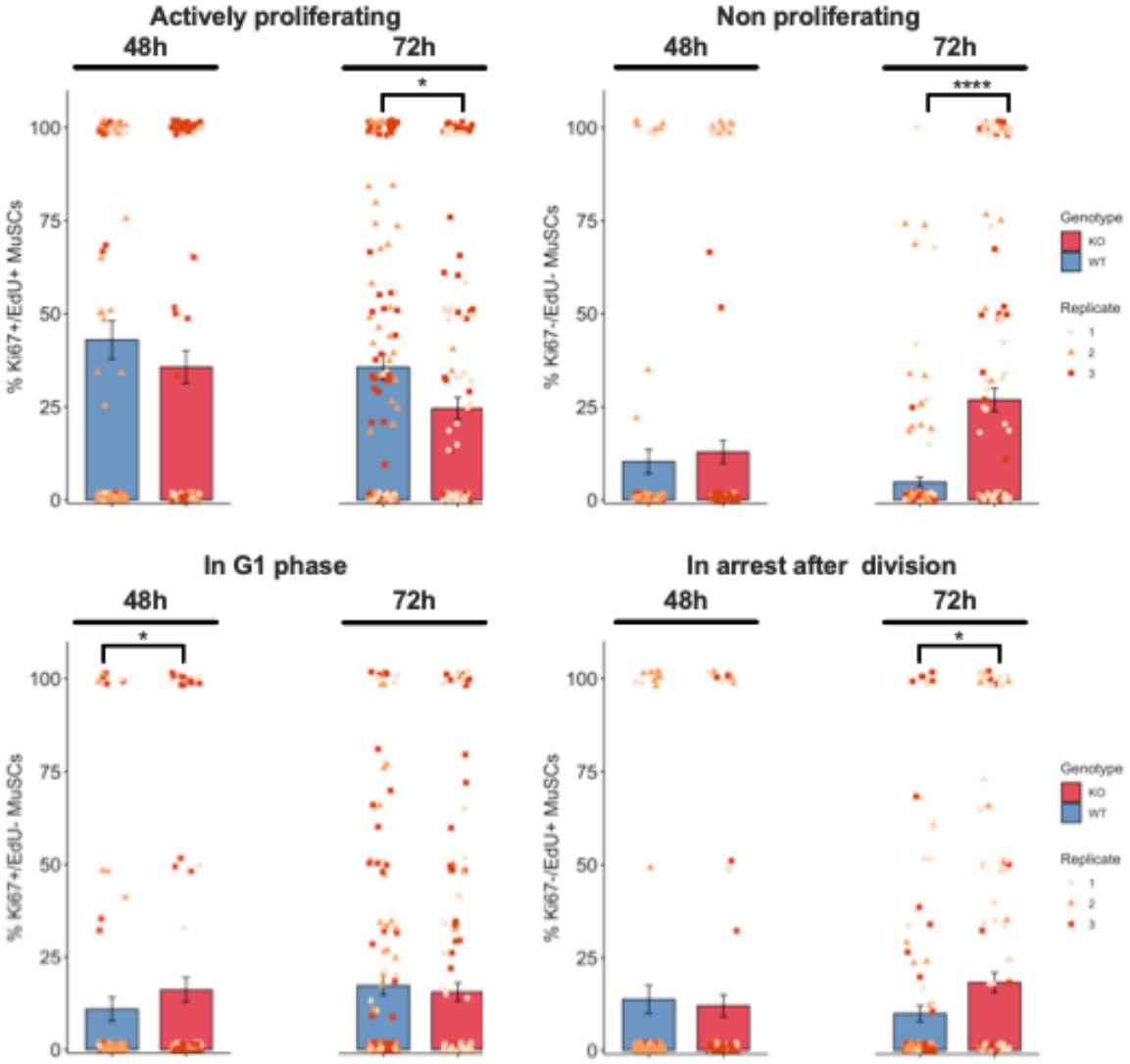

Actively proliferating
48h
72h
*
% Ki67+/EdU+ MuSCs
Non proliferating
48h
72h
****
% Ki67-/EdU- MuSCs
In G1 phase
48h
72h
*
% Ki67+/EdU- MuSCs
In arrest after division
48h
72h
*
% Ki67-/EdU+ MuSCs
Genotype
KO
WT
Replicate
1
2
3

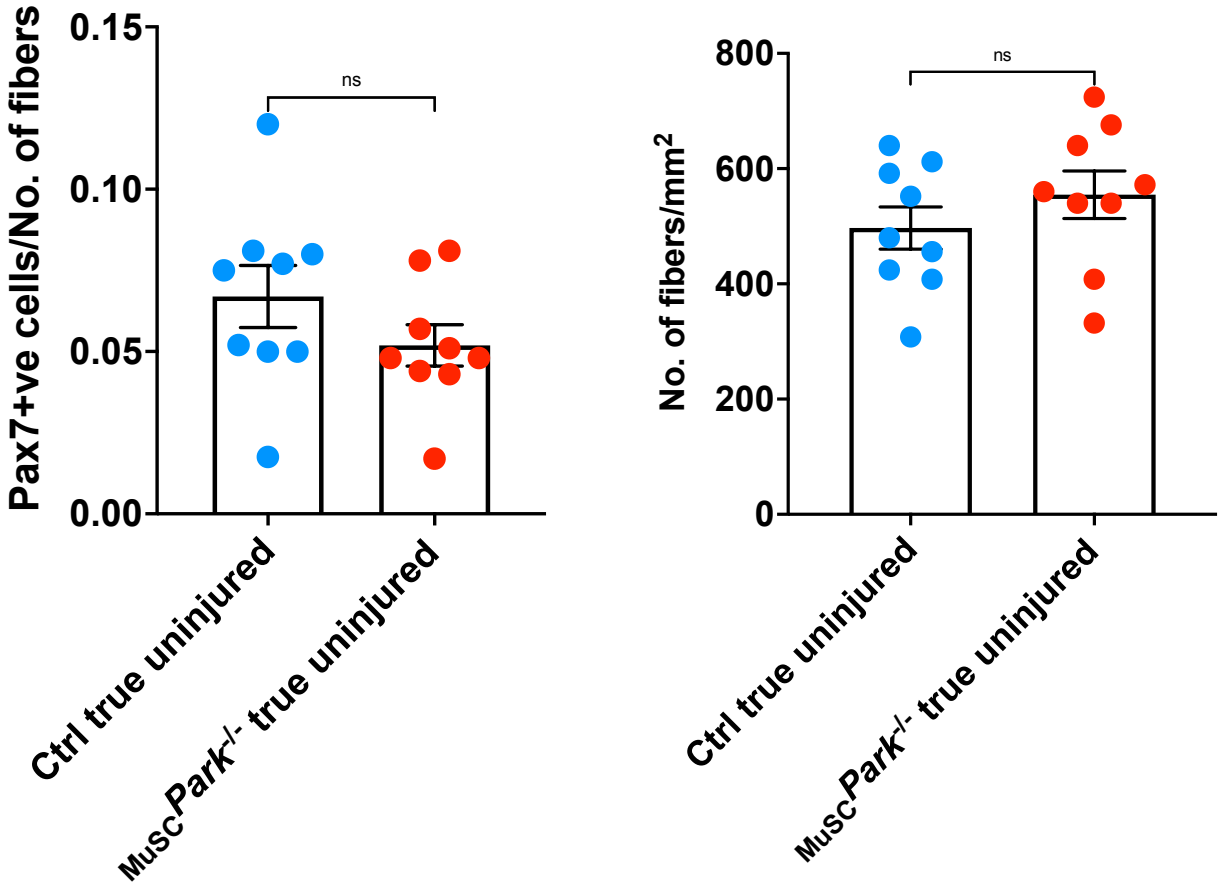
Figure 7. Parkin deficiency in vitro impairs MuSC proliferation and cell-cycle progression.

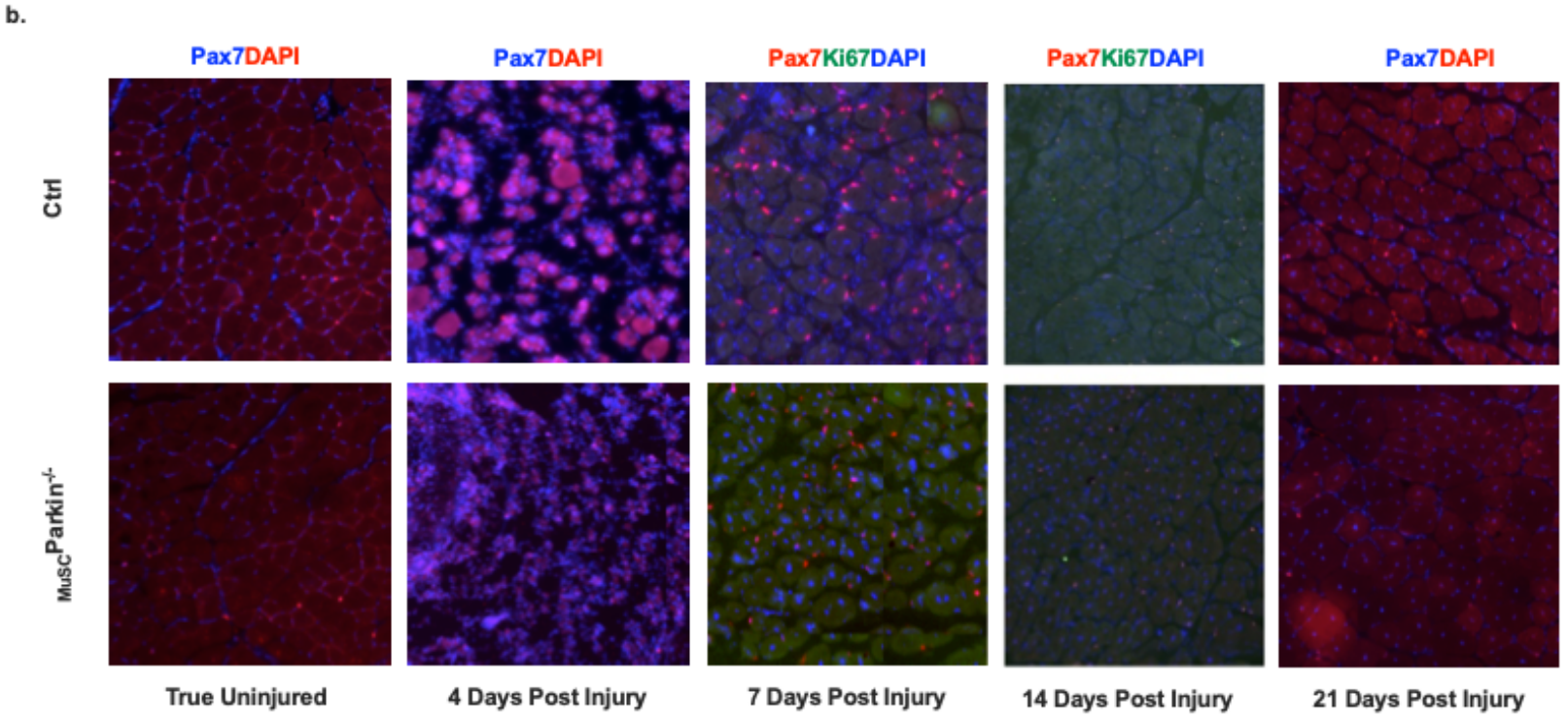
a. Representative epifluorescence images of control and $_{MuSC}$Parkin$^{-/-}$ satellite cells on single EDL fibers at 48 hours and 72 hours post-isolation. b. Mean MuSC cluster size in control vs $_{MuSC}$*Parkin*$^{-/-}$ satellite cells at different isolation timepoints. c. Percentage of cells per cluster in control vs $_{MuSC}$*Parkin*$^{-/-}$ MuSCs at T48 and T72 post isolation. Data is presented as mean ± SEM for n=5 fibers from 3 mice per genotype. Statistics were realised using a two-way ANOVA test. Statistical significance is displayed as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

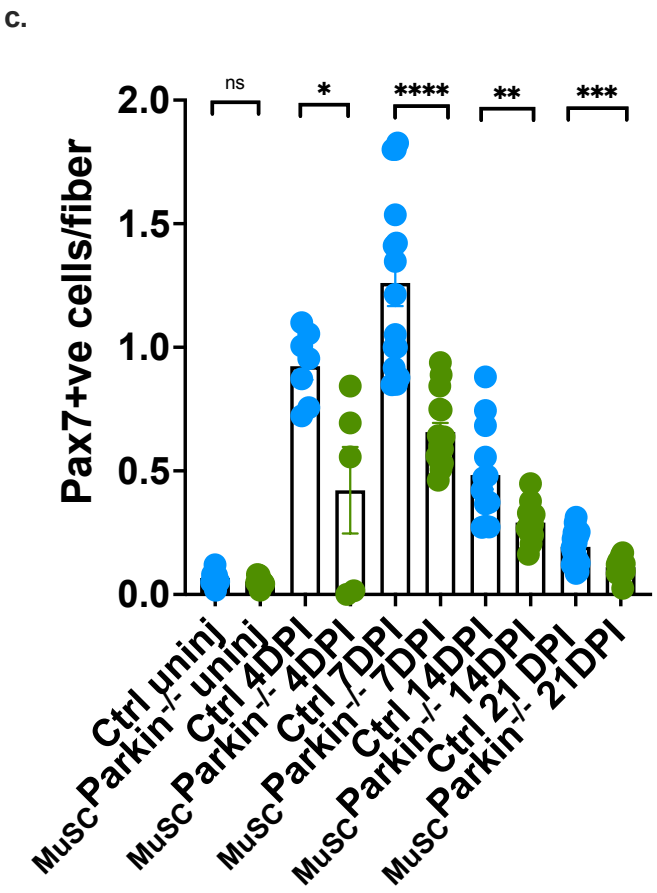
5.5 Parkin deficiency *in vivo* impairs proliferation and self-renewal of MuSCs.

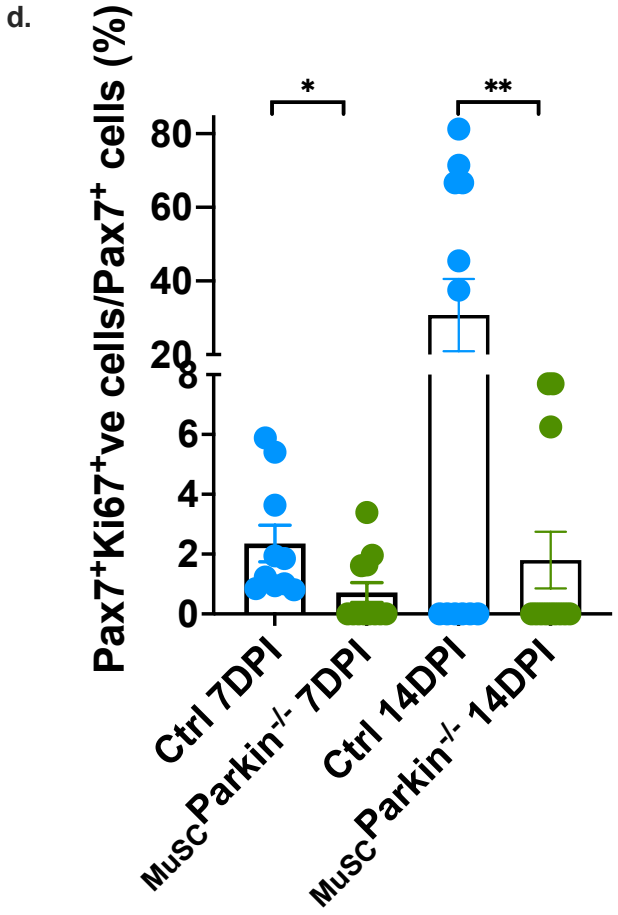
To investigate the in vivo consequences of Parkin deficiency on MuSC behaviour, the cardiotoxin injury (CTX) model was used in the Tibialis Anterior (TA) muscle. In an uninjured state, no differences in the number of MuSCs and the number of fibers per region of interest were found between the control and $_{MuSC}Park2^{-/-}$ TAs suggesting that Parkin deficiency in MuSCs does not overtly affect the MuSC state and survival under baseline conditions **(Fig. 8a)**. Post-injury, at 4-, 7, 14-DPI, a significant decrease in the number of quiescent ($Pax7^{+}$) was observed with a significant decline in the self-renewing (Pax7+) cells 21DPI after complete muscle regeneration indicative of a loss of the MuSC pool **(Fig. 8c)**. Furthermore, actively proliferating ($Pax7^{+}Ki67^{+}$) MuSCs were also significantly reduced in Parkin deficient mice consistent with the impaired proliferation phenotype observed in vitro **(Fig. 8d)**. No significant differences in the number of MyoG+ MuSCs post injury **(Fig. 8g)**. Finally, our data also showed a decrease in the regenerating fibers at 4DPI in $_{MuSC}Park2^{-/-}$ TAs assessed by emHC immunostaining with no differences observed at 14DPI. Collectively, consistent with the in vitro data, these results suggest that Parkin deficiency in MuSCs leads to a perturbed regenerative response which may involve earlier altered MuSC fate decisions with diminished proliferation, and impairments in MuSC self-renewal pool.

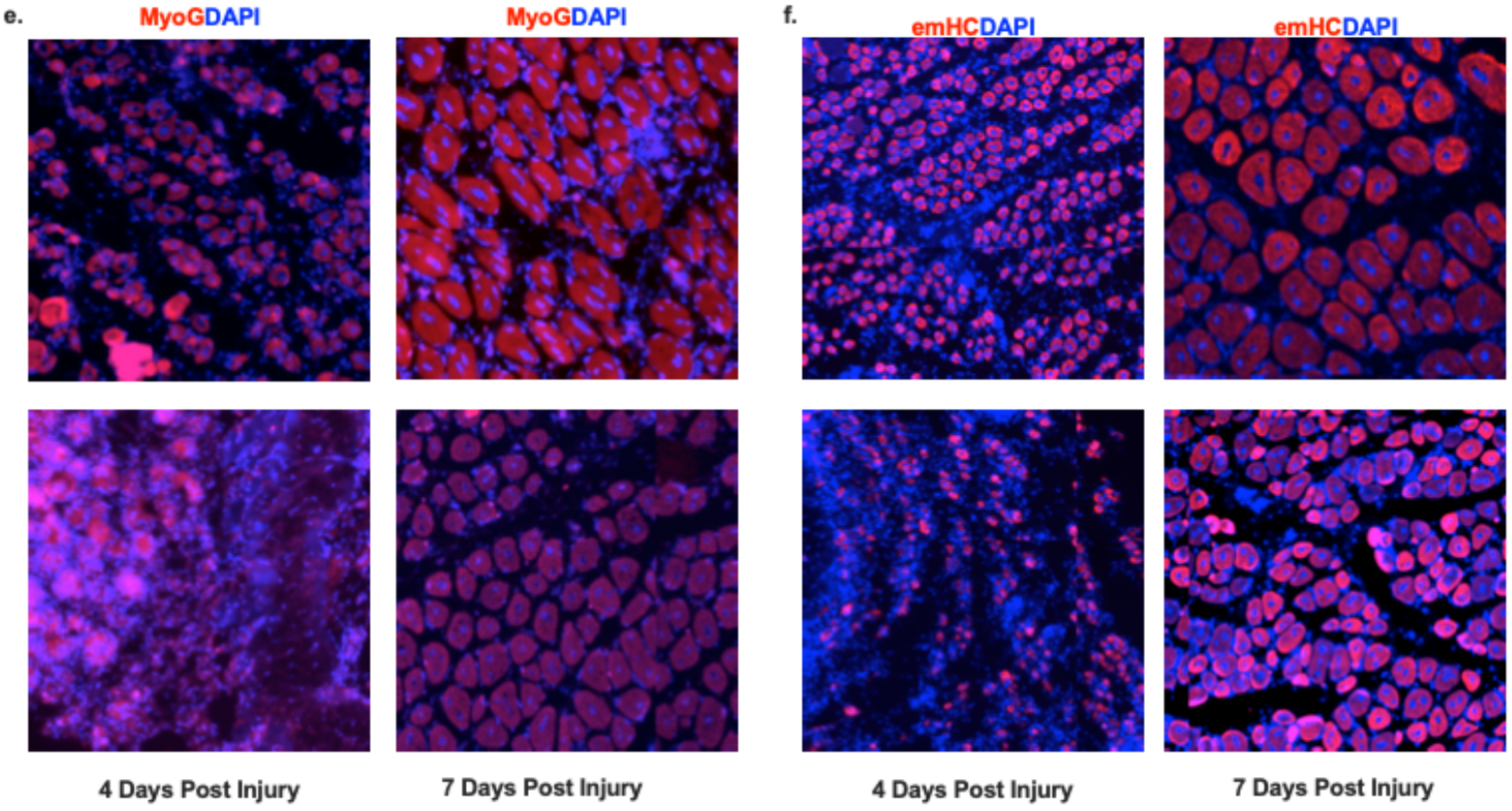
a.

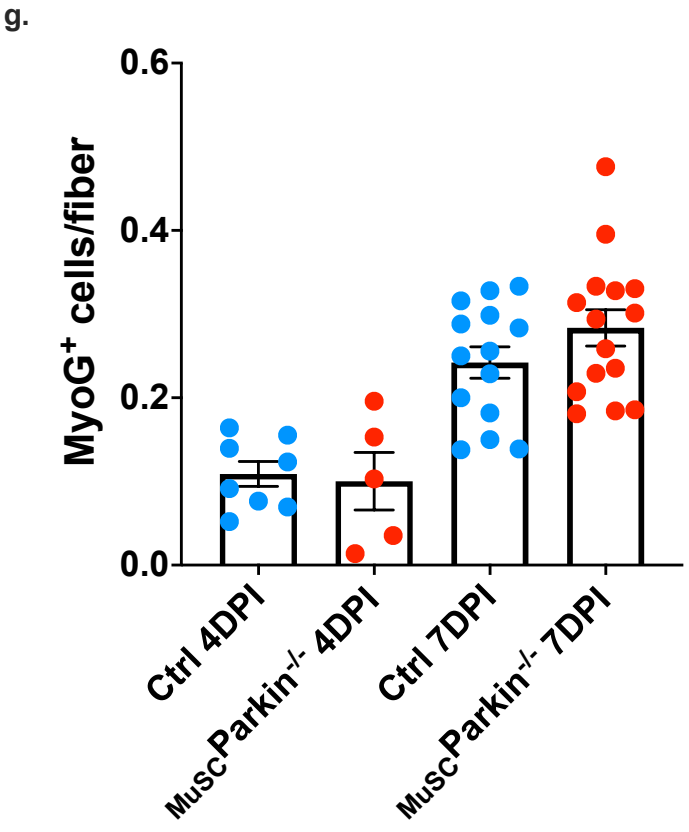

Pax7+ve cells/No. of fibers
0.15
0.10
0.05
0.00
ns
Ctrl true uninjured
MuSC Park-/- true uninjured
No. of fibers/mm2
800
600
400
200
0
ns
Ctrl true uninjured
MuSC Park-/- true uninjured


b.
Pax7DAPI
Pax7DAPI
Pax7Ki67DAPI
Pax7Ki67DAPI
Pax7DAPI
Ctrl
MuSCParkin-/-
True Uninjured
4 Days Post Injury
7 Days Post Injury
14 Days Post Injury
21 Days Post Injury


c.
Pax7+ve cells/fiber
2.0
1.5
1.0
0.5
0.0
ns
*
****
**
***
Ctrl uninj
MuSCParkin-/- uninj
Ctrl 4DPI
MuSCParkin-/- 4DPI
Ctrl 7DPI
MuSCParkin-/- 7DPI
Ctrl 14DPI
MuSCParkin-/- 14DPI
Ctrl 21 DPI
MuSCParkin-/- 21DPI


d.
Pax7+Ki67+ve cells/Pax7+ cells (%)
80
60
40
20
8
6
4
2
0
*
**
Ctrl 7DPI
MuSCParkin-/- 7DPI
Ctrl 14DPI
MuSCParkin-/- 14DPI


e.
MyoGDAPI
MyoGDAPI
f.
emHCDAPI
emHCDAPI
4 Days Post Injury
7 Days Post Injury
4 Days Post Injury
7 Days Post Injury


g.
MyoG+ cells/fiber
0.6
0.4
0.2
0.0
Ctrl 4DPI
MuSCParkin-/- 4DPI
Ctrl 7DPI
MuSCParkin-/- 7DPI

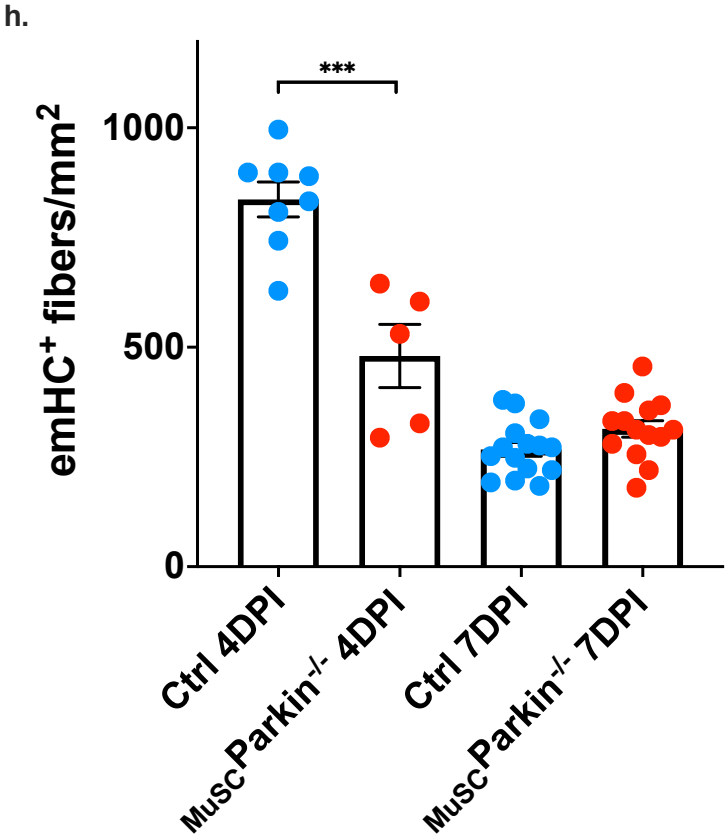
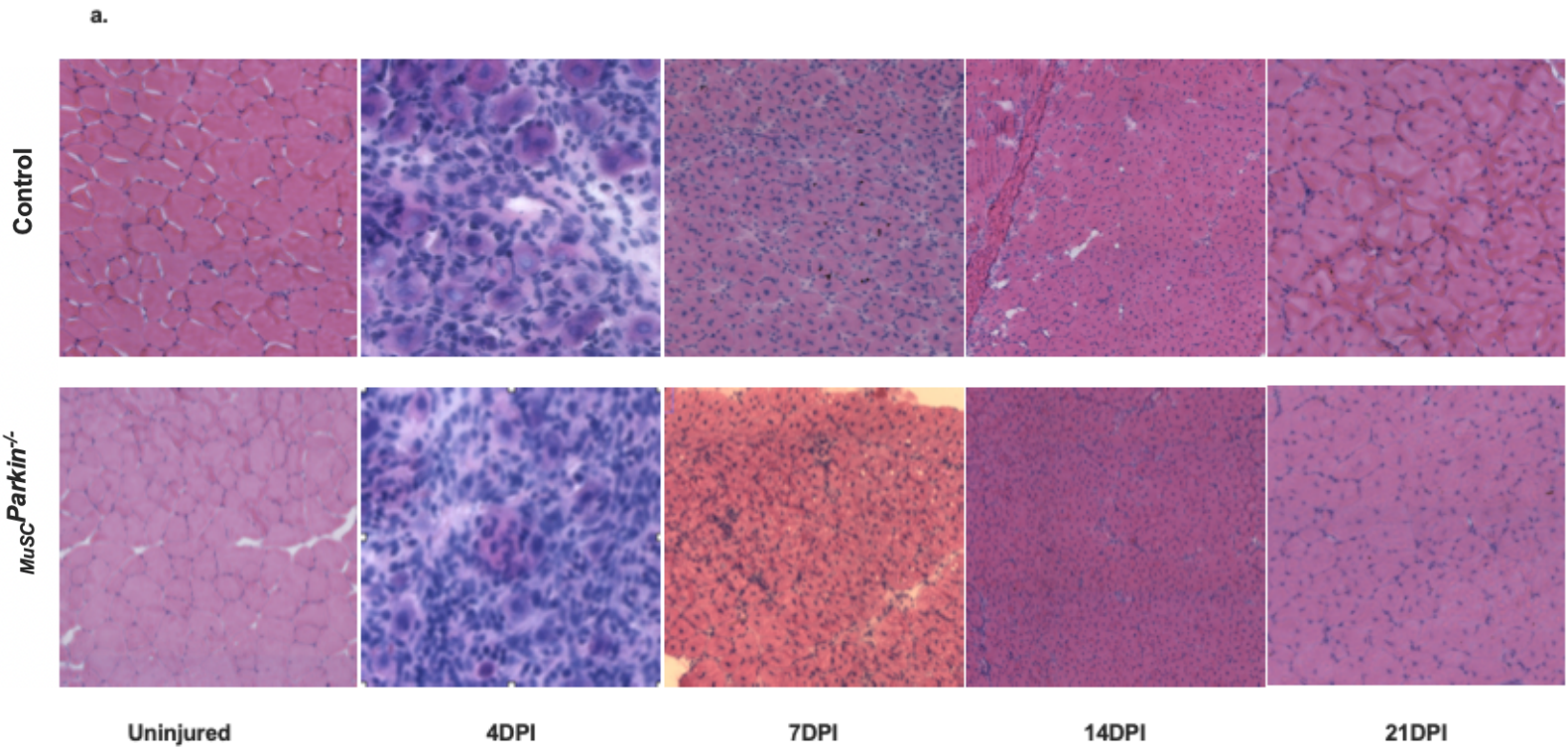

h.
***
emHC+ fibers/mm2
1000
500
0
Ctrl 4DPI
MuSCParkin-/- 4DPI
Ctrl 7DPI
MuSCParkin-/- 7DPI

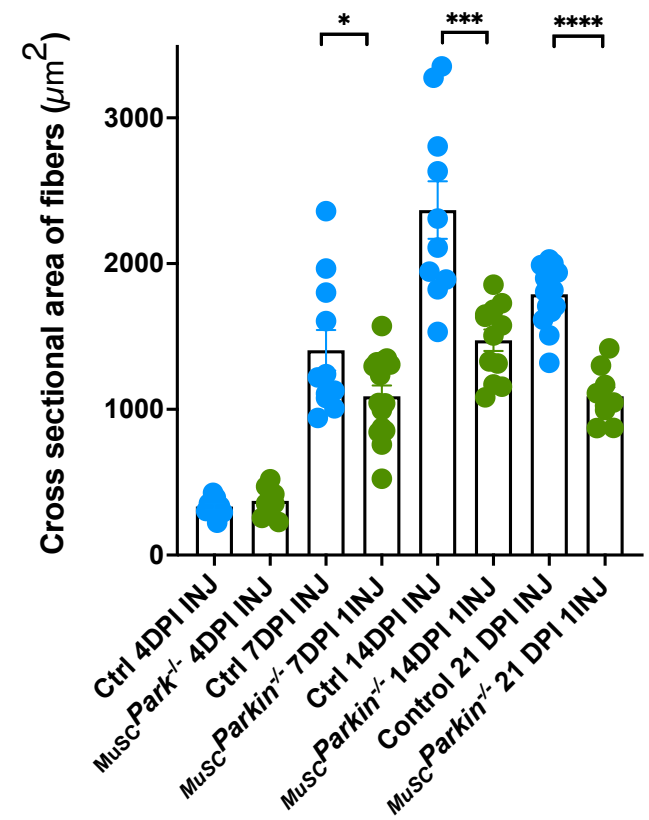
Figure 8. Loss of Parkin in vivo promotes commitment, impairs proliferation of MuSCs and depletes the MuSC pool.

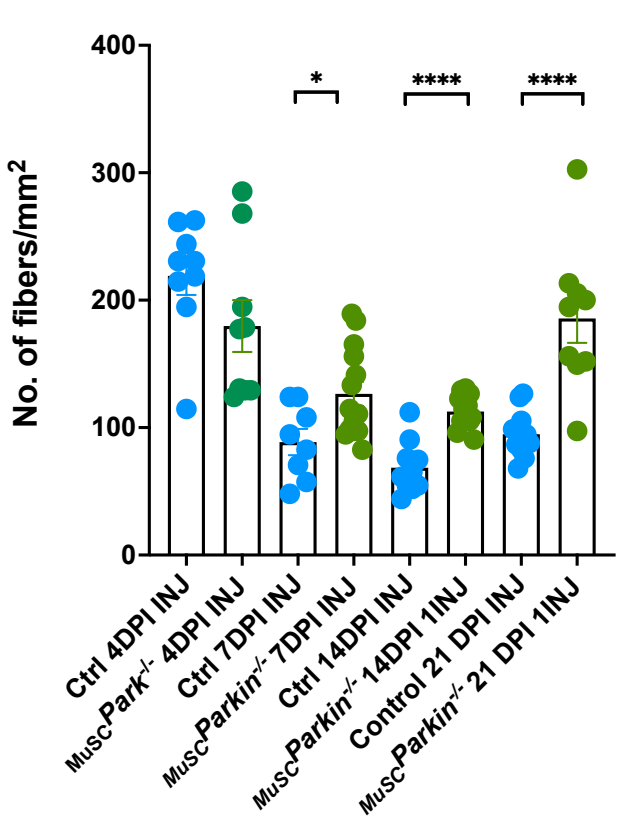
a. Immunostaining analysis of control vs $_{MuSC}Parkin^{-/-}$ uninjured mice stained with MuSC markers Pax7. b. Representative immunostaining images of TA muscle sections of control and $_{MuSC}Parkin^{-/-}$ mice in an uninjured state and, 4-, 7-, 14- and 21-days post injury (DPI) stained with MuSC marker Pax7 and Pax7/Ki67. Magnification: 20X. c. Immunostaining analysis of control vs $_{MuSC}Parkin^{-/-}$ mice stained with MuSC markers Pax7. d. Immunostaining analysis of control vs $_{MuSC}Parkin^{-/-}$ mice stained with MuSC markers Pax7/Ki67. e. Representative immunostaining images of TA muscle sections of control and $_{MuSC}Parkin^{-/-}$ injured mice stained with MuSC marker MyoG, at 4DPI and 7DPI. Magnification: 20X. f. Representative immunostaining images of TA muscle sections of control and $_{MuSC}Parkin^{-/-}$ injured mice stained with MuSC marker eMHC at 4DPI and 7DPI. Magnification: 20X. g. Immunostaining analysis of control vs $_{MuSC}Parkin^{-/-}$ mice stained with MuSC markers MyoG. h. Immunostaining analysis of control vs $_{MuSC}Parkin^{-/-}$ mice stained with MuSC markers emHC. Results represent mean ± SEM for n= 4-5 mice per group, ROI= 3 per mouse. Statistics were realised using an unpaired t test. Statistical significance is displayed as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

5.6 Loss of Parkin impairs MuSC regeneration upon injury.

To study the importance of Parkin-dependent mitophagy on muscle regeneration, we used the CTX injury model of the TA muscle in mice harboring a knockout of the Parkin gene in their MuSCs. The muscles were isolated, fixed and stained with hematoxylin and eosin (H&E) for analysis. No differences in the mean myofiber size of the uninjured muscle of the control and $_{MuSC}Park2^{-/-}$ mice were observed at baseline indicating that a Parkin deficiency in MuSCs does not lead to spontaneous muscle abnormalities. No significant differences in the number of myofibers/mm^2 was found at 4DPI however, at 7- ,14- and 21- days post injury significant increase in the number of myofibers/mm^2 was found in the Parkin knockout group **(Fig. 9b)**. Moreover, no significant differences in the cross-sectional area (CSA) of the myofibers was observed at 4DPI however, significant decrease of the CSA was observed in the $_{MuSC}Parkin^{-/-}$ group at 7- ,14- and 21- days post injury indicating impairments in the capacity of myofiber regeneration **(Fig. 9c)**. Finally, quantification of myofiber distribution showed no significant differences at 4DPI and 7DPI however a significant increase in the smaller myofibers was found at 14DPI and 21DPI with an overall trend towards smaller fibers in the $_{MuSC}Park2^{-/-}$ mice **(Fig. 9d).**


a.
Control
MuSCParkin-/-
Uninjured
4DPI
7DPI
14DPI
21DPI


b.
Cross sectional area of fibers (μm2)
0
1000
2000
3000
*
***
****
Ctrl 4DPI INJ
MuSCPark-/- 4DPI INJ
Ctrl 7DPI INJ
MuSCParkin-/- 7DPI 1INJ
Ctrl 14DPI INJ
MuSCParkin-/- 14DPI 1INJ
Control 21 DPI INJ
MuSCParkin-/- 21 DPI 1INJ


c.
No. of fibers/mm2
0
100
200
300
400
*
****
****
Ctrl 4DPI INJ
MuSCPark-/- 4DPI INJ
Ctrl 7DPI INJ
MuSCParkin-/- 7DPI 1INJ
Ctrl 14DPI INJ
MuSCParkin-/- 14DPI 1INJ
Control 21 DPI INJ
MuSCParkin-/- 21 DPI 1INJ

d.

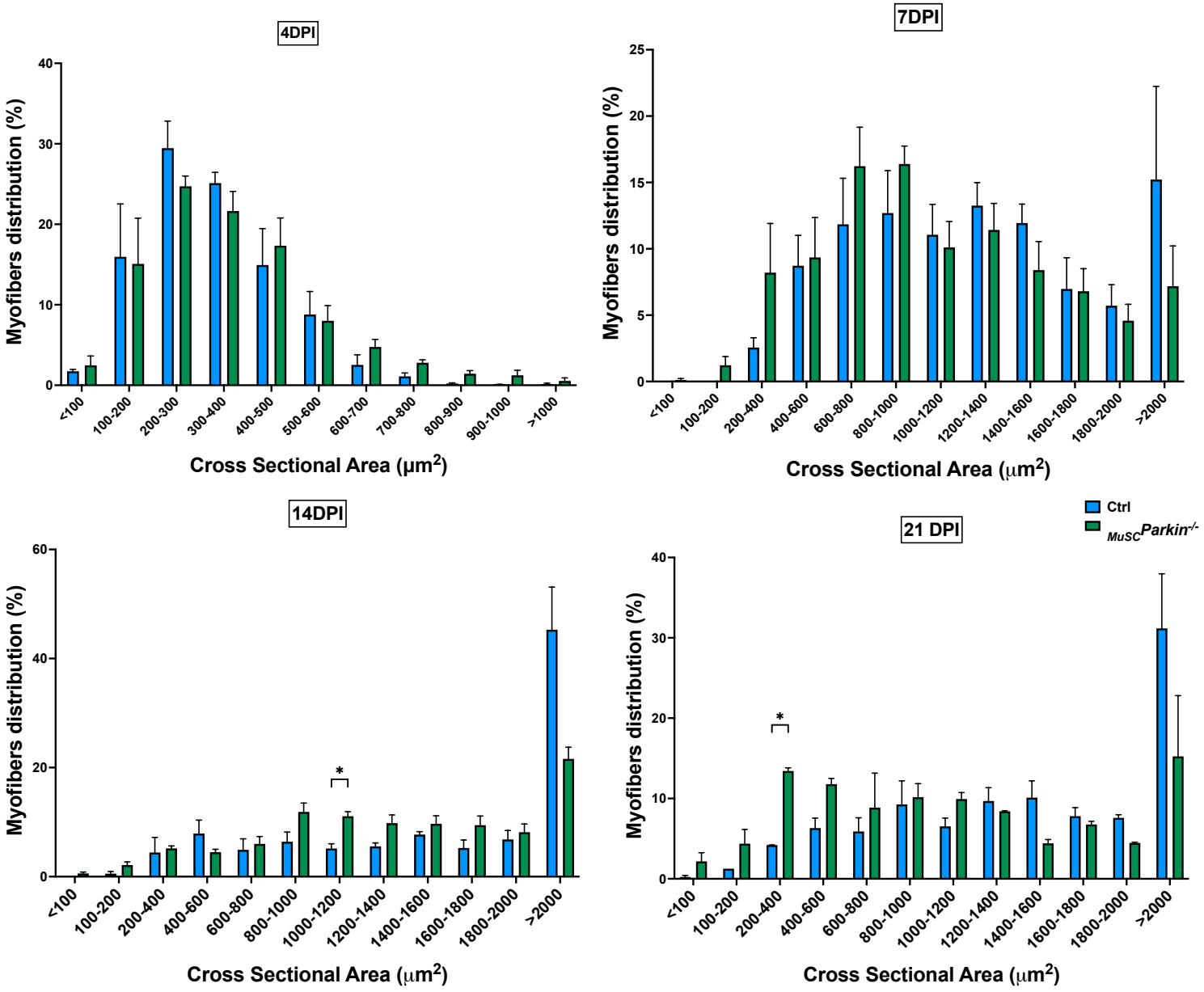

4DPI
Myofibers distribution (%)
Cross Sectional Area (µm²)
<100
100-200
200-300
300-400
400-500
500-600
600-700
700-800
800-900
900-1000
>1000
7DPI
Myofibers distribution (%)
Cross Sectional Area (µm²)
<100
100-200
200-400
400-600
600-800
800-1000
1000-1200
1200-1400
1400-1600
1600-1800
1800-2000
>2000
14DPI
Myofibers distribution (%)
Cross Sectional Area (µm²)
*
<100
100-200
200-400
400-600
600-800
800-1000
1000-1200
1200-1400
1400-1600
1600-1800
1800-2000
>2000
21 DPI
Ctrl
MuSCParkin-/-
Myofibers distribution (%)
Cross Sectional Area (µm²)
*
<100
100-200
200-400
400-600
600-800
800-1000
1000-1200
1200-1400
1400-1600
1600-1800
1800-2000
>2000

Figure 9. Loss of Parkin in vivo impairs muscle regeneration.

a. Representative Hematoxylin and Eosin (H&E) images of uninjured, 4DPI, 7DPI, 14DPI and 21DPI TA muscles of control vs $_{MuSC}Parkin^{-/-}$ mice. Magnification: 20X. b. Quantification of CSA of TA muscle fibers (µm2) at 4DPI, 7DPI, 14DPI and 21DPI CTX of control vs $_{MuSC}Parkin^{-/-}$ injured mice. c. Quantification of number of fibers/mm^2 at 4DPI, 7DPI, 14DPI and 21DPI CTX of control vs $_{MuSC}Parkin^{-/-}$ mice. d. Quantification of myofiber size distribution at 4DPI, 7DPI, 14DPI and 21DPI of control vs $_{MuSC}Parkin^{-/-}$ injured mice. Results represent mean ± SEM for n= 5 mice per group, ROI= 3 per mouse. Statistics were realised using an unpaired t test. Statistical significance is displayed as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars represent mean ± SEM.

6 DISCUSSION:

6.1 Regulation of mitophagy in MuSCs

Studies on autophagy have unveiled the critical role of this bulk recycling process in the maintenance of stem cell homeostasis and function. In particular, autophagy was shown to be essential for preserving the global integrity of cellular constituents, including mitochondria. However, the role of specific autophagy mechanisms, such as mitophagy, are still poorly understood. To gain insight into the role of mitophagy in MuSCs, our lab initiated work to characterize mitophagy in MuSCs under various physiological states. Through analysis of publicly available transcriptomics datasets [143], comparing quiescent and activated MuSC states, previous work done in our lab found that Parkin was enriched in the MuSC quiescent state and downregulated as the MuSCs became activated which suggests that Parkin-mitophagy pathway may play a role in the regulation of MuSC fates[144]. Quantification of mitophagy through the mitophagy reporter mitoQC mouse model and colocalization of lysosomes and mitochondria in wildtype mice showed that mitophagy is active in quiescent stem cells and transiently downregulated upon activation[144]. Moreover, genetic inactivation of PINK1 at the germline level resulted in a mild but significant reduction of mitophagy in quiescent MuSCs, suggesting that PINK1/Parkin dependent pathway plays an important regulatory role[144]. Through confocal imaging and immunostaining analysis of MuSCs, we show that Parkin-dependent mitophagy is active in the quiescent and activated state of MuSCs and MuSC specific inactivation of Parkin, decreases mitophagy to a comparable extent. However, major differences in mitophagy between the quiescent and activated states in our study were not observed, which contrasts with our previous data. Although the reasons for this remain unclear, we suspect that it might be due to experimental factors including lower quality of isolation and longer isolation time which might have dampened the differences in mitophagy amongst the different MuSC states. These aspects are currently being investigated and experiments are being repeated to allow for more conclusive results. Together with previous work performed in our lab, our

results indicate that mitophagy is prominent in quiescent MuSCs and is partly regulated by the PINK1/Parkin mitophagy pathway.

6.2 Impact of altered mitophagy on MuSC fate decisions

It is known that mitochondrial remodelling is important in regulating the transition of stem cells from quiescence to activation[101], however the role of mitochondrial quality control, in MuSC lineage progression has not been studied. Our results in the germline PINK1 knockout mouse model showed that a loss of PINK1 increased MuSC commitment at the expense of self-renewal[144]. Using our MuSC specific approach, in this study we show that inactivation of Parkin, the partner PINK1 in the ubiquitin-dependent mitophagy pathway, has comparable effects where the MuSCs prematurely exit their quiescent state and are poised towards earlier activation and commitment at the expense of self-renewal. These results reinforce the idea that the PINK1/Parkin mitophagy pathway is crucial in balanced MuSC fate decisions. Katajisto *et al.* have previously shown that a knock-down of Parkin decreases the population of stem cells with high quiescence properties, and reduced mitophagy poises stem cells towards commitment[74]. Mitophagy plays a crucial role in the metabolic/mitochondrial remodelling process for the maintenance of self-renewal and proper differentiation[145,146]. Given the results obtained with our PINK1 mouse model and previous studies in stem cells, Reactive Oxygen Species (ROS) is one potential mechanism that may require further investigation[144]. Our PINK1 model revealed that increased mitochondrial ROS production may act as a signal to MuSCs promoting their activation and differentiation at the expense of self-renewal[144]. Furthermore, it has been shown by Khacho *et al.*, that the ROS signalling pathway affects stem cell identity[101]. Papa *et al.* also showed that increased ROS levels act as signals driving stem cells' exit from quiescence, triggering a loss in their self-renewing capacity and causing impairments in their lineage progression[105]. Moreover, studies have also shown that mitophagy can rescue stem cells from ROS-induced apoptosis by eliminating dysfunctional mitochondria[72,147–150]. In line with these studies, we speculate that impaired mitophagy may lead to the

accumulation of dysfunctional mitochondria within the MuSCs, increasing oxidative stress and activating the ROS signalling pathway, causing a premature exit of MuSCs from quiescence and initiating their activation and differentiation. Although ROS could be a possible mechanism that may affect MuSC fate, it may not be the sole factor. Experiments looking into mitochondrial phenotyping and RNA sequencing may explain other critical attributes of mitochondria that are affected and how Parkin deficiency affects the transcriptomes of cells in their quiescent and activated state.

Quiescent MuSCs are known to have low metabolic rates with limited wear and tear[69] therefore it is unclear why the prominence of mitophagy observed in the quiescent state is due to mitochondrial damage and dysfunction. As PINK1/Parkin mitophagy can be triggered due to mitochondrial depolarization[69], a possible mechanism may be that the low mitochondrial membrane potential in the quiescent state promotes mitophagy as a preventative mechanism. The primary intent of mitophagy in the quiescent state may be to not accumulate any damaged mitochondria, rather than clearing out damaged mitochondria. In the case, if mitophagy is inhibited or defective, mitochondria may become polarized, emitting increased ROS, causing a reduction of their quiescence, and promoting them towards activation and commitment.

6.3 Impact of altering mitophagy signalling on MuSC proliferation

In our previous work in the germline PINK1 mouse model, no proliferative defect in the EDL experiments were found[144]. This was also evident in cultured myoblasts and following *in vivo* activation with cardiotoxin[144]. This contrasts with results obtained in Parkin deficient MuSCs in the present study. In this study, our data indicates that in the absence of Parkin, MuSC proliferation is impaired with alterations in the cell cycle progression. These differences observed amongst the mouse models could be linked to the different experimental model used. Inactivation of PINK1 at the germline has been associated with developmental compensation[151,152] which if present in the MuSCs could have dampened

the proliferation phenotype. Furthermore, this could also suggest that the proliferation is linked to other effects of Parkin that are independent of mitophagy. Parkin is a cytosolic protein with effects in this cellular compartment however a small amount of Parkin can translocate to the nucleus where it also exerts some biological effects[153]. Parkin is shown to be a regulator of cyclin-cyclin dependent kinase (CDK) complexes and is essential in DNA damage repair mechanisms[138–140]. As Parkin is involved in these mechanisms, inhibiting Parkin may impair the cell's ability to repair DNA damage efficiently triggering cell cycle arrest and limiting cell proliferation. Furthermore, Parkin is also suggested to have a role in the PI3K/AKT signalling pathway which regulated biological processes including cell proliferation, apoptosis, and cellular metabolism[150,154]. Parkin inhibition in the MuSCs may disrupt this pathway, negatively impacting MuSC proliferation. Studies have also shown that nuclear Parkin regulated transcription factors that control key pathways contributing to stem cell regulation i.e., FOXO3, PPAR, ERR etc.[155,156] thus the proliferation defect observed might not be due to solely defective mitophagy.

6.4 Impact of altered mitophagy on muscle regeneration

Our results in the germline PINK1 knockout mouse model showed that after two and three successive cycles of injury, a gradual reduction in the cross-sectional area of the regenerated fibers was observed[144]. Using a MuSC specific approach, we show that inactivation of Parkin leads to a significant reduction in the cross-sectional area of the regenerated fiber even after a single injury. These results indicate that the Pink1/Parkin mitophagy pathway is important for muscle regeneration. Esteca *et al.* reported that in a germline Parkin knockout mouse model, the cross-sectional area of newly formed myofibers was reduced post injury[157]. Furthermore, Peker *et al.* also demonstrated that Parkin is necessary to maintain proper myotube size and suggested that Parkin has a role in muscle growth and development[158]. The impaired muscle regeneration response observed in our study may occur due to the altered fate decisions and impaired proliferation of the Parkin knockout MuSCs as deficient proliferation has been linked to being

deleterious for tissue maintenance[70]. Due to their inability to proliferate and the promotion of their differentiation, we speculate that post activation insufficient MuSCs progeny is present, which rapidly fuse into myotubes, leading to smaller myofibers causing a rapid decline in the muscle regenerative capacity.

7 CONCLUSION

Through previous experiments done in the lab, our data and, the analysis of the literature we provide evidence that the Pink1-Parkin mitophagy pathway plays a role in MuSC fate regulation. We observed that mitophagy is active in the MuSC quiescent state and any impairments cause altered MuSC fates. Our data shows that impairments in the Pink1-Parkin mitophagy pathway poise MuSCs towards earlier activation and commitment, impairing their self-renewal and proliferative capacities, which lead to an extensive loss in the muscle regenerative capacity. Furthermore, we also speculate that mitophagy in a quiescent state of MuSCs may act as a preventative quality control mechanism to inhibit the cells from accumulating depolarized mitochondria.

Although the focus of this study was on the Pink1-Parkin mitophagy pathway and its effects on MuSC fate, alternative mitophagy pathways including BNIP3/NIX mediated mitophagy, FUNDC1-dependent mitophagy etc. also play a role in stem cell maintenance, activation, differentiation, and homeostasis[124–134]. To better understand the effects of mitophagy on MuSC fate decisions and muscle regeneration, the effects of other mitophagy pathways should be explored alongside the Pink1-Parkin mitophagy pathways. Furthermore, in our study we observe that mitophagy is reduced but still active in $_{MuSC}$Park$^{-/-}$ mice. This may occur due to the compensatory mechanisms of Pink1-Parkin independent mitophagy pathways removing defective mitochondria from within the MuSCs leading to an incomplete ablation of mitophagy.

Moreover, further work needs to be performed, exploring what metabolites, proteins, and pathways are disrupted in MuSCs when Parkin is deleted. Metabolomics, proteomics, and RNA sequencing experiments on the MuSCs of $_{MuSC}$Park$^{-/-}$ needs to be performed. Moreover, to explore any mitochondrial dysfunction in MuSCs in response to the Parkin deletion and impaired mitophagy, ROS measurements, ATP measurements, MMP assessment and mitochondrial respiration experiments would need to be conducted. Exploring and understanding the underlying mechanism that Parkin is involved in will allow for better insight pertaining why MuSC fates, proliferation, and muscle regeneration are affected when Parkin is removed.

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