NANYANG
TECHNOLOGICAL
UNIVERSITY

**ROLE OF MITOPHAGY IN
MUSCLE GROWTH AND METABOLISM**

NESIBE PEKER
SCHOOL OF BIOLOGICAL SCIENCES
2017

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A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of Doctor of
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From a priori to a posteriori...

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ABBREVIATIONS

4EBP1:	4E binding protein 1
AAV:	Adeno-associated virus
ADP:	Adenosine diphosphate
AICAR:	5-aminoimidazole-4-carboxamide ribonucleotide
AIDS:	Acquired immune deficiency syndrome
AKT:	Protein kinase B
AMP:	Adenosine monophosphate
AMPK:	5' AMP-activated protein kinase
Ant/Rot:	Antimycin A/Rotenone
Apaf-1:	Apoptotic protease activating factor 1
APC/C:	Anaphase promoting complex/cyclosome
ATG:	Autophagy-related gene
ATP:	Adenosine triphosphate
Atp5:	ATP synthase subunit 5
Atp6:	ATP synthase subunit 6
atrogene:	Atrophy-related gene
bHLH:	Basic helix-loop-helix
BAK:	BCL-2 antagonist/killer 1
BAX:	BCL-2 associated X protein
BCL-2:	B-cell lymphoma 2
BSA:	Bovine serum albumin
Ca ²⁺ :	Calcium ion
CaCl ₂ :	Calcium chloride
CCCP:	Carbonyl cyanide m-chlorophenyl hydrazine
CDH1:	Cadherin 1
CDC20:	Cell division cycle 20
CEE:	Chick embryo extract
CHOP:	C/EBP homologous protein
CMA:	Chaperone-mediated autophagy
CoI:	Cytochrome c oxidase subunit 1
COPD:	Chronic obstructive pulmonary disease
Cox4:	Cytochrome c oxidase subunit 4

CQ:	Chloroquine
CSA:	Cross-sectional area
Cyt <i>c</i> :	Cytochrome <i>c</i>
Cytb:	Cytochrome <i>b</i>
DAVID:	Database for Annotation, Visualization and Integrated Discovery
DMD:	Duchenne muscular dystrophy
DMEM:	Dulbecco's modified eagle medium
DML:	Dorsomedial
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DRP1:	Dynamic related protein 1
DUB:	Deubiquitinating enzyme
EDL:	<i>M. extensor digitorum longus</i>
EDTA:	Ethylenediaminetetraacetic acid
eIF2 α :	Eukaryotic translation initiation factor 2- α
ER:	Endoplasmic reticulum
ETC:	Electron transport chain
FACS:	Fluorescence-activated cell sorting
FAO:	Fatty acid oxidation
Fbxl22:	F-box and leucine rich repeat 22
FCCP:	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FGF:	Fibroblast growth factor
FIS1:	Fission 1
FoxO:	Fork-head box-O
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
Gas:	<i>M. gastrocnemius</i>
GPX:	Glutathione peroxidase
GR:	Glucocorticoid receptor
GRX:	Glutaredoxin
GWAS:	Genome-wide association study
h:	hour
H&E:	Hematoxylin and eosin

H ₂ DCFDA:	2',7'-dichlorodihydrofluorescein diacetate
H ₂ O ₂ :	Hydrogen peroxide
HFD:	High fat diet
HGF:	Hepatocyte growth factor
HPG:	L-Homopropargylglycine
HRP:	Horse radish peroxidase
HSP60:	Heat shock protein 60
IBR:	In-between-RING
IFM:	Indirect flight muscle
IGF:	Insulin growth factor
IMF:	Intermyofibrillar
IRS1:	Insulin receptor 1
KCl:	Potassium chloride
kDa:	kilo dalton
KEGG:	Kyoto Encyclopedia of Genes and Genomes
LC3-I:	Nonlipidated LC3
LC3-II:	Lipidated LC3
LC3:	Microtubule-associated protein 1A/1B-light chain 3
LPS:	Lipopolysaccharides
MAMs:	Mitochondria-associated membranes
MDV:	Mitochondria-derived vesicles
Mff:	Mitochondrial fission factor
MFI:	Mean fluorescence intensity
MFN:	Mitofusin
MgCl ₂ :	Magnesium chloride
MIM:	Mitochondrial inner membrane
ml:	millilitre
mM:	millimolar
MOM:	Mitochondrial outer membrane
MPC:	Muscle progenitor cell
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRF:	Myogenic regulatory factor
mtDNA:	Mitochondrial DNA
mTOR:	Mammalian target of rapamycin

MuRF1:	Muscle RING finger protein 1
MyHC:	Myosin heavy chain
MyLC:	Myosin light chain
Na ₃ VO ₄ :	Sodium orthovanadate
NaCl:	Sodium chloride
NaF:	Sodium fluoride
NaOH:	Sodium hydroxide
Nd1:	NADH-ubiquinone oxidoreductase chain 1
Nd2:	NADH dehydrogenase subunit 2
Nd4:	NADH dehydrogenase subunit 4
Nd4l:	NADH-ubiquinone oxidoreductase chain 4L
Nd6:	NADH-ubiquinone oxidoreductase chain 6
Ndufa9:	NADH-ubiquinone oxidoreductase subunit A9
Ndufb6:	NADH-ubiquinone oxidoreductase subunit B6
Ndufs3:	NADH-ubiquinone oxidoreductase core subunit S3
Nedd4:	Neuronal precursor cell-expressed developmentally downregulated 4
NF-κB:	Nuclear factor-kappaB
NRF:	Nuclear respiratory factor
nuDNA:	Nuclear DNA
OCR:	Oxygen consumption rate
OCT:	Optimum cutting temperature
Oligo:	Oligomycin
OPA1:	Optic atrophy 1
OXPHOS:	Oxidative phosphorylation
p-:	phosphorylated
p62:	Nucleoporin p62
PANTHER:	Protein Analysis Through Evolutionary Relationships
PARIS:	PARKIN-interacting substrate
<i>Parkin</i> KO:	<i>Parkin</i> knock out
PARL:	Presenilins-associated rhomboid-like protein
Pax:	Paired-box
PBS:	Phosphate buffered saline
PD:	Parkinson's disease

PEG:	Polyethylene glycol
PERK:	Protein kinase RNA-like endoplasmic reticulum kinase
PGC-1 α : P	PAR- γ coactivator-1alpha
PINK1:	PTEN-induced kinase 1
Plk1:	Polo-like kinase 1
PMSF:	Phenylmethane sulfonyl fluoride
POLG:	DNA polymerase gamma
PPAR- γ :	Peroxisome proliferator-activated-gamma
Ppia:	Peptidylprolyl isomerase A
proteostasis:	Protein homeostasis
PVP:	Polyvinylpyrrolidone
Q:	Ubiquinone
QH ₂ :	Ubiquinol
Quad:	M. quadriceps
RIPA:	Radioimmuniprecipitation assay
RLU:	Relative light units
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
s.e.m.:	Standard error mean
SDH:	Succinate dehydrogenase
Sdha:	Succinate dehydrogenase complex subunit A
SDS:	Sodium dodecyl sulfate
siRNA:	Small interfering RNA
SMART:	Simple modular architecture research tool
SNC:	Substantia nigra pars compacta
SOD:	Superoxide dismutase
Sol:	M. soleus
SS:	Subsarcolemmal
SSM:	Subsarcolemmal mitochondria
Suc-LLVY-aminoluciferin:	Succinyl-leucine-leucine-valine-tyrosine-aminoluciferin
TA:	M. tibialis anterior
TEMED:	Tetramethylethylenediamine
Tfam:	Mitochondrial transcription factor A
TGF- β :	Transforming growth factor-beta

TNF α :	Tumor necrosis factor alpha
Trim:	The tripartite motif family
tRNA:	Transfer RNA
TRX:	Thioredoxin
UBL:	Ubiquitin-like domain
ULK1:	Unc-51 like autophagy activating kinase 1
UPS:	Ubiquitin proteasome system
Uqcrc1:	Ubiquinol-cytochrome c reductase core protein 1
Uqcrc2:	Ubiquinol-cytochrome c reductase core protein 2
Uqcrcfs:	Ubiquinol-cytochrome c reductase rieske iron-sulfur polypeptide 1
VLL:	Ventrolateral
Z-LRR-aminoluciferin:	Z-leucine-arginine- arginine-aminoluciferin
Z-nLPnLD-aminoluciferin:	Z-norleucine-proline-norleucine-aspartate aminoluciferin
β -GPA:	Beta-guanadinopropionic acid
μ l:	microliter
μ M:	micromolar
μ m ² :	Square micrometers

ABSTRACT

Skeletal muscle is the largest tissue in the body which accounts for up to 40% of total body weight in humans. Skeletal muscle as a contractile tissue allows for movement during high energy activities, including exercise. As such, mitochondria, which supply energy to skeletal muscle, play a significant role in maintaining skeletal muscle growth and metabolism. Mitochondrial quality is maintained through processes including mitochondrial fusion and fission and mitochondria turnover, which is mediated through a selective type of autophagy, termed mitophagy. Mitochondrial dysfunction and subsequent mitophagy have previously been implicated in the aetiology of diseases that affect skeletal muscle.

Parkinson's Disease is a neurodegenerative disease characterized by tremors, muscle stiffness and muscle weakness. Molecular genetic analysis confirmed that mutations in *PARKIN* and *PINK1* genes, which play major roles in mitochondrial quality control and mitophagy, are frequently associated with Parkinson's Disease. *PARKIN* is an E3 ubiquitin ligase that translocates to mitochondria during loss of mitochondrial membrane potential to increase mitophagy. Although muscle dysfunction is noted in Parkinson's Disease, little is known about the involvement of *PARKIN* in the muscle phenotype of Parkinson's Disease.

In this thesis, a novel role for *Parkin* in regulating skeletal muscle mass, through its primary role in mitochondrial quality control, is described. Initially, C2C12 myotubes were treated with CCCP to recapitulate *PINK1/PARKIN*-mediated mitophagy. Results reveal that CCCP treatment leads to abnormal mitochondrial morphology, mitochondrial dysfunction and notable mitophagy, which further validates the function of CCCP in C2C12 myotubes. In addition to excess mitophagy, CCCP treatment resulted in pronounced myotube atrophy and degradation of myofibrillar proteins *in vitro*. Consistent with previous observations made in other muscle wasting conditions, CCCP results in reduced levels of phosphorylated FOXO3a (p-FOXO3a) protein and a concomitant increase in the expression of the atrophy-related genes *Atrogin-1* and *Murf1*. Collectively, these results suggest that *PINK1/PARKIN*-mediated mitophagy upon CCCP treatment leads to mitochondrial dysfunction and myotube atrophy *in vitro*.

Next, the effect of *Parkin* knock down on mitochondrial function and C2C12 myotube area was investigated. C2C12 cells were transfected with either non-targeting (control) or *Parkin*-specific siRNA. Results revealed preservation of mitochondrial

mass in *Parkin*-siRNA transfected myoblasts as compared to control. Also, knock down of *Parkin* results in mitochondrial dysfunction and reduced levels of critical OXPHOS proteins. Moreover, knock down of *Parkin* prevented increased expression of mitochondrial fusion and fission markers. Strikingly, knock down of *Parkin* resulting in myotubular atrophy *in vitro*. In agreement with this, *Parkin* knock down resulted in increased levels of the muscle-specific E3 ligase MURF1 and enhanced proteasome activity.

Mitochondrial number and function was further assessed in a *Parkin* KO mouse model. Results revealed preservation of mitochondrial number, a significant decrease in levels of critical OXPHOS proteins and reduced mitochondrial respiratory capacity in *Parkin* KO primary cultures. Also, a reduction in the levels of mitochondrial fission markers in *Parkin* KO Gas muscle and *Parkin* KO primary cultures, further suggests that loss of *Parkin* results in impaired mitochondrial turnover both *in vivo* and *in vitro*. Moreover, histological analysis revealed a significant reduction in myofiber cross-sectional area of M. *tibialis anterior* muscle of 4-week-old *Parkin* KO mice, which is consistent with loss of muscle mass. Reduced muscle mass was supported by increased levels of MURF1 protein in *Parkin* KO muscle.

To identify gene expression changes that may be responsible for the phenotype observed in *Parkin* KO mice, microarray analysis was performed. Consistent with mitochondrial dysfunction, genes involved in AMPK signalling, which is activated in response to an increase in ADP:ATP ratio, were upregulated in *Parkin* KO Gas muscle. In addition, increased expression of genes that are involved in the ubiquitin proteasome protein degradation system suggests that increased protein catabolism is responsible for the skeletal muscle atrophy noted in *Parkin* KO skeletal muscle.

Taken together, these data underscore an important function of *Parkin* in skeletal muscle. Specifically, maintenance of *Parkin* expression is critically important for maintaining skeletal muscle mass, through proceeding regulation of mitochondrial quality control.

SUMMARY

Parkinson's Disease (PD) is a neurodegenerative disease characterized by tremors, muscle stiffness and muscle weakness. Molecular genetic analysis confirmed that mutations in *PARKIN* and *PINK1* genes, which play major roles in mitochondrial quality control and mitophagy, are frequently associated with PD. Although muscle dysfunction is noted in PD, little is known about the involvement of *PARKIN* in the muscle phenotype of PD. In this study, results show that the mitochondrial uncoupler CCCP promotes PINK1/PARKIN-mediated mitophagy in myogenic C2C12 cells. As a result of this excess mitophagy, CCCP treatment of myotubes leads to the development of myotube atrophy *in vitro*. Surprisingly, siRNA-mediated knock down of *Parkin* results in accumulation of dysfunctional mitochondria. In addition, knock down of *Parkin* also led to myotubular atrophy *in vitro*. Consistent with these *in vitro* results, *Parkin* knockout muscles showed impaired mitochondrial function and smaller myofiber area, suggesting that *Parkin* function is required for post-natal skeletal muscle growth and development. These findings open new perspectives through investigation of PD from skeletal muscle angle, and may lead to discovery of new therapeutic targets for the treatment of PD.

1 REVIEW OF LITERATURE

1.1 Skeletal Muscle

1.1.1 Skeletal Muscle and Muscle Cell Structure

The largest tissue in the body is skeletal muscle, which accounts for approximately 38% and 30% of the total body weight of humans in men and women, respectively¹. In addition to controlling locomotion of the body, skeletal muscle is essential for maintenance of posture, as well as contraction and protection of internal organs. Skeletal muscle has specialised structures that can generate tension and contract, and it is the cumulative force generated by each muscle cell that is responsible for muscle contraction. These muscle cells or single muscle fibers (myofibers) are long cylindrical shaped structures, which range in length from 1mm to 40 mm. Unlike the typical structure of mammalian cells, single muscle fibers are multinucleated, and in fact, up to 100 nuclei can reside within a single cell².

When sections of skeletal muscle are observed under a microscope, they appear as long packaged myofibrillar proteins attached to tendons through the help of a special connective tissue called the epimysium. Myofibrils are rod-shape structures that consist of actin, myosin, titin and other cytoskeletal proteins and each muscle fiber contains many myofibrils³. A second layer of connective tissue termed the perimysium encloses each muscle fascicle, which consists of a bundle of many muscle fibers. Also, blood vessels are spread between fascicles to nourish muscle cells (**Figure 1.1**). Muscle fibers are embedded in sarcoplasm (cytosol) and enclosed by the sarcolemma (plasma membrane). Neuronal stimuli pass across sarcolemma in the form of calcium ions. Special structures called transverse tubules (T-tubules) facilitate the spread of calcium deep into the muscle fibers by invaginating through the sarcoplasmic reticulum (endoplasmic reticulum)¹. Also, muscle cells have a heterogeneous mitochondrial population composed of subsarcolemmal (SS) mitochondria, located beneath the sarcolemma, and intermyofibrillar (IMF) mitochondria, which are present between myofibrils. These mitochondrial subpopulations exhibit distinct energetic profiles and respond differently to physiological stimuli¹.

Myofibrils are composed of bundles of long Myosin (thick) and Actin (thin) myofilaments which are organized into repeating units termed sarcomeres. Sarcomeres are the basic contractile unit of skeletal muscle and display a characteristic light and dark banding pattern under electron microscopy³ (**Figure 1.2**).

Structure of a Skeletal Muscle

Figure 1.1: Schematic representation of skeletal muscle structure

Single muscle fibers are ensheathed by a layer of connective tissue termed the endomysium. Bundles of muscle fibers, termed fascicles, are further enclosed by a second layer of connective tissue called the perimysium. Blood vessels invaginate through fascicles to provide nutrients and chemicals to each muscle cell. Finally, a third layer of connective tissue, termed the epimysium, encapsulates the entire skeletal muscle, which is connected to bone via tendons. This image was adapted from <https://en.wikipedia.org/wiki/Endomysium>. (This image is in the public domain. No copyright clearance is required).

Within the sarcomere, light bands alternate with dark bands, which correspond to the I band (*isotropic*) and A band (*anisotropic*), respectively. A bands are formed through polymerization of myosin filaments, while I bands lack myosin and are comprised of actin. Actin and myosin filaments overlap with each other, except in the H zone where only myosin filaments reside. The sarcomere has additional accessory proteins that play an important role in maintaining sarcomeric structure and function. The crosslinking protein α -actinin anchors actin filaments to the Z line. Titin supports myosin filaments and maintains sarcomeric resting tension, while nebulin contributes to assembly of actin filaments at the Z line. The unique organization of the sarcomere renders it excitable and contractile and indispensable for generation of movement¹.

1.1.2 Muscle as a Contractile Tissue

The sliding filament model, which explains how muscle movement occurs at the molecular level was first hypothesized in 1954 by Andrew F. Huxley and Rolf Niedergerke⁴. The latest model, developed by James Spudich and Michael Sheetz, proposes that myosin filaments slide along actin filaments during contraction⁵. During contraction, energy in the form of ATP is used to promote the dissociation of myosin from actin filaments, which are normally tightly attached to myosin in the absence of ATP. Subsequent hydrolysis of ATP into ADP and inorganic phosphate (P_i) results in a conformational change of the myosin head, which moves the myosin filament to a new position further along the actin filament. Further hydrolysis of ATP rotates the myosin head to its original position, which results in movement of actin filaments toward the center of the sarcomere^{1,2}.

Figure 1.2: Structure of muscle sarcomere

Thick filaments consist of myosin, whereas myofibrillar protein actin forms thin filament. Z disc limits sarcomere width with M line at the center. A band is defined by where only thick filaments are observed. I band refer to the region lacking myosin that disappears when contraction reaches maximum. This image was adapted from Wikipedia, <https://en.wikipedia.org/wiki/Sarcomere> (This image is in the public domain. No copyright clearance is required).

1.1.3 The Development of Muscle

Skeletal muscle development, or myogenesis, is a highly ordered process that is regulated by distinct programmes during prenatal (embryonic), perinatal and postnatal growth⁶.

1.1.3.1 Embryonic and Perinatal Myogenesis

Early developmental signalling initiates the stratification of un-patterned embryos into three germ layers, termed the endoderm, mesoderm and ectoderm. The very first muscle progenitor cells are derived from the mesoderm, which further develops into the paraxial mesoderm, intermediate mesoderm and lateral mesoderm sublayers. Differential expression of critical developmental genes leads to the formation of somites from the paraxial mesoderm⁷. While factors secreted from the lateral plate, neural tube and ectoderm maintain the mesenchymal cell population located at paraxial mesoderm, other signalling factors secreted from the notochord and neural tube promote the migration of cells, through sonic hedgehog (shh) signalling, to form sclerotome at the rostral most site of the embryo⁸. The dorsal part of the somite gives rise to dermomyotome. With the exception of head muscles, skeletal muscles of the body are derived from the dermomyotome⁹. Further signalling cascades results in the polarization of the dermomyotome into dorsomedial (DML) and ventrolateral (VLL) lips, which give rise to deep back musculature and lateral trunk muscle, respectively. Muscle progenitor cells (MPCs), also, reside in dermomyotome. MPCs in the dermomyotome, which is located on the dorsal side of the somite, have high expression of the paired-box transcription factors *Pax3* and *Pax7* and maintain low expression of the basic helix-loop-helix (bHLH) transcription factor *Myf5*^{10,11}. MPCs in dermomyotome express high levels of certain transcription factors, paired-box-3 (*Pax3*) and paired-box-7 (*Pax7*). Later in development, *Pax3* and *Pax7* expressing cells delaminate from the dermomyotome and move to the myotome, which harbours muscle stem cells or satellite cells. Although loss of *Pax3* is lethal in mice, *Pax7*-null mice survive into adulthood. However, the satellite cell population is reduced in *Pax7*-null mice and severe muscle degeneration is noted in adulthood, which in turn results in lethality, suggesting that muscle stem cells that express *Pax7* are required for developmental growth¹². When satellite cells leave their niche in the myotome, they lose expression of *Pax3* and start expressing the myogenic regulatory factor-5 (*Myf5*). Activated satellite cells co-expressing *Pax7* and *Myf5* then move to developing limb buds where they undergo serial

cell division to give rise to myoblasts. However, a unique fraction of these dividing cells may lose expression of myogenic markers and maintain expression of *Pax7*, which enables the muscle stem cell population to self-renew. Interestingly, cells that maintain self-renewal capacity are deployed between the basal lamina and sarcolemma of myofibers during postnatal myogenesis and become the main pool of reserve muscle stem cells for the adult skeletal muscle system.

Migrating MPCs from the dermomyotome to developing limb buds begin to express high levels of the basic bHLH transcription factors *Myf5* and *MyoD*¹³ and initiate a second wave of myogenesis. In fact, previous studies have revealed that actively dividing myoblasts indeed express *Myf5* and *MyoD*, suggesting that both transcription factors play a critical function in cell cycle regulation¹⁴. The exact mechanisms through which myoblasts align and fuse with each other to form large fully functional muscle tissue remain to be completely delineated (**Figure 1.3**).

Figure 1.3: Skeletal muscle development

During embryonic development, dermomyotome gives rise to myotome where muscle progenitor cells reside. Embryonic myogenesis starts with fusion of muscle progenitor cells or myoblasts into primary myotubes or primary muscle fibers. At later stages of embryonic development, primary myotubes fuse and form secondary myotubes that surround primary muscle fibers. After birth, primary and secondary muscle fibers fuse and form mature muscle fibers. (Journal of Applied Physiology 104-3, 579–587 (2008) doi:10.1152/jappphysiol.01091.2007) Permission to use this copyrighted image is granted by RightsLink online copy right clearance centre. (See Appendix 6.1)

1.1.3.2 Postnatal Myogenesis and Muscle Regeneration

Satellite cells are a population of muscle stem cells that are located at the peripheral region of adult muscle fibers, between the basal lamina and plasma membrane of each myofiber, which is known as the satellite cell niche¹⁵. Postnatal myogenesis and regulation of skeletal muscle mass is primarily attributed to the satellite cell population in skeletal muscle. Satellite cells represents about 2-7% of all muscle nuclei in adult mice, while at birth it is reported that the number of satellite cells can represent as much as 30% of total muscle nuclei. Satellite cells are found in quiescent state and express high levels of *Pax7* in adult muscle. Upon muscle damage, satellite cells exit quiescence and become activated. Upon activation satellite cells begin to express *MyoD* along with *Pax7* and proliferate. Previous studies have shown that the growth factors fibroblast growth factor (FGF) and insulin growth factor (IGF) stimulate the proliferation of satellite cells, which underscores the important contribution of growth factors to satellite cell activation^{16,17}. Mechanical stretch of muscle, or exercise, can also lead to activation of satellite cells through nitric oxide synthesis and subsequent release of hepatocyte growth factor (HGF) and the secreted glycoprotein, follistatin¹⁸. Follistatin antagonizes myostatin (reviewed in *Section 1.4.4.3*), which is a potent negative regulator of myogenesis, suggesting that follistatin may play a role in activation of satellite cells¹⁹.

The inherent self-renewal capacity of satellite cells enables them to preserve their numbers, which would otherwise be depleted due to environmental damage. It has been shown that satellite cells undergo asymmetric cell division not only to maintain their self-renewal potential but also to give rise to committed myogenic cells²⁰. Asymmetric division of satellite cells gives rise to two daughter cells, *Pax7*⁺/*MyoD*⁺ cells, which are committed to differentiation; and *Pax7*⁺/*MyoD*⁻ cells, which are satellite cells²¹. *Pax7*⁺/*MyoD*⁻ cells reside in the basal lamina, and indicate quiescent and self-renewed populations of satellite cells. *Pax7*⁺/*MyoD*⁺ cells reside in the myofiber plasma membrane and fuse to form multinucleated myofibers²². Interestingly, recent studies have revealed that other mesenchymal cell types, such as bone and brown adipose tissue, can form as a result of asymmetric division of satellite cells²³. The satellite cell microenvironment maintains the self-renewal capacity of satellite cells and controls commitment of satellite cells into different lineages²⁴. Departure of satellite cells from their niche on adult myofibers results in a reduction in their self-renewal capacity, indicating that the satellite cell niche provides extrinsic factors to preserve the satellite

cell population²⁴. Also, it is important that satellite cells reside in close proximity to blood capillaries to be able to readily receive essential nutrients²⁴.

Skeletal muscle has the innate ability to repair itself in response to injury. Skeletal muscle regeneration in adult vertebrates is mediated by the activation and migration of satellite cells to the site of injury. In response to injury, satellite cells exit a quiescent state and become active within 24 hours after injury. Activated satellite cells generate myoblasts that either fuse with existing myofibers or fuse with each other to form new myofibers at the site of injury, within two days post injury²⁵. Although the mechanism(s) through which muscle trauma activates quiescent satellites cell is not completely understood, it is known that sphingosine-1-phosphate plays a critical role in the entry of satellite cells into the cell cycle, thereby differentiation. In agreement with this, it has been shown that inhibition of sphingosine-1-phosphate halts the process of skeletal muscle regeneration²⁶.

Skeletal muscle regeneration is a highly-ordered process. Skeletal muscle trauma stimulates an inflammatory response in muscle fibers. Severe injury results in necrosis of muscle fibers, which secrete factors that promote recruitment of macrophages to the site of injury. Macrophages rapidly proliferate within 24 hours after injury²⁷. In addition, migration of macrophages from the epimysium and perimysium towards the site of injury leads to the secretion of several chemoattractants, which in turn recruit neutrophils. Neutrophils proliferate rapidly within a few hours and secrete pro-inflammatory cytokines and proteases that result in degradation of myofibrillar proteins. Also, injury activates satellite cells which in turn give rise to myoblasts migrating to the site of injury. These myoblasts may form new myofibers through fusing with each other or contribute to the existing myofiber population through fusing with existing myofibers. Lastly, damaged muscle tissue is repaired through formation of extracellular matrix around the site of injury. Formation of fibrotic tissue termed as fibrosis, supports the muscle regeneration by acting as scaffold for newly formed myofibers²⁸.

1.1.4 Muscle Fiber Types

Muscle fibers are classified based on their myofibrillar protein composition and metabolic activity. In 1873, Louis A. Ranvier identified muscle fibers that appeared visibly redder in rabbit skeletal muscle, which contracted slower, when compared to

paler muscle fibers²⁹. This pioneering work led to the classification of muscle fibers into two distinct types. Specifically, Type I fibers (slow-twitch fibers), which are rich in myoglobin, and as such have a characteristic deep red color and Type II fibers (fast-twitch fibers), which appear paler³⁰. One of the earliest methods to distinguish muscle fiber types was to perform a succinate dehydrogenase (SDH) activity assay, which is a histochemical staining method that correlates well with mitochondrial content of each muscle fiber³¹. This suggests that muscle fibers exhibit distinct energetic characteristics. Type I fibers, which appear as a dark blue color with SDH staining, are rich in mitochondria, and as such utilize more oxygen for aerobic metabolism, when compared to Type II fibers³². Although Type I fibers (slow-twitch fibers) take three times as long as Type II fibers to reach maximum contraction, Type I fibers are able to maintain function during prolonged activities, like standing or endurance exercise³³. The majority of muscle fibers in the human body are Type II fast-twitch fibers. Unlike slow-twitch fibers, fast-twitch fibers are glycolytic in nature (anaerobic) and are immediately excited, but fatigue faster due to a reduced mitochondrial reservoir³⁴.

1.2 Muscle Wasting in Skeletal Muscle

1.2.1 Different Types of Muscle Wasting

As described above, skeletal muscle has the ability to repair itself in response to injury and trauma. However, under certain situations skeletal muscle tissue is lost, resulting in changes in skeletal muscle flexibility, tension and structure. Skeletal muscle tissue is lost in response to numerous conditions, including prolonged periods of muscle disuse, during muscle aging and in response to chronic illness, which are termed disuse atrophy, sarcopenia and cachexia, respectively. In addition to these conditions, mutations in certain genes may result in skeletal muscle atrophy, for example, mutations in the *dystrophin* gene are associated with the development of Duchenne-muscular dystrophy (DMD), which is a disease that leads to muscle weakness and muscle atrophy.

1.2.1.1 Disuse Atrophy

A sedentary lifestyle makes muscle weakness inevitable. Lack of physical activity leads to loss of muscle tone and eventually muscle weakness. Previous studies have reported that lack of physical activity, due to the microgravity of spaceflight, results in muscle atrophy in astronauts³⁵. Also, conditions that limit physical activity,

such as immobilization due to casting³⁶, spinal cord injury³⁷ and prolonged periods of bedrest are directly linked to muscle weakness. Although there are several factors leading to disuse atrophy, reduced anabolic activity and/or increased catabolism appear to be the most robustly altered processes. Notably, immobilized muscle (due to casting) displays increased protein degradation, as opposed to decreased proteins synthesis, in a mouse model, within as little as 4-6 days³⁸. In addition to rodents, limb suspension in humans results in a 50% reduction in global protein synthesis³⁹. Moreover, lesions in lower motor neurons can lead to denervation, which leads to loss of muscle activity and subsequent muscle atrophy. In addition, damage to neurons of the spinal cord or the brainstem can cause paralysis, dystonia and eventually muscle atrophy due to denervation of affected muscles⁴⁰.

1.2.1.2 Sarcopenia

The term “Sarcopenia” is derived from the Greek words “*sarx*” and “*penia*” meaning flesh and poverty. In 1989, Rosenberg *et al.* (2011), described Sarcopenia as muscle wasting during aging⁴¹. Individuals suffering from Sarcopenia lose up to 25% of their total muscle mass after the age of 45⁴². Also, sarcopenia results in additional metabolic changes, such as fat accumulation, fibrosis and insulin resistance, in affected individuals⁴¹. Besides a noticeable decline in total muscle mass, changes in muscle fiber type have been associated with the progression of sarcopenia^{43,44}, with a shift from Type II fibers to Type I fibers reported during sarcopenia⁴⁵. Interestingly, Type II fibers are more susceptible to atrophy due to aging, and as such the cross-sectional area (CSA) of Type II fibers decreases with age⁴⁶.

1.2.1.3 Cachexia

Cachexia refers to the loss of skeletal muscle mass that occurs in response to severe chronic illness, such as AIDS, cancer, COPD, congestive heart failure and chronic kidney disease (CKD). Individuals suffering from cachexia lose 5% or more of total body weight over 12 months and suffer from fatigue, muscle weakness and lower appetite⁴⁷. Cachexia diverges from anorexia, as nutritional intervention is not able to overcome the weight loss and loss of skeletal muscle mass noted in these individuals⁴⁸. Cancer cachexia is highly prevalent, with 50% to 80% of all cancer patients affected by cachexia⁴⁹. More importantly, almost 20% of all cancer-related deaths occurs as a result of cachexia as opposed to the direct tumor burden⁵⁰. Cachexia is a multifactorial

syndrome, yet inflammatory cytokines secreted into the bloodstream, in response to for example tumor formation, play a major role in the development of the cachectic phenotype⁵¹. Previous studies in cancer patients have underscored the importance of the proinflammatory cytokine, tumor necrosis factor alpha (TNF α) in the progression of cachexia⁵². *In vitro* experiments have revealed that TNF α is sufficient to induce atrophy through upregulating the muscle-specific E3 ligases *Atrogin-1* and *Murf1* (Reviewed in *Section 1.3.2.2.1*), that mediate degradation of myofibrillar proteins^{53,54}.

1.2.1.4 Muscular Dystrophy

Muscular dystrophy refers to a group of diseases with hereditary muscle weakness and atrophy, which is associated with mutations in genes that are critical for muscle integrity and function⁵⁵. Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy⁵⁶. In 1861, French scientist Guillaume Benjamin Duchenne initially described the disease in detail⁵⁷ and therefore the disease is referred to as, Duchenne muscular dystrophy or DMD. DMD is an X-linked disorder, therefore males are affected more than females, with an incidence of 1 in every 3500 births⁵⁵. Further genetic studies have revealed that most patients carry mutations in the *dystrophin* gene, which leads to truncation of, or complete loss of, dystrophin protein⁵⁵. The absence of dystrophin protein leads to muscle weakness and increased muscle degeneration. Also, fat accumulation and increased fibrosis have been observed in skeletal muscle of DMD patients. Affected males become wheelchair bound by early adulthood and prematurely die in their early 20s due to failure of cardiac or respiratory musculature function⁵⁸. Dystrophin forms a complex with glycoproteins and functions to anchor the cytoskeleton to the extracellular matrix⁵⁹. Mutations in the dystrophin complex leads to deformation of the cytoskeleton and results in muscle atrophy⁶⁰. Histochemical staining of muscle biopsies from DMD patients has revealed an elevated calcium concentration in the sarcoplasm due to leaky ryanodine receptors (RyR) that allow release of excess Ca⁺² from sarcoplasmic reticulum into the sarcolemma. Ca⁺² overload in sarcolemma results in activation of calcium-dependent calpains (Reviewed in *Section 1.3.2.1.1*), which in turn leads to break down of sarcomeric proteins and muscle wasting⁶¹.

1.3 Proteostasis in Skeletal Muscle Mass

As observed in various type of muscle atrophy, loss of muscle mass results from a shift in the balance of anabolic and catabolic stimuli, whereby protein synthesis is unable to keep up with the accelerated breakdown of myofibrillar proteins⁶². Therefore, understanding protein homeostasis (proteostasis) is crucial to unravelling mechanisms that lead to muscle wasting.

1.3.1 Protein Synthesis in Muscle Mass Maintenance

Although loss of muscle mass is mainly regulated by proteolytic pathways, since protein synthesis and degradation pathways share common mediators, the role of protein synthesis in muscle mass maintenance cannot be underestimated. The quality of protein synthesis is controlled by two different processes, namely, translational efficiency, which is defined as the protein translation per unit amount of mRNA and translational capacity, which is limited by ribosomal content of the cell⁶³.

1.3.1.1 mTOR Pathway

Mammalian target of rapamycin (mTOR) is considered as the main regulator of protein synthesis, which functions through increasing translation efficiency⁶⁴. Moreover, recent studies have shown that rapamycin-mediated inhibition of mTOR suppresses increased ribosomal RNA content observed during hypertrophy, suggesting that mTOR may play a role in regulating not only translation efficiency but also capacity⁶⁵. During hypertrophy, activation of insulin-like growth factor-1 IGF-1 leads to upregulation of Akt/mTOR, which in turn leads to increased protein synthesis⁶⁶ (**Figure 1.4**). In addition, it was further shown that levels of AKT and mTOR were comparable between wild type and *Igf-1* overexpressing transgenic mice, suggesting that either additional pathways may play a role in regulating proteins synthesis, or that the Akt/mTOR pathway may have additional partners in regulating protein synthesis, during mechanical loading. Recent studies have shown that the release of membrane bound lipid phosphatidic acid (PA), upon eccentric muscle contraction, leads to activation of mTOR in an IGF-1 independent manner⁶⁷. Moreover, mTOR promotes translation efficiency by phosphorylating, and thereby activating several translation initiation factors, such as ribosomal protein S6 kinase (p70S6K1), 4E-binding protein (4EBP1) and eukaryotic initiation factor 4 (eIF4), to facilitate formation of the 48S pre-

initiation complex⁶⁸. Also, mTOR plays a role in initiation of translation by shuttling eukaryotic initiation factor 2B (eIF2B) to the translation pre-initiation complex, further supporting that mTOR increases global protein synthesis upon its activation⁶⁹.

1.3.1.2 β -catenin/c-Myc Pathway

Besides translational efficiency, translational capacity is modified upon hypertrophy. Previous reports have revealed upregulation of β -catenin and its downstream target c-myelocytomatosis oncogene (*c-Myc*), by up to 4- and 3-fold, respectively, in overloaded skeletal muscle, independent of mTOR⁷⁰. Dephosphorylation of β -catenin leads to its translocation to the nucleus, which in turn drives expression of *c-Myc* gene. *c-Myc* regulates transcription of many genes involved in cell growth, proliferation and apoptosis⁷¹. Importantly, inhibition of β -catenin augments hypertrophy observed upon mechanical loading, further suggesting that ribosomal content is increased upon hypertrophy stimulus in a β -catenin/c-Myc-dependent manner⁷⁰.

1.3.2 Protein Breakdown in Muscle Mass Maintenance

A decrease in protein synthesis may not necessarily result in loss of muscle mass due to the post-mitotic characteristics of mature myofibers, suggesting that loss of muscle mass is reliant on protein degradation pathways. Several proteolytic systems are present in skeletal muscle, namely the calcium-dependent calpain system, the lysosomal protease system, the ATP-dependent ubiquitin proteasome system and the autophagy pathway, which function together to ensure healthy protein turnover is maintained. The calcium-dependent calpain system, the lysosomal protease system, the ATP-dependent ubiquitin system and autophagy are reviewed in the following sections.

Figure 1.4: *mTOR* pathway in skeletal muscle protein synthesis

Certain hormones and growth factors (e.g. insulin and IGF-1) lead to activation of mTOR pathway through PKB/Akt. Also, leucine enhances phosphorylation thereby activity of mTOR. In stress conditions, low ATP levels result in activation of AMPK which in turn inhibits activity of mTOR. Activated mTOR promotes protein synthesis through ribosomal protein S6 kinase (p70S6K), 4E-binding protein (4E-BP1) and eukaryotic initiation factor 4G (eIF4G). (This image is in the public domain and adapted from <http://www.efdeportes.com>. No copyright clearance is required)

1.3.2.1 Lysosomal Proteases and Calcium Dependent Calpains

1.3.2.1.1 Calpains

Calpains are calcium-dependent cysteine proteinases that are ubiquitously expressed in mammals. Since constant activation of proteolytic mechanisms is detrimental to the cell, calpains remain in an inactive state until their catalytic site is uncapped when Ca^{2+} levels reach a certain threshold^{72,73}. Muscle tissue expresses three isoforms of calpains; calpain 1, 2 and 3, which were detected to be significantly upregulated in response to various muscle wasting conditions⁷⁴⁻⁷⁶. Calpains are not able to directly degrade intact myofibrillar proteins but mediate their depolymerization and disassembly. However, calpains may interfere with organization of titin, vimentin and troponin, which are located at Z-disc⁷⁷, leading to their dissociation from the sarcomere, breakdown of sarcomeric structure and release of sarcomeric proteins myosin and actin into the cytosol⁷⁸.

1.3.2.1.2 Cathepsins

Lysosomes are highly acidic (pH=4-5) membrane-bound vesicles that contain various hydrolases namely proteases, phosphatases, lipases and nucleases⁷⁹, as such, lysosomal proteases facilitate the degradation of macromolecules in cells. In addition, lysosomal proteases are implicated in the turnover of cytosolic compartments and organelles. Cathepsins are lysosomal proteases that have been shown to be upregulated during muscle atrophy, although their contribution to the etiology of muscle wasting remains controversial⁸⁰. Importantly, substances that inhibit cathepsins are not able to attenuate myofibrillar protein degradation during muscle wasting, suggesting that cathepsins may not be sufficient to induce muscle atrophy alone^{81,82}. Studies have revealed that cathepsins do not participate in breakdown of myofibrillar proteins, but play an important role in turnover of membrane associated proteins such as ligands, transporters and channels, which may explain why cathepsin inhibitors fail to rescue the muscle wasting phenotype⁸³.

The lysosomal pathway and ubiquitin proteasome system (reviewed in *Section 1.3.2.2*) collaborate during muscle atrophy. Ubiquitination, one of the prime post-translational modifications that qualifies proteins for degradation. Mono- or di-ubiquitination facilitates degradation of protein substrates through lysosomes, while further ubiquitination is required for activation of the proteasomal pathway⁸⁴. The

ubiquitin proteasome system is unable to degrade intact myofibrils and in fact relies on the function of the lysosomal pathway, such that disassembled myofibrillar proteins can be subsequently degraded by the ubiquitin proteasome pathway, which will be described in the following section (*Section 1.3.2.2*).

1.3.2.2 Ubiquitin-Proteasome System

The Ubiquitin proteasome system (UPS) is a multienzymatic system that regulates turnover of proteins involved in biological processes in the cell. The initial step of the UPS begins with activation of ubiquitin, which is followed by sequential ligation of ubiquitin protein to a specific protein substrate and subsequent transfer of the protein substrate to the proteasome.

Proteasome is a giant protein complex where degradation of target protein takes place. Specifically, E1 enzymes are responsible for activating ubiquitin at its C-terminus through the hydrolysis of ATP⁸⁵. Subsequently, ubiquitin is then transferred to the cysteine residue of an E2 conjugating enzyme, which conjugates ubiquitin to an E3 ubiquitin ligase, which are responsible for catalyzing the ligation of ubiquitin to the substrate protein. Although there are two E1 enzymes and several E2 enzymes identified to date, a large number of E3 ligases, which is currently estimated to be around 650, exist in mammals, which suggests that E3 ligases have a high level of substrate specificity^{86,87}. E3 ligases are classified into three distinct classes; HECT domain E3 ligases, RING-finger E3 ligases and U-box E3 ligases. E3 ligases with a HECT domain harbor two catalytically active domains at their N-terminal and C-terminal. Specific protein substrates bind to the N-terminal HECT domain, whereas the C-terminal HECT domain forms a thioester link with active ubiquitin⁸⁸. RING domain E3 ligases represent the majority of E3 ligases in the mammalian system. Unlike HECT domain E3 ligases, RING domain E3 ligases are not directly involved in ubiquitination, but facilitate the interaction of E2 enzymes with the substrate protein⁸⁹. U-box E3 ligases represent a newly characterized class of E3 ligase family, with structural and functional similarities to RING domain E3 ligases⁹⁰.

Figure 1.5: Ubiquitin proteasome system machinery

The ubiquitin proteasome system (UPS) is tightly controlled through the sequential action of several enzymes. Initially, an E1 ubiquitin-activating enzyme activates ubiquitin by hydrolysing ATP and transfers activated ubiquitin to the E2 conjugating enzyme. E3 ligases subsequently bind to protein substrates and E2 enzymes, allowing association of ubiquitin with its specific substrate. Multiple rounds of E3-mediated ligation of ubiquitin to target substrate proteins results in their polyubiquitination and subsequent recognition by the proteasome. Polyubiquitination of the substrate, which is reversible via the action of de-ubiquitinating enzyme (USP) activity, results in recognition and degradation of the substrate protein by the proteasome. Digestion of the substrate is further carried out in the cytoplasm through the activity of the peptidases, which in turn results in either turnover of amino acids in the cytosol or sequestration of peptides to the cell membrane to act as antigens. Figure is adapted from the illustration of Josh Gramling—Gramling Medical Illustration, *Journal of the American Society of Nephrology*, 17: 1807–1819, 2006. doi: 10.1681/ASN.2006010083. Permission to use this copyrighted image is granted by RightsLink online copy right clearance centre (See Appendix 6.2)

Sequential enzymatic activities of E1, E2 and E3 enzymes leads to the eventual conjugation of ubiquitin to lysine residues in target protein substrates and subsequent recognition of the ubiquitinated substrate by the proteasome. The Proteasome is a multi-complex system that consists of a 19S regulatory subunits and a 20S core particle. The 20S subunit controls the proteolytic activity of the proteasome, yet it is found in inactive state unless it is activated by the 19S regulatory subunit. Assembly of 19S subunit with the 20S core particle allows removal of polyubiquitin chains; resulting in protein unfolding and degradation upon passing of the protein through the barrel-like structure formed by the complex⁸⁷. This process is regulated by ATPases that reside in the 19S regulatory subunit, which function to hydrolyze ATP to provide energy for the process. After entry of substrate protein into the 20S core complex, which harbors 6 proteolytic sites, target proteins are degraded into peptides that range from 3 to 25 amino acids in length (**Figure 1.3**). These peptides are released into the cytosol, where they are further digested through the activity of cytosolic peptidases and are subsequently reutilized to synthesize new proteins or as a source of energy.

The expression of E1 enzymes is very low in skeletal muscle and is not affected during muscle atrophy (presumably because it is the common enzyme shared by many processes). In contrast, several E2 conjugating enzymes show differential expression during muscle wasting⁹¹. Importantly, gene expression analyses, using different muscle wasting models, ranging from cancer cachexia to diabetes mellitus, has revealed a set of common differentially expressed genes that have later been referred to as atrophy-related genes or “atrogenes”⁹². Of these genes, two muscle-specific ubiquitin E3 ligases *Atrogin-1/Mafbx* and *Murf1* have been shown to be differentially expressed^{66,93}, which are reviewed in the following section (*Section 1.3.2.2.1*).

1.3.2.2.1 Atrogin-1/MAFbx and MuRF1

The E3 ligases *Atrogin-1* and *Murf1* were both identified in a screen for muscle-specific E3 ligases associated with several forms of skeletal muscle wasting⁹². Subsequent *In vivo* studies have revealed that loss of *Atrogin-1* and *Murf1* protect mice from denervation-associated loss of muscle mass⁶⁶. Similarly, *Atrogin-1* knockout mice showed resistance to starvation-mediated muscle wasting, whereas knockout of *Murf1* restored dexamethasone-induced atrophy in mice^{94,95}. ATROGIN-1 has been shown to target the myogenic differentiation protein MyoD and eukaryotic initiation factor-3

subunit f (eIF3f), a positive regulator of protein synthesis^{96,97}. Studies have shown that MuRF1 seems to facilitate degradation of myofibrillar proteins, such as MyHC, MyLC and troponin⁹⁸⁻¹⁰⁰.

The UPS is challenged by the accumulation of excess unfolded protein aggregates or damaged cytosolic compartments. In fact, protein aggregates and damaged cytosolic compartments impair proteasome function and are as such removed through activation of an alternative response machinery known as autophagy¹⁰¹.

1.3.2.3 Autophagy

The word autophagy is derived from combination of Greek words “auto” and “phagein” meaning self-eating. Autophagy is a process that mediates turnover and removal of proteins, cytosolic compartments and organelles in a lysosome-dependent manner¹⁰². The autophagy-lysosome pathway has gained recent attention, as it has been recently discovered that the autophagy-lysosome system is involved in the development of a vast range of diseases^{103,104,105}. Autophagy has been categorized into three different subtypes; chaperone-mediated autophagy (CMA), micro-autophagy and macro-autophagy. In CMA, damaged proteins are accompanied by cytosolic chaperones and carried to the lysosomal complex, where damaged proteins are then unfolded and degraded by the lysosomal complex¹⁰². Micro-autophagy refers to the removal of cytosolic components by the lysosomal membrane directly. However, macro-autophagy refers to the engulfment of cytoplasmic compartments by an intermediary double-membrane that later fuses with the lysosomal membrane with the help of specific and non-specific receptors¹⁰⁶. Whether micro-autophagy and CMA take part in muscle wasting is yet to be determined, however, recent studies have emphasized the role of macro-autophagy in muscle atrophy (will be referred as autophagy henceforth).

Glick *et al.*, describes the autophagy process in 5 major steps: 1) formation of a phagophore around the specific target, 2) conjugation of autophagy-related protein 5 (ATG5) to autophagy-related protein 12 (ATG12), 3) recruitment of microtubule-associated protein 1 light chain 3 (LC3/MAP1LC3) to the phagophore, 4) capture of target cargo by receptor proteins and 5) fusion of the lysosomal membrane with the phagophore and subsequent degradation of target molecules¹⁰⁷. Early events of autophagy initiates with interaction of autophagy-related protein 1 (ATG1) with autophagy-related protein 13 (ATG13) and autophagy-related protein 17 (ATG17)¹⁰⁸. The activity of ATG13 is regulated by its phosphorylation status, which is controlled by

the kinase mTOR, suggesting that phagosome formation is dependent on nutrition and growth factors¹⁰⁹. Also, it has been shown that ATG5 knock out mice exhibit atrophy phenotype in muscle, presumably due to accumulation of damaged proteins, suggesting that autophagy is required for maintenance of skeletal muscle function and mass¹¹⁰. After conjugation of ATG5 to ATG12, non-selective autophagosome engulfment is mainly controlled by the activity of microtubule associated protein light chain 3 beta (LC3B), which is cleaved by autophagy-related protein 4 (ATG4). Cleaved LC3B or LC3-I is activated further in an ATP-dependent manner and a lipid moiety is attached through the action of ATG3, termed lipidation. Since autophagy promotes lipidation of LC3B, the levels of lipidated LC3B (LC3B-II) are typically assessed as a means to monitor the extent of autophagy¹¹¹. While LC3B targets the phagosome (vacuole that contains phagocytosed molecules), it needs an adaptor protein on the lysosomal vesicle to allow recruitment of the lysosome to the cargo carrying phagocytosed molecules. Ubiquitin-binding protein/Sequestosome 1 (p62/SQSTM1) was the first protein identified to have an adaptor function to allow fusion of the cargo with the lysosome during autophagy¹¹². Although previous studies have found that p62/SQSTM1 facilitates degradation of proteins by interacting with LC3B, its role is challenged by a new study that suggests that p62/SQSTM1 is dispensable for selective autophagy^{112,113}.

Given that previous works have reported increased autophagy rate in different muscle wasting conditions, including diet-induced muscle wasting¹¹⁴, denervation¹¹⁵, disuse atrophy¹¹⁶, cancer cachexia¹¹⁷ and aging¹¹⁸, targeting autophagy machinery may have promising therapeutic benefit for muscle atrophy. Interestingly, myofibrillar protein degradation due to overexpression of the transcription factor *Atrogin-1*, and its upstream regulator forkhead box-O3 (*Foxo3*), is attenuated by LC3B-siRNA¹¹⁹.

1.3.3 Systemic Mediators of Muscle Wasting

The sections above have described macro-responses that function to maintain homeostasis during muscle atrophy. The section below describes three systemic hormones and growth factors that have been shown to be activated during muscle atrophy. These systemic factors are; IGF-1, glucocorticoids and myostatin.

1.3.3.1 IGF-1/Akt Pathway

Insulin-like growth factor-1 (IGF-1) promotes anabolic metabolism in muscle. Previously, ectopic expression of IGF-1 in muscle has been shown to attenuate sarcopenia in old mice and leads to hypertrophy in younger mice¹²⁰. In response to increased levels of IGF-1, the downstream target AKT is phosphorylated and activated. Activation of AKT negatively regulates FOXO transcription factors by phosphorylating them and preventing their nuclear entry. Inhibition of FOXO transcription factors prevents the upregulation of downstream target atrogenes, including *Atrogin-1* and *Murfl*¹²¹ (Reviewed in *Section 1.3.2.2.1*). Also, AKT promotes protein synthesis via mTOR signaling¹²²(Reviewed in *Section 1.3.1.1*). Previous studies have revealed hypertrophy in transgenic mice expressing IGF-1¹²⁰ or AKT¹²³ in skeletal muscle, which further support the role of IGF-1/AKT signaling in regulation of skeletal muscle mass through activation of protein synthesis.

1.3.3.2 Glucocorticoids

Glucocorticoids (GCs) are steroid hormones that are widely used to suppress the inflammation associated with chronic inflammatory diseases¹²⁴. GCs bind to glucocorticoid receptors (GR), which in turn dimerizes and translocates to the nucleus where it binds to glucocorticoid response element (GRE). Binding of GR to GRE increases the expression of genes involved in anti-inflammatory response¹²⁴. GCs are found to be increased in the bloodstream of individuals undergoing skeletal muscle atrophy^{125,126}. GCs also have a fiber type-specific effect, with a greater impact noted on Type II fast-twitch fibers, when compared to slow-twitch fibers¹²⁷. Importantly, GCs have been shown to induce protein break down and inhibit protein synthesis in skeletal muscle, which in turn leads to loss of muscle mass¹²⁸. Dexamethasone is a synthetic GC, which is commonly used as immunosuppressant. Mechanistically, Waddell *et al.*, showed that dexamethasone treatment results in increased expression of *Murfl* through a mechanism involving increased activation of the transcription factor FOXO1. Subsequent loss-of-function experiments by the same group revealed that mutation of the GR prevents dexamethasone-mediated *Murfl* expression¹²⁹.

1.3.3.3 Myostatin

Myostatin is a member of TGF- β superfamily and has been characterized as negative regulatory of muscle growth¹³⁰. Myostatin, as a secreted growth factor, binds

to a receptor complex on the plasma membrane, which consists of a Type I (Alk4/5) and Type II receptor (ActRIIB)¹³¹. Interaction of Myostatin with its Type II receptor leads to a conformational change on the receptor, which in turn facilitates heterodimerization of type II receptor with type I receptor¹³¹. This interaction triggers the kinase activity of type I receptor, which leads to activation of various downstream signaling pathways, including the canonical Smad signaling pathway^{132,133}. Mechanistically, Myostatin has been shown to regulate the growth and differentiation of myoblasts^{131 134}. Myostatin negatively regulates myoblast proliferation through upregulating p21, which leads to cell cycle arrest¹³⁵. In addition, myostatin hinders MyoD activation and expression via a Smad3-dependent mechanism, which eventually impedes myogenic differentiation¹³⁴. The importance of myostatin has been revealed in knock out mouse studies, where *myostatin*-null mice display a remarkable hypertrophy phenotype¹³⁶. Consistent with this phenotype, blockade of myostatin, through injection of either a soluble form of the ActRIIB receptor (sActRIIB) or an antibody raised against myostatin, results in significant skeletal muscle hypertrophy in mice¹³⁷. Excess levels of Myostatin have been shown to promote skeletal muscle wasting. Similar to other muscle wasting stimuli, myostatin inhibits phosphorylation of FOXO1, through suppression AKT, which leads to enhanced activation of FOXO1 and increased expression of atrogenes^{138,139}. Importantly, loss of myostatin has been shown to attenuate expression of atrogenes and prolong survival of cancer bearer mice¹⁴⁰.

1.4 Mitochondria and Skeletal Muscle

1.4.1 Mitochondria and Their Function

According to the endosymbiotic theory, mitochondria were originally aerobic bacteria that had been taken up into eukaryotic cells ~1.5 billion years ago¹⁴¹. It is further thought that upon this merging, eukaryotic cells became able to exist in oxygen rich environment and utilize oxygen and nutrients to produce energy¹⁴². Therefore, mitochondria are considered the powerhouse of mammalian cells. In addition to energy production mitochondria have important functions in detoxification of reactive oxygen species and regulating apoptosis. Mitochondria are double membrane structures which consist of outer membrane, inner membrane, intermembrane space and matrix. Outer mitochondrial membrane which encloses entire mitochondria has integral membrane

Figure 1.8: Structure of mitochondrion

Mitochondria are double-membrane structures that consist of an outer membrane and inner membrane. Mitochondrial outer and inner membrane are separated by intermembrane space, while mitochondrial inner membrane forms cristae-like structures that invaginate through matrix. Mitochondria have their own DNA and ribosomes that reside in matrix. This image was adapted from Tutorvista, <http://biology.tutorvista.com> (This image is in the public domain. No copyright clearance is required).

proteins which control transport of proteins and molecules across the membrane. Intermembrane space is the space between mitochondrial inner membrane and outer membrane. Inner membrane, which is highly impermeable to proteins, harbors 1/5 of total mitochondrial proteins including critical OXPHOS proteins. Also, inner membrane

has highly organized inner cristae that invaginate through the mitochondrial matrix. Such invaginations increase the surface area of the inner mitochondrial membrane, where electron transport chain (ETC) proteins reside, to maximize energy generation.

Mitochondrial matrix contains

mitochondrial enzymes, which play a critical role in generation of ATP, mitochondrial ribosomes, tRNAs and mitochondrial DNA suggesting that mitochondrial matrix has a critical role in mitochondrial function¹⁴³. Mitochondria contain their own DNA, which encodes for 22 tRNAs and 13 small peptides that play important roles in oxidative phosphorylation. In the mitochondria, DNA is transcribed by the unique polymerase POLG, accompanied by a mitochondrial-specific single stranded binding protein and a helicase known as TWINKLE¹⁴⁴.

1.4.1.1 Electron Transport Chain and Oxidative Phosphorylation (OXPHOS)

The mitochondrial outer membrane (MOM) is responsible for controlling the passing of ions and proteins into the mitochondria through pores located on the membrane. Whereas, the mitochondrial inner mitochondria (MIM) is the location where

electron transport chain¹⁴⁵ proteins are embedded. The ETC consists of five complexes that orchestrate energy production in eukaryotic cells: complex I (NADH:ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (cytochrome *bc*₁ complex), complex IV (cytochrome *c* oxidase) and complex IV or F₁F₀-ATP synthase. The ETC is series of enzymatic reactions involving electrons acceptors and donors that eventually results in the production of energy. Electron transport is controlled by two enzymes, coenzyme-Q10, or ubiquinone (Q) and cytochrome *c*. In the first step of the ETC, NADH enters the ETC cycle at complex I where Q is reduced to ubiquinol or QH₂ through the capture of electrons from the electron carrier, NADH. Complex II balances the electron gradient across the MIM and allows the transport of protons generated by the reduction of NADH by complex I. Electrons carried by QH₂ are subsequently passed to cytochrome *c* in complex III. Reduced cytochrome *c* is then oxidized at complex IV. This process continues until all electrons are passed to the ultimate electron acceptor, oxygen, which in turn forms water molecules. In response to the sequential donation of electrons through the ETC, a proton gradient is created across the mitochondrial inner membrane¹⁴⁶. Electron gradient that is formed due to electrons pumped into mitochondrial intermembrane space drives electrons back through the mitochondrial membrane through mitochondrial complex V, ATP synthase. Coupling of electrons through ATP synthase leads to formation of ATP from ADP and inorganic phosphate (Pi) in this gigantic protein complex¹⁴⁷. The complete process of energy generation through the ETC is referred to as oxidative phosphorylation or OXPHOS¹⁴⁸. However, like every biological system, the ETC is not perfect. Some electrons skip this process and form free radicals through non-enzymatic reactions. The formation of free radicals is potentially deleterious to the cell, which makes energy production by mitochondrial a double-edged sword, as such mitochondrial function needs to be tightly controlled.

1.4.1.2 Detoxification of Reactive Oxygen Species (ROS)

OXPHOS results in the generation of reactive oxygen species (ROS) due to the process of electron capture by mitochondrial complexes. ROS encompasses chemically reactive byproducts containing oxygen, such as hydrogen peroxide (H₂O₂)¹⁴⁹, hydroxyl radical (HO•)¹⁵⁰ and singlet oxygen (¹O₂)¹⁵¹. Superoxide (O₂⁻) is primary and the most abundant precursor of free radicals¹⁵². Final electron transfer to O₂ leads to formation of superoxide that is converted to hydrogen peroxide (H₂O₂) through the action of two different superoxide dismutases, SOD1 in the inner membrane space and SOD2 in the

mitochondrial matrix¹⁵³. Excess H₂O₂ may leak into the intermembrane space or even cytosol. Since excess H₂O₂ leads to oxidative damage of proteins, lipids and mtDNA, mitochondria employ various response mechanisms to prevent deleterious effects of H₂O₂¹⁵⁴. Oxidized proteins and lipids due to high levels of H₂O₂ trigger a repair mechanism in mitochondria that is mediated by glutaredoxin (GRX), thioredoxin 2 (TRX2) and glutathione peroxidase 4 (GPX4)¹⁵⁴. In addition to these enzymes, antioxidant effects of mitochondrial electron carriers ubiquinol and coenzyme Q (Reviewed in *Section 1.4.1.1*) have been implicated^{155,156}.

Figure 1.9: Electron transport chain (ETC) in mitochondrial inner membrane

Electrons captured from NADH enter the system from complex I (NADH-ubiquinone oxidoreductase), which is the largest mitochondrial complex, containing 40 subunits. Complex II (succinate-quinone oxidoreductase) provides an alternative entry site for electrons. Complex I reduces ubiquinone (Q) to ubiquinol (QH₂). QH₂ is then utilized by complex III (cytochrome *bc*₁ complex) to reduce cytochrome *c*, which in turn reduces oxygen at complex IV (cytochrome *c* oxidase) to form water. 10 protons are pumped across the mitochondrial inner membrane for each NADH molecule oxidized, which generates proton motive force that drives complex V, F₁F₀-ATP synthase, resulting in ATP formation from ADP and Pi (Nature Reviews Molecular Cell Biology 16, 375–388 (2015) doi:10.1038/nrm3997). Permission to use this copyrighted image is granted by RightsLink online copy right clearance centre (See Appendix 6.3)

1.4.1.3 Mitochondrial Biogenesis

Given that mitochondria are the major energy production site in the cell, the contribution of mitochondria to cellular biogenesis is undeniable. Studies in mice have identified peroxisome proliferator-activated (PPAR)- γ coactivator-1 (PGC-1 α) as the main regulator of mitochondrial biogenesis¹⁵⁷. Studies have further demonstrated that PGC-1 α regulates mitochondrial biogenesis by activating the nuclear respiratory transcription factors NRF1 and NRF2¹⁵⁸. Importantly, these two transcription factors are direct upstream regulators of *Tfam*, which promotes transcription of mtDNA from two distinct promoter sites¹⁵⁹. The importance of NRFs to *Tfam* regulation are clear, as mutations in the NRF binding site of the *Tfam* promoter, not only hinders binding of NRFs to *Tfam*, but also reduces *Tfam* promoter activity¹⁶⁰. In muscle cells, overexpression of *Pgc-1 α* has been confirmed to increase mitochondrial biogenesis, as assessed by uncoupling protein 2 (*Ucp2*) upregulation, which that is presumably driven by NRF1 binding on *Tfam* promoter¹⁵⁸.

Although PGC-1 α is able to control the transcription of several mitochondrial genes that contribute to mitochondrial biogenesis, mitochondria need to be able to crosstalk with cytosolic pathways to be able to respond to the energy status and nutrient availability of the cell. Energy crisis in the cell has been found to be regulated by an AMP-responsive protein, AMP-activated kinase (AMPK)¹⁶¹. In an unstressed state, ATP:ADP ratio remains high due to active catabolic processes. High ATP:ADP ratio drives adenylate kinase to synthesize more ADP which results in a low AMP:ATP ratio, which maintains AMPK in an inactive state. However, in response to stress conditions, such as hypoxia, inadequate carbon sources or exposure to metabolic inhibitors, AMPK becomes activated through the lowering of the ADP:ATP ratio in the cell¹⁶². Further examination of AMPK in rodent skeletal muscle has revealed that feeding mice with a β -guanadinopropionic acid (β -GPA) diet (which mimics exercise training) not only leads to activation of AMPK, but also increased mitochondrial biogenesis through PGC-1 α and NRFs¹⁶³. In addition, studies have revealed increased activity of mitochondrial enzymes citrate synthase, malate dehydrogenase and cytochrome *c* upon 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR)-induced AMPK activation in rats¹⁶⁴. Activation of AMPK has also been shown to be responsible for the development of muscle atrophy during hind limb unloading¹⁶⁵. In addition, sustained activation of AMPK in slow twitch fibers results in atrophy that is associated with a

sarcopenia-like phenotype in a rat model¹⁶⁶. Such negative regulation of muscle mass by AMPK could potentially result from its role as an upstream regulator of FOXO transcription factors, as described under *Section 1.3.4.1*. In fact, a recent study suggests that as a consequence of impaired mitochondrial biogenesis, AMPK is activated and subsequently promotes protein degradation through FOXO transcription factors¹⁶⁷.

1.4.1.4 Apoptosis

Apoptosis refers to programmed cell death that can be brought about by infection, irreparable damage, severe energy deprivation or DNA damage due to antioxidants¹⁶⁸. The apoptotic signaling pathway relies on the activity of pro-apoptotic factors and cysteine proteases, known as caspases, that eventually lead to the release of cytochrome c (Cyt c)¹⁶⁹. In healthy conditions, caspase-3/7 and caspase-9 activity is blocked by XIAP (Inhibitors of Apoptosis), while apoptotic protease activating factor-1 (APAF-1) is inactivated through its auto-inhibitory function¹⁷⁰. Also, anti-apoptotic factor B-cell lymphoma-2 (BCL-2) inhibits activity of BCL-2 associated X protein (BAX) and BCL-2 antagonist/killer 1 (BAK). Upon activation of apoptosis, BCL-2 inhibitory effect is released and thereby, BAX and BAK become activated¹⁷¹. Subsequent activation of BAX and BAK proteins promotes the release of several apoptotic agents, mainly Cyt c, Omi/HtrA2 and Smac/DIABLO¹⁷². In addition, active Cyt c in the cytosol initiates apoptosome formation by interacting with APAF-1, which in turn results in caspase activation¹⁶⁸. Apoptosome is a tetrameric protein complex that induces activity of caspases, which leads to systematic degradation of cellular components and thereby, cell death. In skeletal muscle, fructose-induced oxidative stress has been associated with decreased mitochondrial membrane potential, poor mitochondrial biogenesis and eventually apoptosis¹⁷³. Also, mitochondrial dysfunction and subsequent apoptosis has been shown to be responsible for selective cell death in muscles of human and rodent juvenile Parkinsonism models¹⁷⁴.

1.4.2 Importance of Mitochondria for Skeletal Muscle

Muscle is highly dynamic tissue with contractible and excitable properties, as such muscle energy expenditure should be tightly controlled. Since muscle cell ATP consumption can increase 100-fold during contraction¹⁷⁵, mitochondria are critical for meeting the energy demand of skeletal muscle. Mitochondria supply ATP not only

during contraction but also for basal metabolism of skeletal muscle. It is also known that defective mitochondrial activity leads to muscle weakness and exercise intolerance¹⁷⁶. As described above, muscle wasting can occur in response to either chronic or acute stimuli; while acute muscle wasting predominantly affects slow twitch fibers, chronic muscle wasting targets Type-II fibres¹⁷⁷. Mitochondria, not only generate the ATP required for contraction of muscle and cell metabolism, but also have a role in cell signaling, ROS production, biogenesis, and apoptosis, as reviewed in *Section 1.4.1.2, 1.4.1.3 and 1.4.1.4*, respectively. Mitochondria can have specialized functions based on their location within skeletal muscle. Mitochondria residing beneath the sarcolemma, are termed subsarcolemmal mitochondria (SSM); whereas mitochondria located between myofibrils are known as intermyofibrillar mitochondria (IMF)¹⁷⁸. The different metabolic nature of SSM and IFM allows them to respond distinctively to various conditions. These two mitochondrial subpopulations differ in protein and lipid content, and thereby function. For example, SSM are shown to have more branched cristae, probably enabling these mitochondria to more efficiently produce energy¹⁷⁹. The close proximity of SSM to nuclei and cell membranes allows SSM to generate a more rapid response to cellular challenges, such as during exercise¹⁸⁰. IFM in skeletal muscle reside in close proximity to the sarcoplasmic reticulum, which enables the two organelles to crosstalk to maintain Ca^{2+} homeostasis. Also, previous studies have shown that IFM have greater mitochondrial enzyme activity and respiration, when compared to SSM¹⁸¹. Interestingly, skeletal muscle exposed to prolonged activity or disuse stimulate increased turnover of SSM relative to IFM populations of mitochondria¹⁸².

1.4.3 Mitochondrial Quality Control

Mitochondria form a tubular network throughout the cell that is tightly controlled through the action of various quality control systems. Mitochondrial homeostasis can influence the fate of cells, in fact, the apoptosis pathway is centralized around mitochondrial function. Mitochondrial damage due to unfolded mitochondrial proteins, metabolic stress, and exposure to mitochondrial uncouplers triggers proteolytic systems in mitochondria, which result in degradation of mitochondrial components. The following sections describe different mechanisms that maintain mitochondrial quality.

1.4.3.1 Mitochondrial Proteasome System

Mitochondria respond to the presence of unfolded proteins, that cannot be refolded by chaperones, through activating either cellular or mitochondrial proteolytic machinery. Also, the presence of unassembled or disassembled mitochondrial complex subunit proteins leads to activation of mitochondrial proteasome system. Mitochondrial proteases that reside in the MIM regulate the unfolded protein response, whereas cytosolic proteases have been shown to contribute to degradation of mitochondrial proteins in an ATP-dependent manner^{183,184} (**Figure 1.5**). AAA+ (ATPases associated with diverse cellular activities) proteases are mitochondrial proteases that play a central role in mitochondrial quality control through their proteolytic activities. Of these mitochondrial proteases, caseinolytic mitochondrial matrix peptidase chaperone subunit (mtClpXP) and Lon peptidase 1 (LON/PIM1) that are highly conserved from bacteria to eukaryotes mediate degradation of oxidized proteins in the mitochondrial matrix^{185,186}. Recent studies have revealed the additional mitochondrial proteases HTRA2/OMI and presenilins-associated rhomboid-like protein (PARL), which are associated with neurodegenerative diseases^{187,188}. Although the exact target of HTRA2/OMI remains elusive, mice lacking HTRA2/OMI show accumulation of unfolded proteins in mitochondria that results in mitochondrial dysfunction and neuronal apoptosis¹⁸⁹.

1.4.3.2 Mitochondria Derived Vesicles (MDV)

Mitochondria derived vesicles (MDVs) are a recently characterized class of mitochondrial quality control. In response to oxidative stress, selective cargo that carries damaged proteins and lipids is delivered to lysosomes for ultimate degradation¹⁹⁰ (**Figure 1.5**). Interestingly, it has been recently shown that MDVs require PTEN-induced kinase (PINK1) and PARKIN, which are primary regulators of mitophagy¹⁹¹. However, contrary to this, increased ROS has been shown to induce the MDV pathway¹⁹². Results suggest that MDVs preserve mitochondrial quality, through maintaining mitochondrial membrane potential, by selective degradation of proteins, prior to the initiation of mitophagy¹⁹¹.

Figure 1.10: Mitochondrial quality control

Unfolded mitochondrial proteins trigger either mitochondrial AAA+ proteases in the intermembrane space and mitochondrial matrix or selective removal of those proteins through fission. If mitochondrial damage overwhelms these two quality control mechanisms, then the entire mitochondrion is isolated from the healthy mitochondrial network and degraded by autophagy machinery (Frontiers in Cellular Neuroscience (2016) 10, 24; doi:10.3389/fncel.2016.00024). Permission to use this copyrighted image is granted by Creative Commons Attribution Licence centre (See Appendix 6.4).

1.4.3.3 Mitophagy

When mitochondrial damage overwhelms the mitochondrial quality control mechanisms described above, autophagosomes mediate the removal of mitochondria in a systematic and selective manner^{193,194} (**Figure 1.6**). Mitochondrial autophagy or mitophagy maintains a healthy pool of mitochondria, such that damaged mitochondria are prevented from fusing with healthy mitochondria¹⁹⁵. Once mitochondria are damaged, several proteins label the mitochondria for recruitment to autophagosomes, enabling the removal of defective mitochondria. In steady-state situations, mitophagy can function to impart beneficial effects on cell development. For example, erythrocytes lose their mitochondria during maturation through mitophagy¹⁹⁶. Although the reason has not been completely identified, it has been shown that mitophagy-mediated removal of mitochondria in red blood cells helps to keep ROS production in an optimal level¹⁹⁷. Another example of mitophagy in health occurs in sperm where mitochondria are removed by egg fertilization¹⁹⁸. Mitophagy is also activated in response to various stress conditions, most notably in the presence of mitochondrial uncouplers¹⁹⁹, such as carbonyl cyanide m-chlorophenyl hydrazine (CCCP), photo irradiation²⁰⁰ and in response to mtDNA deletions²⁰¹.

Figure 1.11: Graphical model of mitochondrial quality control

Proteostasis is maintained by the mitochondrial ubiquitin proteasome system (UPS^{mt}) within the mitochondria. Impaired proteostasis machinery leads to elevated levels of ROS, which turn activates mitochondrial antioxidant enzymes, such as superoxide dismutases (SODs), peroxiredoxins (PRXs) and glutathione peroxidases (GPXs). When excessive ROS overwhelms the detoxification process, damage of mitochondrial DNA (mtDNA), proteins and lipids occurs, which eventually activates DNA repair mechanisms and lipases. If the aforementioned control mechanisms fail to repair the mitochondrial damage, depolarized mitochondria undergo fission and complete removal by autophagosomes through the process termed mitophagy. Persistent failure in repair mechanisms eventually results in cellular apoptosis (Nature Reviews Drug Discovery 12, 465–483 (2013), doi:10.1038/nrd4023). Permission to use this copyrighted image is granted by RightsLink online copy right clearance centre (See Appendix 6.5)

1.4.3.3.1 PINK1/PARKIN-Mediated Mitophagy

The quality of mitochondria is maintained through mitochondrial fusion and fission events. In healthy cells, fusion and fission events are maintained in an equilibrium. However, for engulfment of mitochondrion by autophagosomes, single mitochondrion need to be isolated from the network. Mitochondrial fusion is orchestrated by the highly conserved GTPases, mitofusion protein 1 (MFN1) and 2 (MFN2) in the MOM, while optic atrophy 1 (OPA1) controls mitochondrial fusion events in the MIM²⁰². In addition to mitochondrial fusion there are proteins that regulate mitochondrial fission. Dynamic related protein 1 (DRP1) forms a complex in the mitochondrial outer membrane and facilitates mitochondrial budding with the help of the Drp1 receptor proteins fission 1 (FIS1), mitochondrial fission factor (MFF), mitochondrial dynamics proteins MID49 and MID51¹⁹⁴. Recent studies have consistently reported that mitochondrial depolarization leads to degradation of mitochondrial fusion markers OPA1¹⁶¹, MFN1¹⁶² and MFN2²⁰³⁻²⁰⁶.

Mitochondrial quality and mitophagy are primarily controlled by crosstalk between P-TEN-induced kinase (PINK1) and PARKIN (**Figure 1.7**). In a fly model, similar phenotypes of muscle degeneration, hyperfused mitochondria and impaired mitochondrial function, have been observed upon loss of *pink1* and *parkin*, indicating that these proteins act on the same pathway²⁰⁷. Also, previous studies have shown that *parkin* overexpression partially rescues the phenotype observed upon *pink1* knockdown, while *pink1* overexpression cannot mask *parkin* knockdown phenotype, suggesting that PARKIN is downstream target of PINK1²⁰⁸. PINK is a serine/threonine kinase with an N-terminal mitochondrial targeting sequence, serine/threonine kinase domain and a transmembrane helix²⁰⁹. PARKIN is a RING-Between-RING (RBR) type E3 ligase with ubiquitin-like domain (UBL) at the N-terminal, 3 RING domains (RING0, RING1, RING2), in-between-RING (IBR) and repressor element of PARKIN (REP)²¹⁰.

Upon mitochondrial depolarization, PINK1 activates PARKIN through phosphorylation of PARKIN at Ser65²¹¹. Recently, a new mechanism has been proposed whereby PINK1 phosphorylates ubiquitin to hyperactivate PARKIN catalytic activity²¹²⁻²¹⁴. According to the proposed model, PINK1 phosphorylation of ubiquitin leads to recruitment of PARKIN to the OMM and further activation of PARKIN catalytic activity. Hyperactivation of PARKIN is achieved by relieving auto-inhibition induced by both the RING0 and UBL domain of PARKIN, which obstruct the catalytic cysteine (Cys431) in the PARKIN RING2 domain and RING1 E2 ligase binding

domain, respectively²¹³. Phosphorylation of PARKIN at Ser65 results in a conformational change in PARKIN structure, thereby releasing repression of the autoinhibitory domain²¹⁵. Mitochondrial membrane associated and activated PARKIN leads to degradation of downstream targets MFN1, MFN2 and several other mitochondrial proteins^{205,216}. Activation of PARKIN leads to the activation of two different ubiquitination cascades on mitochondria, namely lysine48 (K48) and lysine63 (K63) ubiquitination. Polyubiquitination at K48 results in degradation of the substrate through proteasome, whereas polyubiquitination at K63 has been reported to be proteasome-independent²¹⁷. Due to preference of autophagy adaptors for K63, autophagic degradation upon K63 ubiquitinated substrates has been implicated^{218,217,219}. Ubiquitinated proteins then targeted by an intermediary protein p97/vasolin-containing protein (VCP) for degradation through proteasome²²⁰. Degradation of mitofusin proteins through the AAA-ATPase activity of p97/VCP and proteasomal system, and subsequent activation of mitochondrial fission machinery, results in disassembly of unhealthy mitochondria from the healthy mitochondrial pool²²¹. Finally, the autophagy adaptors p62/SQSTM1 and LC3 sequester impaired mitochondria and direct them to autophagosomes for degradation by lysosomal vesicles^{222,223}. Importantly, loss of autophagy-related genes has been shown to block mitophagy in CCCP treated cells, underscoring the importance of crosstalk between mitophagy and the canonical autophagy pathway¹⁹⁹. Although initial studies predicted that p62/SQSTM1 mediates autophagosome formation around mitochondria, a recent study has shown that it may be dispensable for mitophagy¹¹³.

Besides mitochondrial turnover, PARKIN takes part in regulating mitochondrial biogenesis. A recent study showed that PARKIN increases proteasomal degradation of PARKIN interacting substrate (PARIS), which represses *Pgc1a* expression, thereby leading to reduced mitochondrial biogenesis^{224,225}. Moreover, it is proposed that mitochondrial ubiquitin ligase 1 (MUL1) and PARKIN may work in parallel to promote mitophagy, with both factors converging on MFN2 degradation²²⁶.

Since mitophagy is an infrequent event in the cell, researchers promote mitophagy through mitochondrial uncouplers. Mitochondrial uncoupling agents have been shown to reduce mitochondrial membrane potential, which in turn results in mitophagy²²⁷. Although mitochondrial uncouplers are widely used in mitophagy research, they have limitations due to their nonspecific and even detrimental effects in the cell²²⁸. Rotenone is a parkinsonian toxic which results in a milder reduction in

mitochondrial membrane potential that is insufficient to induce PINK1/PARKIN-mediated mitophagy²²⁹. In addition, other parkinsonian toxins, such as MPP⁺ and 6-hydroxydopamine (6-OHDA), have been shown to regulate mitophagy through activation of extracellular-signal-regulated protein kinase 2 (ERK2) that is PINK1/PARKIN-independent^{230,231}. Due to non-specific effects of uncoupling agents, carbonyl cyanide m-chlorophenyl hydrazine (CCCP) and carbonyl cyanide-p-(trifluoromethoxy) phenylhydrazone (FCCP) are most commonly used uncouplers in this context. FCCP and CCCP treatment induce recruitment of PARKIN and LC3 to mitochondria within minutes suggesting that their effect is specific to PINK1/PARKIN-mediated mitophagy^{199,232}. Given that CCCP treatment model has significantly contributed to parkinsonian studies conducted using neurons and HeLA cells^{113,199,233}, its effect on skeletal muscle has remained unstudied. Therefore, in this study, myotubes were treated with CCCP to understand the most basic mechanism of PINK1/PARKIN-mediated mitophagy in C2C12 myotube model.

Figure 1.12: Mitochondrial turnover and mitophagy

Mitochondrial fusion is controlled by Mfn2 and Mfn1 while fission is Opa1 and Fis1 dependent. Mitochondrial depolarisation recruits certain E3 ligases to mitochondria. These E3 ligases ubiquitinate specific mitochondrial proteins. Then autophagosomes engulf mitochondria for autophagy. Alternatively, mitochondria may succumb to mitophagy directly through BNIP3L/NIX proteins (Expert Rev Mol Med. 2010 Apr; 12: e12, doi: 10.1017/S1462399410001456). Permission to use this copyrighted image is granted by RightsLink online copy right clearance centre (See Appendix 6.6)

1.5 Parkinson's Disease and Mitochondrial Dysfunction

Parkinson's disease (PD) is the second most common neurodegenerative disease of our era, which is reported to be affecting 7 million people around the world²³⁴. PD was first characterized by James Parkinson in 1817 in his book "*An Essay on the Shaking Palsy*". In this initial characterization, PD was described by symptoms including involuntary tremors, muscle weakness, abnormal gait and postural instability. The studies by James Parkinson not only led to clinical recognition of the disease, but also provided a greater understanding of the pathophysiology of PD. Importantly, these initial studies have allowed subsequent studies to be undertaken to decipher the molecular mechanism(s) of PD. In the early 1980s, drug addicts who developed progressive parkinsonism-like phenotype captured wide interest from clinicians and scientists. Studies revealed that the neurotoxic drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leads to Parkinsonian symptoms in these drug users²³⁵. Further studies revealed that MPTP results in reduced mitochondrial ATP production and elevated ROS levels through inhibiting complex I, suggesting that mitochondrial dysfunction could be a causative factor for PD^{236,237}. Although PD has been thought to be a sporadic disease resulting from neurotoxic agents and additional environmental risk factors²³⁸, a family history of young individuals affected by PD drove scientists to search for genetic background of this severe neurodegenerative disease. Genetic studies have identified mutations in six genes which are associated with the autosomal recessive form of PD: *PARKIN* (*PARK2*), *PINK1* (*PARK6*), *DJ-1* (*PARK7*), *SCNA* and *LRRK2*^{239,240}. Interestingly, further studies have revealed that these disease associated genes are involved in mitochondrial quality control and function, which further support the mitochondrial dysfunction theory of PD that was first established following the identification of MPTP-induced parkinsonism²³⁵.

Mutations in the *PARKIN* gene are the most common cause of familial PD and in fact, mutation of *PARKIN* is responsible for almost 80% of early onset PD²⁴¹. *PARKIN* mutations were first identified in autosomal recessive juvenile Parkinsonism phenotype observed in Japanese families²³⁹. Unlike human PD, *Parkin* knock out mice display reduced locomotor activity when they are placed in a new environment, suggesting a cognitive decline in those mice²⁴². In contrast with this study, Perez *et al.*²⁴³ have shown no significant alteration in locomotor activity or anxiety-depression-like behaviors in *Parkin* knock out mice. Previous studies have reported that *Parkin* knock out mice have impaired dopamine release in substantia nigra and reduced synaptic

excitability, although nigral degeneration is not as massive as in human PD patients^{244,245}.

As mentioned above, mutations in *Parkin* are linked to mitochondrial dysfunction and development of PD. Interestingly, in the fly model system, knockout of *parkin* leads to reduced climbing activity and abnormal wing posture, suggesting that flight muscle function is affected upon loss of *Parkin*¹⁷⁴. Also, a recent study has documented the presence of a hyperfused mitochondrial network and increased cell death in *parkin* knock out drosophila²²⁶. In addition, another study has reported increased sensitivity to mitochondrial toxins and β -amyloid protein A β in *Parkin* knock out primary myotubes, which was restored by lentiviral-mediated overexpression of *Parkin*, suggesting that *Parkin* has a protective role in skeletal muscle²⁴⁶. A further study has revealed that denervation-induced atrophy is alleviated in *Parkin* knock out mice, which is presumably due to its involvement in autophagy-mediated denervation²⁴⁷. In addition to skeletal muscle atrophy, *Parkin* KO myotubes display reduced insulin sensitivity and impaired respiratory capacity²⁴⁸. Consistent with loss of muscle mass and muscle weakness, previous studies have demonstrated that PD patients also have impaired mitochondrial function in muscle tissue, with a reduction in mitochondrial complex activity noted²⁴⁹. Although we know that *Parkin* is central to the mitochondrial quality control mechanism, it remains to be determined whether or not *Parkin*-mediated mitophagy plays an important role in regulating muscle function and strength. Moreover, no study to date has identified the causative factors responsible for the skeletal muscle atrophy phenotype noted in PD patients and rodent PD models.

1.6 Aim of The Project

PD is the second most prevalent neurodegenerative disease and affects 1% of the aging population worldwide²⁵⁰. The pathology of PD is characterized by a loss of dopaminergic neurons in substantia nigra pars compacta (SNc) and Lewy bodies in the brains of PD patients²⁵¹. Previous reports have documented reduced mitochondrial complex I and complex IV activity in skeletal muscle of PD patients^{249,252-256}. In addition, further studies have attributed impaired motor activity, loss of skeletal muscle mass, muscle weakness, fatigue and resistance to exercise to mitochondrial dysfunction in skeletal muscle of PD patients²⁵⁷⁻²⁵⁹. Changes in muscle mass and function are noted in individuals with PD and that these changes are linked to mitochondrial dysfunction. However, the molecular mechanisms behind the skeletal muscle phenotype in PD is not well understood.

Due to a robust phenotype observed in *pink1* and *parkin* mutants, our current understanding of familial PD has come from studies performed in the drosophila fly model system. In drosophila, loss of *parkin* results in abnormal wing posture and reduced motor activities, due to a hyperfused mitochondrial network observed in their indirect flight muscles (IFM)^{174,226}. A recent study has identified protective role of *Parkin* in skeletal muscle. Rosen *et al.*, (2006) revealed increased sensitivity of primary myoblasts isolated from newborn *Parkin* KO mice to mitochondrial toxins and β -amyloid protein A β , which was restored by lentiviral mediated overexpression of *Parkin*²⁴⁶. In addition, it has been shown that *Parkin* KO mice are resistant to denervation-induced muscle atrophy, presumably due to involvement of *Parkin* in autophagy-mediated denervation^{247,260}. PD is associated with muscle weakness and impaired mitochondrial function in skeletal muscle. Moreover, given that mutations of the *PARKIN* gene have a causative role in PD, *Parkin* is important for controlling mitophagy and has a potential role in skeletal muscle, I hypothesize that the E3 ligase *Parkin* may play a critical role in maintaining skeletal muscle mass and function through regulation of mitochondrial turnover and function. To test this hypothesis the following objectives were undertaken:

Objectives:

- Investigate whether CCCP promotes PINK1/PARKIN mediated mitophagy in myoblasts.
- Determine the changes in myotube phenotype induced upon CCCP treatment.
- Assess mitochondrial function and turnover in *Parkin* knock down and knock out models.
- Define the role of *Parkin* in regulation of skeletal muscle mass using *in vitro* and *in vivo* *Parkin* loss-of-function models.
- Elucidate potential mechanism(s) responsible for regulation of skeletal muscle mass and function in *Parkin* knock down and knock out models.

2 MATERIALS AND METHODS

2.1 Materials

Materials used in this study have been described in the following section with details of compositions, companies and catalogue numbers provided.

Compositions of all materials used in this thesis is provided in the following section.

2.1.1 DNA Electrophoresis

Details of reagents used for DNA gel electrophoresis are provided in the following tables.

Table 2.1: TAE Buffer

TAE Buffer	Concentration/Amount/Volume
Tris-HCl (pH 7.6)	40 mM
EDTA	1 mM
Acetic Acid	20 mM

Table 2.2: 1% DNA Gel

1% DNA Gel	Concentration/Amount/Volume
Ultra Pure Agarose	1 g
TAE Buffer	100 ml

2.1.2 RNA Electrophoresis

Reagents used for RNA gel electrophoresis are given in the tables below.

Table 2.3: MOPS Buffer (10X)

MOPS Buffer (10X)	Concentration/Amount/Volume
MOPS	200 mM
Sodium Acetate	50 mM
EDTA	20 mM

Table 2.4: 1.2% RNA Gel

1% DNA Gel	Concentration/Amount/Volume
Ultra Pure Agarose	1.2 g
MOPS Buffer (1X)	10 ml
37% Formaldehyde	18 ml
Top up with MQ water to	100 ml

Table 2.5: RNA Loading Dye (2x)

RNA Loading Dye (2X)	Concentration/Amount/Volume
MOPS (10X)	10% (v/v)
Deionised Formaldehyde	20% (v/v)
Deionised Formamide	50% (v/v)
Bromophenol Blue	0.02% (w/v)
Glycerol	5% (v/v)
EDTA (pH 8.0)	1 mM
Ethidium Bromide	40 µg/ml

2.1.3 Western Blot Analysis

The following tables outline details of reagents used for total protein extraction and subsequent western blot analysis to detect protein levels.

Table 2.6: Phosphate-Buffered Saline (PBS)

PBS (1X)	Concentration/Amount/Volume
Oxoid™ Phosphate-Buffered Saline Tablet	1 tablet
Top up with MQ water to	100 ml

Table 2.7: Radioimmunoprecipitation Assay (RIPA) Buffer

RIPA Buffer	Concentration/Amount/Volume
Nonidet P-40 (NP-40)	1% (v/v)
SDS	0.1% (w/v)
Sodium Deoxycholate	0.5% (w/v)
Sodium Fluoride	50 mM
Top up with PBS to required volume	

Table 2.8: Complete RIPA Buffer

Complete RIPA Buffer	Concentration/Amount/Volume
RIPA Buffer	7 ml
Sodium Orthovanadate	1 mM
PMSF	1 mM
cOmplete™ Mini Protease Inhibitor	1 tablet
PhosSTOP™	4 tablets

Table 2.9: 16% Bis-Acrylamide Resolving Gel

16% Resolving Gel	Concentration/Amount/Volume
30% Acrylamide/Bis Stock	4.27 ml
1.5M Tris-HCl (pH 8.8)	2 ml
SDS	80 µl
Ammonium Persulfate	80 µl
TEMED	8 µl
MQ water	1.6 ml

Table 2.10: 6% Bis-Acrylamide Stacking Gel

6% Stacking Gel	Concentration/Amount/Volume
30% Acrylamide/Bis Stock	1 ml
0.5M Tris-HCl (pH 6.8)	1.25 ml
SDS	50 µl
Ammonium Persulfate	50 µl
TEMED	5 µl
MQ water	2.6 ml

Table 2.11: Protein Loading Dye (4X) for Bis-Tris Gel

Protein Loading Dye for Bis-Tris Gel (4X)	Concentration/Amount/Volume
LDS Sample Buffer (4X)	75% (v/v)
β-mercaptoethanol	25% (v/v)

Table 2.12: Protein Loading Dye (2X) for Tris-Glycine Gel

Protein Loading Dye For Tris-Glycine Gel (2X)	Concentration/Amount/Volume
Tris-Glycine SDS Sample Buffer (2x)	90% (v/v)
β-mercaptoethanol	10% (v/v)

Table 2.13: MES SDS Running Buffer

MES SDS Running Buffer	Concentration/Amount/Volume
NuPAGE® MES SDS Running Buffer (20X)	50 ml
MQ water	950 ml

Table 2.14: Tris-Glycine SDS Running Buffer

Tris-Glycine SDS Running Buffer	Concentration/Amount/Volume
Novex™ Tris-Glycine SDS Running Buffer (10X)	100 ml
MQ water	900 ml

Table 2.15: Western Blot Wet Transfer Buffer

Transfer Buffer	Concentration/Amount/Volume
Tris Base	25 mM
Glycine	192 mM
Methanol	20% (v/v)
Top up with MQ water to required volume	

Table 2.16: Tris-Buffered Saline with Tween-20 (TBS-T)

TBS-T	Concentration/Amount/Volume
Tris-HCl (pH 7.5)	20 mM
NaCl	150 mM
Tween-20	0.1% (v/v)
Top up with MQ water to required volume	

Table 2.17: Polyvinylpyrrolidone (PVP) Blocking Solution

PVP Blocking Solution	Concentration/Amount/Volume
PVP	1% (w/v)
Polyethylene Glycol (PEG)	1% (w/v)
Bovine Serum Albumin (BSA)	0.3% (w/v)
Top up with TBST to required volume	

Table 2.18: 5% Milk Blocking Solution

5% Milk Blocking Solution	Concentration/Amount/Volume
Skimmed Milk Powder	5% (w/v)
Top up with TBST to required volume	

Table 2.19: 5% Bovine Serum Albumin (BSA) Blocking Solution

5% BSA Blocking Solution	Concentration/Amount/Volume
BSA	5% (w/v)
Top up with TBST to required volume	

2.1.4 MitoTracker Red CMXRos Confocal Analysis

Details of the fixatives used for MitoTracker red staining analysis are given below.

Table 2.20: 4% Paraformaldehyde Solution

4% Paraformaldehyde	Concentration/Amount/Volume
Paraformaldehyde	40 g
PBS	800 ml
NaOH	As required to dissolve PFA
HCl	As required to reach pH 6.9
Top up with MQ water to	1000 ml

Table 2.21: 2% Paraformaldehyde Solution

2% Paraformaldehyde	Concentration/Amount/Volume
4% Paraformaldehyde Solution	250 ml
MQ water	250ml

2.1.5 Hematoxylin and Eosin (H&E) Staining

Details of reagents used for H&E staining are listed in the tables below.

Table 2.22: 20:2:1 Fixative Solution for H&E Staining

20:2:1 Fixative	Concentration/Amount/Volume
70% ethanol	20 ml
37% Formaldehyde	2 ml
Acetic Acid	1 ml

Table 2.23: Scott's Tap Water

Scott's Tap Water	Concentration/Amount/Volume
Sodium Bicarbonate	3.5 g
Magnesium Sulphate	20 g
MQ Water	1000 ml

2.1.6 Seahorse XF²⁴ Analysis

Medium and reagents used to assess MitoStress Stress, Glycolysis Stress and Fatty Acid Oxidation (FAO) using the XF²⁴ seahorse instrument are provided in the following tables.

Table 2.24: Seahorse Assay Medium for MitoStress Test Kit

Seahorse Assay Medium	Concentration/Amount/Volume
Sodium Pyruvate	1 mM
Glucose	25 mM
Glutamine	2 mM
Top up with Seahorse XF Base Medium (See Table 2.34 for details) to required volume	

Table 2.25: Seahorse Fatty Acid Oxidation (FAO) Assay Substrate-Limited Medium

Seahorse FAO Substrate-Limited Med.	Concentration/Amount/Volume
Glucose	0.5 mM
Glutamine	1 mM
Carnitine	0.5 mM
FBS	1%
Top up with Seahorse XF Base Medium (See Table 2.34 for details) to required volume	

Table 2.26: Seahorse FAO Assay Medium (KHB Medium)

Seahorse FAO Substrate-Limited Med.	Concentration/Amount/Volume
NaCl	111 mM
KCl	4.7 mM
Calcium Chloride	1.25 mM
Magnesium Sulphate	2 mM
Sodium Phosphate Monobasic	1.2 mM
Glucose	2.5 mM
Carnitine	0.5 mM
HEPES	5 mM
Top up with MQ water to required volume	

Table 2.27: Seahorse FAO Assay Palmitate-BSA Solution

Seahorse FAO Substrate-Limited Med.	Concentration/Amount/Volume
Sodium Palmitate	1 mM
BSA (Fatty acid free)	0.17 mM
NaCl	150 mM
Top up with MQ water to required volume	

2.1.7 SMARTPool Small interfering RNA (siRNA) Resuspension

Details of the 5X concentrated siRNA buffer used to resuspend lyophilized siRNA stock vials are provided in the following table.

Table 2.28: siRNA Buffer (5X)

siRNA Buffer (5X)	Concentration/Amount/Volume
KCl	300 mM
HEPES (pH=7.5)	30 mM
MgCl ₂	1 mM
Top up with DEP-C treated water to required volume	

2.1.8 Antibodies

Primary and secondary antibodies used in this thesis are listed below. Details of antibody companies, catalogue number, dilution, host species (species the antibody was raised in) and blocking solution are provided.

Table 2.29: Primary Antibodies

<i>Antibody Name</i>	<i>Company</i>	<i>Catalogue Number</i>	<i>Dilution</i>	<i>Host</i>	<i>Blocking Solution</i>
p-AMPK α (Thr172)	Cell Signaling	2535	1:1000	rabbit	5% milk
AMPK α	Cell Signaling	2532	1:1000	rabbit	5% milk
p-eIF2 α (Ser51)	Cell Signaling	9721	1:500	rabbit	5% BSA
CHOP	Cell Signaling	5554	1:200	rabbit	5% BSA
p-mTOR (Ser2448)	Cell Signaling	2971	1:1000	rabbit	5% BSA
mTOR	Cell Signaling	2972	1:1000	rabbit	5% milk
p-FOXO3a (Ser253)	Cell Signaling	9466	1:1000	rabbit	5% BSA
FOXO3a	Cell Signaling	7497	1:500	rabbit	5% milk
p-FOXO1 (Ser256)	Cell Signaling	9461	1:1000	rabbit	5% milk
FOXO1	Cell Signaling	2880	1:1000	rabbit	5% milk
DRP1	Cell Signaling	8570	1:1000	rabbit	5% milk
PINK1	Abcam	ab23707	1:1000	rabbit	5% milk
PARKIN	Abcam	ab130867	1:1000	mouse	5% milk
COXIV	Abcam	ab14744	1:500	mouse	PVP
OXPHOS	Abcam	ab110413	1:1000	mouse	5% milk
MFN1	Abcam	ab57602	1:1000	mouse	5% milk
PARIS	Abcam	130867	1:1000	rabbit	5% milk
HSP60	Abcam	ab46798	1:10000	rabbit	5% milk
MURF1	Regeneron	(Gift)	1:500	mouse	5% milk
MFN2	Santa Cruz	sc-100560	1:1000	mouse	5% milk
p-AKT1/2/3 (Ser 473)	Santa Cruz	sc-7985	1:1000	rabbit	5% milk
AKT1/2/3	Santa Cruz	sc8312	1:1000	rabbit	5% milk
PERK	Cell Signaling	sc-13073	1:1000	rabbit	5% milk
ATROGIN-1	Cell Signaling	PAB15627	1:500	goat	5% milk
LC3	Novus Bio.	NB100-2220	1:500	rabbit	5% milk
PGC-1 α	Millipore	ab3242	1:1000	rabbit	5% milk
FIS1	BioVision	3491R	1:1000	rabbit	5% milk
MyHC	DSHB	MF20	1:10000	mouse	5% milk

MyLC	DSHB	T14C	1:10000	mouse	5% milk
GAPDH	Santa Cruz	sc-32233	1:10000	mouse	5% milk
α -TUBULIN	Sigma	T9026	1:10000	mouse	5% milk

Table 2.30: Secondary Antibodies

<i>Antibody Name</i>	<i>Company</i>	<i>Catalogue Number</i>	<i>Dilution</i>	<i>Host</i>	<i>Blocking Solution</i>
Anti-mouse HRP	Bio-Rad	1706516	1:5000	goat	same as primary
Anti-rabbit HRP	Bio-Rad	1706515	1:5000	goat	same as primary
Anti-goat HRP	Bio-Rad	1721034	1:2000	rabbit	same as primary

2.1.9 Oligonucleotides

Oligonucleotides were designed using NCBI primer designing tool (Primer-BLAST, NCBI, <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and were purchased from Integrated DNA Technologies (IDT, Singapore). Pre-designed gene-specific SMARTpool siRNA, which target four different regions of the gene of interest and amplify a 150-200 base pairs region, were purchased from Dharmacon RNAi Technologies (Lafayette, CO, USA). Sequences of oligonucleotides and SMARTpool siRNA are provided in the tables below.

Table 2.31: Oligonucleotide Sequences

<i>Gene Symbol</i>	<i>Accession No/Reference</i>	<i>Forward Primer Sequence (5'-3')</i>	<i>Reverse Primer Sequence (5'-3')</i>
<i>Mfn1</i>	261	ATTGGGGAGGTGCTGTCTC	TTCGGTCATAAGGTAGGCTTT
<i>Mfn2</i>	261	AGATGTCCCTGCTCTTTTCTC	TGTGTTCCCTGTGGGTGCTT
<i>Opa1</i>	261	GATGACACGCTCTCCAGTGAAG	CTCGGGGCTAACAGTACAACC
<i>Drp1</i>	261	CGGTTCCCTAAACTTCACGA	GCACCATTTCATTTGTACACG
<i>Fis1</i>	261	AAGTATGTGCGAGGGCTGTT	GGCAGAGAGCAGGTGAAGG
<i>Mff</i>	261	TCACATTTGGTGAGTGGGGC	TTTCCGGGACCCTCATTCCG
<i>Nd1</i>	262	TGCACCTACCCTATCACTC	ATTGTTTGGGCTACGGCTC
<i>Nd2</i>	262	ATGAGTAGGCCTGGAATT	ATCAGAAGTGAATGGGGG
<i>Nd4</i>	262	ATAATTATAACTAGCTCAATCTGC	TCGTAGTTGGAGTTTGCCTAG
<i>Nd4L</i>	262	CTCACCATAGCCTTCTCAC	CGTAATCTGTTCCGTACGTG

<i>Nd6</i>	262	TGTATGAGGTTGATGATGTT GG	CCGCAAACAAAGATCAC CC
<i>Cytb</i>	262	CTTTGGGTCCCTTCTAGGAG TCTG	GCTGTGGCTATGACTGC GAACAG
<i>CoI</i>	262	GCTGAAGGAGAAGGAGAA	ATACACATAGCTCTTCT CCC
<i>Atp6</i>	262	CCACACACCAAAAGGACGA ACATGA	CGGACTGCTAATGCCAT TGGTTG
<i>Ndufa9</i>	262	CATTACTGCAGAGCCACT	ATCAGACGAAGGTGCAT GAT
<i>Ndufb6</i>	262	ATAACTTTTTGCGGGACGGG	CAGGAAAATCTCTCATT GGTG
<i>Ndufs3</i>	262	AGGAACATGGCGGCGGCTGC	ATTTCAGCCACATACTC TCC
<i>Sdha</i>	262	CATACTGTTGCAGCAGCACA GG	CCACCAAATGCACGCTG ATA
<i>Uqcrc1</i>	262	CCTACAGCACTCGAGAGCAC	AGGTGTGCCCTGGAATG CTG
<i>Uqcrc2</i>	262	TCCCTCAAAGTTGCCCC	GCAAGACGTAGTAAATG TGAG
<i>Uqcrfs</i>	262	GATGTCAAGGTGCCCGACTT	GATCTCGATCTTCGACA TGG
<i>Cox4</i>	262	GAGCACATGGGAGTGTGTG	CTGTCTTCCATTCAATTG TGC
<i>Atp5</i>	262	GGTCATCCTTTGTTGGTGC	GAGAATTCCACCATCCC TTC
<i>Parkin</i>	XM_006523 336.3	AAACCGGATGAGTGGTGAGT	AGCTACCGACGTGTCCT TGT
<i>Pink1</i>	NM_026880.2	GCTGATCGAGGAGAAGCAG	GATAATCCTCCAGACGG AAGC
<i>Nedd4</i>	NM_010890 .3	CTCTCGGAGGACGAGGTATG	CTCTCGGAGGACGAGGT ATG
<i>FoxO3</i>	NM_019740.2	CCTACTTCAAGGATAAGGGC GAC	GGAGCTCCAGGTACGGA TCA
<i>MuRF1</i>	NM_00103904 8.2	ACGAGAAGAAGAGCGAGCT G	GCCTTCATTCTGAACGC GCATG
<i>Atrogin-1</i>	NM_026346.3	GCGTGTTCTCTGGCAACATA	CTTGGCACTTGAGAGGA AGG
<i>Trim72</i>	NM_00107993 2.3	GGAGTGCTCAGACCAGAAG G	GAGAAGAGGTGCAGGG ACTG
<i>mtDNA (CoI)</i>	263	GCCCCAGATATAGCATTCCC	GTTTCATCCTGTTCTGCT CC
<i>nuDNA (18S)</i>	263	TAGAGGGACAAGTGGCGTTC	CGCTGAGCCAGTCAGTG T
<i>Ube2c</i>	NM_026785.2	GCCGGCACCGTATATGAAGA	GTGGGTGCGTTGTAAGG GTA
<i>Fbxl22</i>	NM_175206.4	ACCCTTTCTGGGCATTCACG	CACAGCAGACACTCCCG ATT
<i>Rnf144a</i>	NM_080563.4	CGGGACCAGGTACTTTGTGT	GCACTAGGGGTCCATCC AAC
<i>Traip</i>	NM_011634.3	ACTAGCAAGAATGGCAGCCC	AGTCCTGAACCCAAGCC CTA
<i>Trim43b</i>	NM_00117088 4.1	CAAGTGTGTGACCCACAAGG	ACCACCACAAGGTCGTT CTC
<i>Trim59</i>	NM_025863.3	AATTGATGAAGTCCGCCAAC	CAGGAACATGGCGTTCT TTT

<i>Zfp598</i>	NM_183149.1	GGTGAGGACTACGAGGAGG T	CTTGTACCTCCAGCTTCC CC
<i>Usp18</i>	NM_011909.2	CAGGAGTCCCTGATTGCGT	GGGCTGGACGAAACATC TCA
<i>Bip</i>	NM_001163 434.1	TGTGTGTGAGACCAGAACCG	TGCCCACATCCTCCTTCT TG
<i>Chop</i>	NM_00129018 3.1	CCTGAGGAGAGAGAACCTG GTC	CTTCGTTTCCTGGGGAT GAGAT
<i>Irela</i>	M_023913.2	TGCTGTTAGCAAGAGGACGG	GGCCTGAACCAATTCTG GGA
<i>Gapdh</i>	NM_008084	GATGATGACCCGTTTGGCTC C	ACGCTCGTGGAAGAAA AGA
<i>Ppia</i>	NM_008907.1	GCATACGGGTCTGGCATCT TGTCC	GTGGCAAAGTGAGATTG TTGCC

Table 2.32: SMARTpool siRNA Sequences

<i>siRNA</i>	<i>Catalogue Number</i>	<i>Sequence (5'-3')</i>	
Non-silencing control	D-001206-13	UAGCGACUAAACACAUC AA	UAAGGCUAUGAAGAGAU AC
		AUGUAUUGGCCUGUAUU AG	AUGAACGUGAAUUGCUC AA
Murine <i>Parkin</i> -specific	M-065413-01	CGAAUCACCUGACGGUU CA	ACACGUCGGUAGCUUUG AA
		ACUCAACGAUCGGCAGU UU	GAGGAAUGCGUGCUGCA AA

2.1.10 Chemical, Reagent and Kit Inventory List

Details of chemicals, reagents and kits used in this study are listed in following tables.

Table 2.33: List of Chemicals Used in This Thesis

<i>Chemical</i>	<i>Company</i>	<i>Catalogue Number</i>
2-propanol (Isopropanol)	Merck KGaA, Darmstadt, Germany	109634
Acetic acid	Merck KGaA, Darmstadt, Germany	100056
Ammonium persulfate	Sigma-Aldrich, St. Louis, MO, USA	A3678
β -mercaptoethanol	Sigma-Aldrich, St. Louis, MO, USA	M6250
Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, MO, USA	A3675
Bromophenol blue	Sigma-Aldrich, St. Louis, MO, USA	114391
Calcium chloride (CaCl ₂)	Sigma-Aldrich, St. Louis, MO, USA	449709
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Sigma-Aldrich, St. Louis, MO, USA	C2759
Chloroform	Merck KGaA, Darmstadt, Germany	102445
Chloroquine	Sigma-Aldrich, St. Louis, MO, USA	C6628

Collagenase	Sigma-Aldrich, St. Louis, MO, USA	C0130
D-glucose	Sigma-Aldrich, St. Louis, MO, USA	G7021
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA	276855
Eosin-Y solution	Merck KGaA, Darmstadt, Germany	109844
Ethanol	Merck KGaA, Darmstadt, Germany	107017
Ethidium bromide	Bio-Rad, Hercules, CA, USA	1610433
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich, St. Louis, MO, USA	E9884
Fatty acid free-BSA	Sigma-Aldrich, St. Louis, MO, USA	3117057001
Formaldehyde	Sigma-Aldrich, St. Louis, MO, USA	252549
Formamide	Sigma-Aldrich, St. Louis, MO, USA	F9037
L-Glutamine	Thermo Fisher Scientific, Waltham, MA, USA	21051-024
Glycerol	Sigma-Aldrich, St. Louis, MO, USA	536407
Glycine	Bio-Rad, Hercules, CA, USA	1610718
Hematoxylin, Gill III	Merck KGaA, Darmstadt, Germany	105174
HEPES	Sigma-Aldrich, St. Louis, MO, USA	H7006
L-Carnitine hydrochloride	Sigma-Aldrich, St. Louis, MO, USA	C0283
Magnesium chloride (MgCl ₂)	Sigma-Aldrich, St. Louis, MO, USA	M8266
Magnesium sulphate	Sigma-Aldrich, St. Louis, MO, USA	M5921
Methanol	Merck KGaA, Darmstadt, Germany	106009
MOPS	Sigma-Aldrich, St. Louis, MO, USA	M1254
PMSF	Sigma-Aldrich, St. Louis, MO, USA	P7626
Polyethylene Glycol (PEG)	Sigma-Aldrich, St. Louis, MO, USA	202444
Polyvinylpyrrolidone (PVP)	Sigma-Aldrich, St. Louis, MO, USA	234257
Potassium chloride (KCl)	Sigma-Aldrich, St. Louis, MO, USA	P9541
Sodium acetate	Sigma-Aldrich, St. Louis, MO, USA	S2889
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO, USA	S5761
Sodium chloride (NaCl)	1st Base, Singapore	BIO-1110
Sodium deoxycholate	Sigma-Aldrich, St. Louis, MO, USA	D6750
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, St. Louis, MO, USA	L3771
Sodium fluoride (NaF)	Sigma-Aldrich, St. Louis, MO, USA	S7920
Sodium hydroxide (NaOH)	Sigma-Aldrich, St. Louis, MO, USA	S0899
Sodium orthovanadate (Na ₃ VO ₄)	Sigma-Aldrich, St. Louis, MO, USA	S6508
Sodium palmitate	Sigma-Aldrich, St. Louis, MO, USA	P9767
Sodium phosphate monobasic	Sigma-Aldrich, St. Louis, MO, USA	S0751

Tetramethylethylenediamine (TEMED)	Bio-Rad, Hercules, CA, USA	1610801
Tris base	1st Base, Singapore	BIO-1400
Tween-20	Fisher Scientific, Waltham, MA, United States	PRH5151
Xylene	Sigma-Aldrich, St. Louis, MO, USA	214736

Table 2.34: List of Reagents Used in This Thesis

<i>Reagent</i>	<i>Company</i>	<i>Catalogue Number</i>
1 kb plus DNA ladder	Thermo Fisher Scientific, Waltham, MA, USA	10787018
30% acrylamide/bis solution	Bio-Rad, Hercules, CA, USA	1610156
BlueJuice™ gel loading buffer	Thermo Fisher Scientific, Waltham, MA, USA	10816015
Chick embryo extract (CEE)	United States Biological, Salem, MA, USA	C3999
cOmplete™ Mini EDTA-free Protease Inhibitor	Sigma-Aldrich, St. Louis, MO, USA	4693159001
DharmaFECT® 1	Dharmacon RNAi Technologies, Lafayette, CO, USA	T-2001
DPX	Sigma-Aldrich, St. Louis, MO, USA	44581
Fetal bovine serum (FBS)	Gibco, Carlsbad, CA, USA	10082147
High Glucose Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Carlsbad, CA, USA	10566016
Horse serum (HS)	Gibco, Carlsbad, CA, USA	16050122
IGEPAL (NP-40)	Sigma-Aldrich, St. Louis, MO, USA	I8896
LDS sample buffer	Thermo Fisher Scientific, Waltham, MA, USA	NP0008
Matrigel®	Corning Inc, corning, NY, USA	354248
MitoTracker® Green FM	Thermo Fisher Scientific, Waltham, MA, USA	M7514
MitoTracker® Red CMXRos	Thermo Fisher Scientific, Waltham, MA, USA	M7512
Novex™ Tris Glycine SDS Running Buffer	Thermo Fisher Scientific, Waltham, MA, USA	LC2675
NuPAGE® 4-12% bis-tris precast gel	Invitrogen, Carlsbad, CA, USA	NP0321BOX
NuPAGE® MES Running Buffer	Thermo Fisher Scientific, Waltham, MA, USA	NP0002
OCT	VWR, Radnor, PA. USA	25608-930
Opti-MEM™	Gibco, Carlsbad, CA, USA	31985070

Oxoid™ Phosphate-Buffered Saline Tablet	Thermo Fisher Scientific, Waltham, MA, USA	BR0014G
Penicillin/Streptomycin	Gibco, Carlsbad, CA, USA	15140122
PhosSTOP™	Sigma-Aldrich, St. Louis, MO, USA	PHOSS-RO
Ponceau S	Sigma-Aldrich, St. Louis, MO, USA	P7170
RPMI, no methionine	Thermo Fisher Scientific, Waltham, MA, USA	A1451701
Seahorse XF Assay Medium	Agilent Technologies, Santa Clara, CA, USA	102365-100
Seahorse XF Base Medium	Agilent Technologies, Santa Clara, CA, USA	102353-100
Seahorse XF Calibrant solution	Agilent Technologies, Santa Clara, CA, USA	100840-000
See Blue Plus2 protein standard	Thermo Fisher Scientific, Waltham, MA, USA	LC5925
SsoFast™ EvaGreen® Supermix	Bio-Rad, Hercules, CA, USA	172-5200
Tris-Glycine lds sample buffer	Thermo Fisher Scientific, Waltham, MA, USA	LC2676
TRIzol®	Thermo Fisher Scientific, Waltham, MA, USA	15596026
UltraPure™ agarose	Thermo Fisher Scientific, Waltham, MA, USA	16500-500
Western Lightning® Plus-ECL	Perkin Elmer, Waltham, MA, USA	NEL105001EA

Table 2.35: List of Kits Used in This Thesis

<i>Kit Name</i>	<i>Company</i>	<i>Catalogue Number</i>
Agilent RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, CA, USA	5067-1511
Click-iT® HPG Alexa Fluor® Protein Synthesis Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA	C10428
iScript™ cDNA Kit	Bio-Rad, Hercules, CA, USA	170-8890
mirVana™ miRNA Isolation Kit	Thermo Fisher Scientific, Waltham, MA, USA	AM1560
Mitochondria Isolation Kit for Cultured Cells	Thermo Fisher Scientific, Waltham, MA, USA	89874
Proteasome-Glo™ 3 Substrate Cell-Based Assay System	Promega, Madison, WI, USA	G1180
QIAamp® DNA Mini Kit	Qiagen, Valencia, CA, USA	51304
Seahorse XF Cell Mito Stress Test Kit	Agilent Technologies, Santa Clara, CA, USA	103020-100

2.2 Methods

2.2.1 Animal Studies

Park2^{tm1Shn} mice in C57BL/6 background were obtained from The Jackson Laboratory (Sacramento, CA, USA). Wild type C57BL/6 mice were obtained from Biological Resource Centre (BRC, Singapore). Mice were housed in the Nanyang Technological University (NTU) Animal House Facility (Singapore), in temperature (20-22°C) and light (12 hours light/dark) controlled rooms. All animal experiments were approved by the Institutional Animal Ethics Committee (IACUC, Singapore) and were conducted only after successful completion of the Responsible Care and Use of Laboratory Animal Course (RCULAC, Singapore).

2.2.2 C2C12 Cell Culture and Treatments

C2C12 mouse myoblasts were obtained from ATCC (Manassas, VA, USA) and have been described previously²⁶⁴. For all studies, C2C12 myoblasts were grown at 37°C in a 5% CO₂ incubator. Myoblast proliferation was maintained with proliferation medium, which consists of high-glucose (4.5 g/L) Dulbecco's Modified Eagle Medium (DMEM; GE Healthcare, Piscataway, NJ, USA), supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (P/S; Gibco, Carlsbad, CA, USA). To induce differentiation of myoblasts into myotubes, myoblasts were seeded at the density of 25000 cells/cm² and grown in low serum differentiation medium, which consists of DMEM supplemented with 2% Horse Serum (HS) and 1% P/S (Gibco, Carlsbad, CA, USA), for 72 h. Myoblasts fully differentiate into myotubes within 72 h, as previously confirmed before²⁶⁵. For CCCP treatment, 72 h differentiated myotubes were treated with either 10 µM CCCP or an equal volume of DMSO vehicle control (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. For chloroquine (CQ) treatment, 72 h differentiated myotubes were treated with either 100 µM CQ or DMSO for an additional 6 h (Sigma-Aldrich, St. Louis, MO, USA).

2.2.3 Transient Transfection of SMARTpool siRNA into C2C12 Myoblasts and Myotubes

Myoblasts were seeded in 6-well plates, at a density of 15000 cells/cm² and maintained in 2 ml/well of proliferation medium lacking antibiotics (without P/S). For

myotube transfection, myoblasts were seeded as 20000 cells/cm² and differentiated for 72 h in differentiation medium. On the day of transfection, excess medium was removed to get a final volume of 1.6 ml/well. Transfection medium was prepared as follows: 5 nmol lyophilized SMARTpool siRNAs were resuspended in 250 µl of 5X siRNA Buffer to obtain a 20 µM stock solution. siRNA solutions were further diluted with DEPC water to a concentration of 5 µM (1st Base, Singapore) just before transfection. A total of 10 µl diluted siRNA solution (25 nM final) and 8 µl DharmaFECT[®] 1 Transfection Reagent per well were added into two different tubes, each containing 200 µl Opti-MEM[™] medium (Dharmacon RNAi Technologies, Lafayette, CO, USA). After incubation for 5 minutes at room temperature, each siRNA-Opti-MEM mixture was added into respective tubes containing DharmaFECT-Opti-MEM dropwise, while gently tapping the bottom of the micro centrifuge tubes. Following 20 minutes incubation at room temperature, the siRNA-liposome solution was added into respective wells dropwise while rocking the plate. Transfected cells were incubated for either 24 h or 48 h and were subjected to a medium change prior to sample collection. Use of antibiotics was avoided during transfections.

2.2.4 Murine Primary Myoblast Isolation

Four- to six-week-old wild type and *Parkin* KO male mice were euthanatized with CO₂. Hind limb muscles were dissected and collected in PBS solution inside conical tubes, with each tube containing hind limb muscle tissue from no more than 2½ mice. Samples were transferred into gentleMACS[™] C-tubes and mechanical digestion was performed using the gentleMACS dissociator (Miltenyi Biotec, Singapore). Muscle samples were dissociated by running program “*m_muscle_01*” preinstalled by the manufacturer twice and separated from lipids (supernatant) by centrifuging at 3000xrpm for 5 minutes. Subsequently, samples were digested for 45 minutes in 0.2% collagenase solution (Sigma-Aldrich, St. Louis, MO, USA) in a 37°C shaking incubator at 120xrpm. During digestion, samples were further dissociated every 15 minutes by running the *m_muscle_01* program. Following digestion, the collagenase-muscle solution was topped up to 50 ml by adding PBS and samples were centrifuged at 3000xrpm for 15 minutes. The supernatant was then discarded and the remaining pellet was resuspended in 10 ml PBS. Pellets were triturated for 5 minutes using a 10 ml serological pipette and subsequently applied to 100 µm cell strainers placed on 50 ml canonical tubes. The

flow-through was then topped up to 50 ml with PBS and centrifuged at 3000xrpm for 15 minutes. The resulting cell pellet was resuspended in 10 ml/mouse proliferation medium (high-glucose DMEM [Gibco, Carlsbad, CA, USA], 20% Fetal Bovine Serum, 10% Horse Serum, 1% Penicillin/Streptomycin and 1% Chick Embryo Extract [CEE] [United States Biological, Salem, MA, USA]). Populations of pure primary myoblasts were generated using a pre-plating technique. Upon initial resuspension of the cell pellet in proliferation medium, cells are plated onto uncoated 10 cm cell culture dishes for 3-4 h, which is referred to as pre-plate 1 (PP1). PP1 is the initial step in the isolation procedure that allows for the separation of fibroblasts from myoblasts, as fibroblasts adhere more rapidly to uncoated cell culture dishes, when compared to myoblast populations. Following PP1, cells are transferred to new 10 cm cell culture dishes coated with a 10% matrigel[®] solution (Corning Inc, corning, NY, USA) dissolved in pre-chilled DMEM and are allow to attach for a further 24 h, which is referred to as pre-plate 2 (PP2). The medium from PP2 plates is then transferred to a third matrigel[®] coated 10 cm cell culture dish and allowed to settle for a further 24 h, which is termed pre-plate 3 (PP3). Cells adhered during PP2 and PP3 are myoblasts and both PP2 and PP3 populations of myoblasts were pooled for downstream studies. Primary myoblasts were maintained and differentiated on matrigel[®] coated plates as described under *Sections 2.2.2 and 2.2.4.*

2.2.5 Tissue Dissection

4-week-old mice were euthanized through CO₂ asphyxiation. *M. quadriceps* (Quad), *M. gastrocnemius* (Gas), *M. tibialis anterior* (TA), *M. soleus* (Sol) and *M. extensor digitorum longus* (EDL) hind limb skeletal muscle were dissected. Tissue weights, body weights and tibia lengths were recorded for each mouse. Tissue samples were snap frozen in liquid nitrogen for subsequent biochemical analyses or embedded in optimum cutting temperature (OCT) compound using liquid nitrogen cooled isopentane (Sigma-Aldrich, St. Louis, MO, USA) for subsequent histological analysis and myofiber cross-sectional area measurement.

2.2.6 Protein Extraction and Western Blot Analysis

To extract total cell protein, cells were lysed in complete RIPA buffer (1% (v/v) IGEPAL, 0.1% SDS, 0.5% (w/v) Na Deoxycholate (w/v), 50 mM NaF, 0.2 mM

Na_3VO_4 , 1 mM PMSF, cOmplete™ Mini EDTA-free Protease Inhibitor, PhosSTOP™ (Sigma-Aldrich, St. Louis, MO, USA)) after washing once with ice-cold PBS. Cells were kept on ice for 30 minutes and triturated 25 times using a 26 g needle assembled onto a 1 ml syringe. To isolate proteins from tissues, approximately 30 mg of each snap frozen tissue was cut on dry ice and homogenized using the Qiagen Tissue Lyser-II at a frequency of 30 seconds⁻¹, for 3x2 minutes (Qiagen, Valencia, CA, USA). Cell lysates and muscle homogenates were then centrifuged at 10000xg for 10 minutes at 4°C, with protein containing supernatants collected into fresh micro centrifuge tubes. Finally, each protein sample was quantified using Bio-Rad (Bradford) protein assay reagent, as per the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). To isolate proteins from mitochondria, mitochondrial fractions were enriched using the Mitochondria Isolation Kit for Cultured Cells, as per the manufacturer's guidelines (Thermo Fisher Scientific, Waltham, MA, USA). Following isolation of mitochondria, proteins from mitochondrial fractions were extracted using RIPA buffer, as described above. Equal amounts of protein (5-25 µg) were resolved using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and NuPAGE® 4-12% bis-tris precast gels (Invitrogen, Carlsbad, CA, USA). For immunoblotting of LC3B/MAPLC3B, 30 µg of total protein was resolved on either 4-12% bis-tris or 18% tris-glycine gels. Following electrophoresis, proteins were transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using either the XCell II SureLock™ wet transfer or Invitrogen iBlot® 2 dry transfer systems (Invitrogen, Carlsbad, CA, USA). Wet transfer was performed under constant voltage (30 V) for 2 h at room temperature, with pre-chilled transfer buffer (25 mM Tris Base, 190 mM Glycine [Sigma-Aldrich, St. Louis, MO, USA], 20% Methanol [Millipore, Billerica, MA, USA]). For detection of MyHC and MyLC, wet transfer was performed using the Trans-Blot® cell module (Bio-Rad, Hercules, CA, USA) at 70 V for 2.5 hours at 4°C. To confirm equal loading of samples, membranes were stained with Ponceau S solution (Sigma-Aldrich, St. Louis, MO, USA). After removing excess Ponceau S stain with TBS-T, membranes were blocked for 1 hour at room temperature and hybridized with specific primary antibodies overnight at 4°C. Details of primary antibodies' sources, catalogue numbers, and dilutions are provided in *Table 2.29*. Following overnight incubation, primary antibodies were removed and membranes were incubated with respective horseradish peroxidase (HRP) conjugated secondary antibodies for 1h at room temperature. Details of secondary antibodies are provided in *Table 2.30*. Immuno-reactive bands were detected using Western Lightning® Plus-enhanced

chemiluminescence (ECL) reagent (Perkin Elmer, Waltham, MA, USA) and either autoradiography films or the ChemiDoc™ Touch Imaging System (Bio-Rad, Hercules, CA, USA). To quantify changes in protein levels, densitometry analysis was performed using either the GS-800 calibrated densitometer or Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

2.2.7 RNA Extraction and Real Time Quantitative PCR (qPCR)

To extract RNA, cells were washed once with 10 ml ice-cold PBS solution after which 1 ml TRIzol® reagent was added per 10 cm² plate (Thermo Fisher Scientific, Waltham, MA, USA). For RNA isolation from tissue, approximately 30 mg of skeletal muscle tissue was homogenized with 1 ml TRIzol® reagent using the Qiagen Tissue Lyser-II instrument, as described in *Section 2.2.6*. Following homogenization, 200 µl chloroform, per 1 ml of TRIzol® originally used, was added and tubes were shaken vigorously for 15 seconds. Following incubation for 3 minutes at room temperature, tubes were centrifuged at 12000xg for 15 minutes at 4°C and the upper aqueous phase was transferred into fresh micro centrifuge tubes. RNA was precipitated by adding 500 µl ice-cold isopropanol, per 1 ml TRIzol® originally used, within 10 minutes at room temperature. Subsequently, samples were centrifuged at 12000xg, for 10 minutes, at 4°C. Pellets were then washed with 1 ml ice-cold 75% ethanol, per 1 ml TRIzol® originally used, and recollected by spinning at 7500xg, for 5 minutes. The 75% ethanol was then removed and pellets were allowed to air dry for 5-10 minutes. The resulting pellets were resuspended in 30-50 µl DEPC water (1st Base, Singapore) and RNA concentration was estimated using the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA electrophoresis was performed using 250-500 ng of each RNA to confirm RNA integrity.

To generate complementary DNA (cDNA), 1 µg RNA was reverse transcribed using 4 µl 5X iScript™ reaction mix (Bio-Rad, Hercules, CA, USA) and 1 µl iScript™ reverse transcriptase enzyme (Bio-Rad, Hercules, CA, USA), using the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Prior to qPCR, cDNA samples were diluted 1:5 through the addition of MQ water. For qPCR analysis, 3 µl of relevant cDNA, 1 µl of 2.5 µM gene specific forward primer, 1 µl 2.5 µM of gene specific reverse primer and 7 µl of SsoFast™ EvaGreen® Supermix was loaded per well into Hard-Shell® 96-well PCR plates. qPCR analysis was performed

using the CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). qPCR conditions were as follows: 98°C 3 minutes, 40 cycles of denaturation, annealing and elongation at 98°C for 15 seconds, 55-60°C for 10 seconds and 72°C for 20 seconds, respectively, followed by a melting curve from 65°C to 95°C in 10 seconds increments of 0.5°C. Gene names and primer sequences are provided in *Table 2.31*. Gene expression was analysed using the $\Delta\Delta C_t$ method and represented as fold change, normalised to the expression of either *Gapdh* or *Ppia*.

2.2.8 Assessment of 20S Proteasome Activity

To determine 20S proteasome activity, C2C12 cells were seeded into clear bottom 96-well black plates at a density of 15000 cells per cm² and maintained and differentiated as described in *Section 2.2.2*. Fully differentiated myotubes were transfected as previously described (*Section 2.2.3*). A volume of 100 µl of the Proteasome-Glo™ cell-based reagent, containing a specific peptide substrate Succinyl-leucine-leucine-valine-tyrosine-aminoluciferin (Suc-LLVY-aminoluciferin), Z-leucine-arginine- arginine-aminoluciferin (Z-LRR-aminoluciferin) and Z-norleucine-proline-norleucine-aspartate-aminoluciferin (Z-nLPnLD-aminoluciferin) was added into wells containing the cells for the assessment of chymotrypsin-like, trypsin-like and caspase-like activities, respectively. 96-well plate was then shaken on a plate shaker for 2 minutes at 700xrpm and incubated at room temperature for 10 minutes. The plate then was read using the GloMax® 96 microplate luminometer and the Proteasome-Glo programme that was pre-installed by manufacturer (Promega, Madison, WI, USA). Relative light units (RLU) detected by the machine were normalized to total protein and represented as percentage of values obtained from control-siRNA transfected cells.

2.2.9 Detection of Nascent Protein Synthesis

To determine nascent protein synthesis, C2C12 myoblasts were seeded at a density of 10000 cell/cm² into 96-well clear bottom black plates, maintained and differentiated as described in *Section 2.2.2*. Following 72 h differentiation, cells were incubated with methionine-free RPMI medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 50 µM of methionine analog, Click-iT® homopropargylglycine (HPG) for 30 minutes. Following incubation at 37°C in a 5% CO₂ incubator, medium was removed and cells were washed once with 100 µl PBS/well.

Cells were then fixed with 100 µl/well of 3.7% formaldehyde solution in PBS for 15 minutes at room temperature. Cell were then washed twice with 3% BSA solution in PBS and permeabilized with 100 µl/well of 0.5% Triton[®] X-100 for 20 minutes at room temperature. Permeabilization solution was removed and cells were washed twice with 100 µl/well 3% BSA. 100 µl/well of Click-iT[®] reaction cocktail was added to each well and the plate was incubated in the dark for 30 minutes at room temperature. After incubation, each well was washed with 100 µl Click-iT[®] reaction rinse buffer. To stain nuclear components, NuclearMask[™] Blue Stain was diluted 1:2000 in PBS. 100 µl/well of diluted NuclearMask[™] Blue Stain solution was added to each well and the plate was incubated in the dark for 30 minutes at room temperature. The solution containing the NuclearMask[™] Blue Stain was then removed and cells were washed twice with 100 µl PBS/well, before a final volume of 100 µl of PBS was added to each well. Fluorescent intensity was measured from 8 wells with appropriate filters for Alexa Fluor[®] 488 (Excitation/Emission: 488 nm/520 nm) and NuclearMask[™] Blue Stain (Excitation/Emission: 355 nm/460 nm) using the Tecan 2000 fluorescent plate reader and iControl software (Tecan Group Ltd, Männedorf, Switzerland). Mean fluorescence intensity (MFI) of Alexa Fluor[®] 488 was normalized to MFI of DNA content that is stained by NuclearMask[™] Blue Stain to obtain nascent protein synthesis. MFI values were plotted as bar graph with error bars indicating $\pm$ s.e.m.

2.2.10 Assessment of Mitochondrial Respiratory Capacity

C2C12 myoblasts and primary myoblasts were seeded at the density of 15000 cell per cm² onto XF^e24 cell culture microplates (Agilent Technologies, Santa Clara, CA, USA). XF^e24 sensor cartridges were hydrated overnight at 37°C in a non-CO₂

Figure 2-1: Representation of mitochondrial respiration parameters.

Areas representing basal respiration, ATP-linked respiration (ATP production), maximum respiration, proton leak, non-mitochondrial respiration and spare respiratory capacity are indicated with different colours. The time points when oligomycin, FCCP and antimycin A & rotenone were injected are indicated with arrows. OCR measurement and rates are indicated with black dots and lines respectively (Translational Psychiatry, 2014 Apr; 4(4): e377.; doi: 10.1038/tp.2014.15). Permission to use this copyrighted image is granted by RightsLink online copyright clearance centre (See Appendix 6.6.).

when drugs injected. For the MitoStress Test Kit, basal respiration was recorded at rate 3, just prior to oligomycin injection, maximum respiration was calculated as the maximum OCR during rate 7, 8 and 9 and proton leak was calculated at rate 6. Spare respiratory capacity and ATP-linked respiration were obtained by subtracting basal respiration from maximum respiration and proton leak values, respectively. Non-mitochondrial respiration (minimum OCR during rate 10, 11 and 12) was subtracted from all respiratory calculations. Each respiration parameter is represented with area highlighted with different colour in Figure 2.1.

incubator by adding 1 ml/well XF calibrant solution into the utility plate. Medium was replaced with Assay Medium (for Seahorse XF Cell Mito Stress Test Kit see Table 2.24 and see Table 2.25 for Seahorse XF Glycolysis Stress Test Kit) and microplates were incubated at 37°C in a non-CO₂ incubator for 30 minutes. Three measurements were recorded before and after sequential injection of 1 µM Oligomycin, 0.5 µM FCCP and 0.5 µM Antimycin/Rotenone solutions (Agilent Technologies, Santa Clara, CA, USA). Protein concentration was estimated for each well containing cells using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA, USA). Figure 2.1 highlights the typical schematic representation of real-time OCR measurement with rates, time points

2.2.11 Measurement of Exogenous Fatty Acid Oxidation

To determine exogenous fatty acid oxidation (FAO), myoblasts were grown and differentiated in XF^e24 cell culture microplates, as described in *Section 2.2.10*. Cells were subjected to glycolytic stress and induced to catabolise fatty acids by replacing growth medium with Substrate-limited Medium (see *Table 2.26*) supplemented with 0.5 mM glucose, 1 mM glutamine and 0.5 mM carnitine and 1% FBS for 6 h. Subsequently, the substrate-limited medium was changed to FAO Assay Medium (see *Table 2.27*) 30 minutes prior to beginning the assay run. Palmitate-BSA conjugation was carried out as follows: The required amount of BSA was dissolved in 150 mM NaCl solution in a 37°C water bath. Palmitate was dissolved in 150 mM NaCl solution at 70°C while stirring. Once the palmitate solution became clear, the palmitate-NaCl solution was added to the BSA-NaCl solution dropwise 87.5 µl/well Palmitate-BSA FAO substrate was added onto the cell and cells were incubated in non-CO₂ incubator at 37°C for 15 minutes prior to assay run. 1 µM Oligomycin, 0.5 µM FCCP and 0.5 µM Antimycin/Rotenone solutions were prepared as described in *Section 2.2.10* and the plate was loaded into XF^e24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA). Quantification of OCR from exogenous fatty acid oxidation was calculated as outlined in *Section 2.2.10*.

2.2.12 MitoTracker Red CMXRos Staining and Confocal Microscopy

C2C12 myoblasts were seeded on 8-well glass chamber slides (Thermo Fisher Scientific, Waltham, MA, USA) at the density of 5000 cells/well. Cells were stained with 200 nM MitoTracker[®] Red CMXRos for 20 minutes at 37°C in a 5% CO₂ incubator following overnight attachment (Thermo Fisher Scientific, Waltham, MA, USA). Cells were washed with proliferation medium followed by fixation with 2% paraformaldehyde for 15 minutes and 4% paraformaldehyde for 10 minutes at 37°C. Slides were mounted with SlowFade[®] Gold Antifade Mountant with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) and covered with glass coverslips (VWR, Radnor, PA, USA). MitoTracker[®] stained myoblasts were visualized using the Olympus BX61 WI microscope and analyzed using the Olympus FV1000 viewer software (Olympus Imaging, Central Valley, PA, USA).

2.2.13 FACS Analysis of MitoTracker® Green FM Stained Myoblasts

C2C12 myoblasts and murine primary myoblasts were seeded at a density of 15000 cells per cm² onto 6-well plates. The next day, myoblasts were stained with 150 nM MitoTracker® Green FM (Thermo Fisher Scientific, Waltham, MA, USA) for 20 minutes at 37°C in the dark, trypsinized and collected in conical tubes. Cell pellets from three biological replicates were resuspended in PBS and FACS analysis was performed immediately using the FITC channel of the FACSCanto flow cytometry system (BD Biosciences, Franklin Lakes, NJ, USA). 10000 events from each of 3 biological replicates were recorded and subtracted from background signal recorded from unstained cells. A total of 10,000 events from 3 replicate wells per experimental group were detected using the FITC channel. Fluorescence intensity was averaged and represented as mean fluorescence intensity (MFI) $\pm$ s.e.m.

2.2.14 Assessment of Cellular ROS Production

To assess cellular ROS production in C2C12 myoblasts, C2C12 cells were seeded onto 96-well clear bottom black microplates at a density of 10000 cells/well and were transiently transfected next day with the DharmaFECT 1 transfection reagent (Dharmacon RNAi Technologies, Lafayette, CO, USA), as described in *Section 2.2.3*. 24 hours' post-transfection, cells were stained with 10 μ M H₂DCFDA for 30 minutes as per manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, USA). To assess cellular ROS generation in primary myotubes, primary myotubes were isolated from hind limb muscle of *Parkin* KO mice and age-matched wild type controls, as described in *Section 2.2.4*. 72 h differentiated primary myotubes were stained with 10 μ M H₂DCFDA for 30 minutes as per manufacturer's guidelines. The H₂DCFDA stain was then removed and cells were washed once with 200 μ l PBS/well, before a final volume of 100 μ l of PBS was added to each well. Fluorescent intensity (Excitation/Emission: 492–495 nm/517–527 nm), which is an indication of ROS levels, was measured in quadruplicate using the Tecan 2000 fluorescent plate reader and iControl software (Tecan Group Ltd, Männedorf, Switzerland).

2.2.15 Genomic DNA Isolation and mtDNA Copy Number Analysis

To determine mtDNA copy number, genomic DNA was extracted using the QIAamp® DNA Mini Kit, according to the manufacturer's instructions (Qiagen,

Valencia, CA, USA). To determine mtDNA copy number qPCR was performed using 5 ng/μl genomic DNA, together with 1 μl of 2.5 μM mitochondrial gene (*mtCoI*) and nuclear gene (*18S*) specific primers and 10 μl SsoFast EvaGreen Supermix per reaction (Bio-Rad, Hercules, CA, USA). The qPCR conditions were as follows: 95°C for 5 minutes, 45 cycles of 95°C for 10 seconds, 60°C for 20 seconds, 72°C 20 seconds followed by melting curve. Details of primers used to determine mtDNA copy number are provided in *Table 2.31*. Results were analysed using the $\Delta\Delta C_t$ method and resulting data was represented as fold change, relative to respective controls.

2.2.16 Hematoxylin and Eosin (H&E) Staining and Myotube Area Analysis

For H&E staining of CCCP treated myotubes, C2C12 myoblasts were seeded and differentiated on Thermanox™ coverslips (Nunc, Rochester, NY, USA) at a density of 20000 myoblasts/cm². After 72 h differentiation, myotubes were treated with 1, 5, and 10 μM CCCP for 24 h. Control myotubes were treated with equal volume of DMSO for 24 h. For H&E staining of transfected myotubes, myoblasts were seeded at the density of 12000 cells/cm² and differentiated on 2 or 4-well tissue culture treated μ-Slides (Ibidi GmbH, Martinsried, Germany). Transfection was performed as described in *Section 2.2.3*. Following treatments and relevant incubation times all myotubes were fixed with 20:2:1 ethanol:formaldehyde:acetic acid solution for 30 seconds at room temperature. H&E staining was performed as outlined in the following table (*Table 2.36*)

Table 2.36: Myotube H&E Staining Protocol

Reagent	Exposure Time
Hematoxylin, Gill III	2 minutes
0.1% HCl	2 seconds
Scott's tap Water	1 minute x 5
Eosin-Y	2 minutes
100% Ethanol	5 minutes x 3
Xylene	5 minutes x 2
DPX mounting	

For staining of transfected myotubes on μ-Slides, xylene and DPX mounting were avoided and images were captured immediately following ethanol dehydration. Myotubes were imaged using the Leica DM6000 B microscope (Leica Microsystems, Wetzlar, Germany), equipped with 10X objective and the area of individual myotubes was determined using ImageJ software (Bethesda, MA, USA). For CCCP treatment the

area of 10 myotubes per image (n=5, per coverslip) across 2-3 biological replicates per treatment group. For *Parkin* knockdown experiments the area of 200 myotubes was measured across 4 biological replicates each for Control-siRNA and *Parkin*-siRNA transfected myotubes. Results were represented as average myotube area.

2.2.17 H&E Staining of Tibialis Anterior (TA) Muscle and Cross-Sectional Area Analysis

Transverse serial sections (10 μ m) were cut from the mid-belly region of snap frozen OCT embedded TA muscles using the Leica CM1950 Cryostat (Leica Microsystems, Wetzlar, Germany). Sections were mounted on Superfrost™ glass microscope slides (Fisher Scientific, Waltham, MA, United States) and stored at -80°C prior to H&E staining. The procedure for H&E staining of skeletal muscle sections is provided in *Table 2.37* below:

Table 2.37: H&E Staining Protocol of Muscle Sections

Reagent	Exposure Time
Hematoxylin, Gill III	1 minute
Rinse with tap water	Until clear
Blue with Scott's tap water	2 minutes
Rinse with tap water	Until clear
Eosin-Y	2 minutes
Rinse with tap water	Until clear
50% ethanol	3 dips
70% ethanol	3 dips
95% ethanol	2 minutes
100% ethanol	2 minutes x 2
Xylene	5 minutes x 2
DPX mounting	

Images were captured as indicated in *Section 2.2.16*. Cross-sectional area of 1000 myofibers from each muscle section were recorded and represented as distribution graph. Entire TA muscle sections were imaged using the Leica DM6000 B microscope (Leica Microsystems, Wetzlar, Germany), equipped with 5X objective. For TA muscle sections, the area of 1000 myofibers were calculated from each muscle section per genotype using ImageJ software (Bethesda, MA, USA) and results were represented as a frequency distribution graph and average myotube area was also calculated.

2.2.18 Total RNA Isolation for Microarray and *In Silico* Analysis

To identify global genes expression differences between wild type and *Parkin* KO mice, total RNA was isolated from Gas muscle of 3-month-old *Parkin* KO and age-matched wild type controls using mirVANA™ miRNA isolation kit, as per manufacturer's guidelines (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Subsequent microarray analysis was performed by Genomax Technologies (Singapore) as per the following: 100 ng/μl RNA was probed with the Low Input AMP labelling Kit, One Color (Agilent p/n 5190-2305) as per manufacturer's instruction (One-color microarray-based gene expression, analysis low input quick amp labelling, version 6.5). 100 ng/μl RNA was converted into cDNA using oligo-dt primers containing a T7 RNA polymerase recognition site. Cyanine 3-CTP labelled cRNA was transcribed using T7 RNA polymerase. 600 ng cRNA was hybridized onto Agilent SurePrint G3 Human GE 8x60K microarray slides at 65°C, at 10xrpm for 17 hours in an Agilent hybridization oven. In silico pathway analysis was performed with significantly upregulated (>1.5 fold) and downregulated (<0.75 fold) genes with a *p*-value cut off <0.05 using Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database integrated in the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (<https://david.ncifcrf.gov>). Protein class analysis and identification of significantly upregulated ubiquitin-conjugating enzyme, E3 ubiquitin ligases and deubiquitinating enzymes (DUBs) was carried out using Protein Analysis Through Evolutionary Relationships (PANTHER) gene list analysis software (<http://www.pantherdb.org>). Expression of significantly upregulated genes were validated using qPCR analysis, as previously described in *Section 2.2.7*.

2.2.19 Statistical Analysis

F-tests were performed on each data set to test for equal variance between groups. Two-tailed Student's T-test were conducted for statistical analysis to assess for differences between two groups, where the calculated F-value from the data sets is less than the F-critical value. Two-way ANOVA was applied for analysis of data from more than two groups, followed by Tukey post-hoc test. **P*<0.05, ***P*<0.01 and ****P*<0.001 denote significance levels. Data was represented as mean ± s.e.m. Outliers were removed following analysis of data using online GraphPad Software outlier calculator

with an $\alpha=0.05$ (GraphPad Software Inc., La Jolla, CA, USA). Researchers were not blinded during experiment plan and analysis. Details of biological replicates for each experiment are indicated in respective figure legends.

3 RESULTS

Some parts from this section have been communicated with American Journal of Physiology-Cell Physiology Journal

3.1 Treatment with CCCP promotes PINK1/PARKIN-mediated mitophagy in C2C12 myotubes

To investigate if induction of mitophagy leads to atrophy of muscle cells, a model of PARKIN-mediated mitophagy was established through treating C2C12 myotubes with CCCP. Previous studies have used 10 μ M CCCP for 24 h to induce PINK1/PARKIN-mediated mitophagy in different cell culture models^{113,266-268}. Moreover, Gomez-Sanchez *et al.* has reported that only 24 h-10 μ M CCCP treatment results in a significant reduction in mitochondrial content and 4-fold increase in PINK1 protein levels²⁶⁹. Therefore, in parallel to previous studies, C2C12 myotubes were treated with 10 μ M CCCP for 24 h. Quantitative real-time PCR (qPCR) analysis revealed increased mRNA expression of both *Pink1* and *Parkin* following CCCP treatment (Fig. 3.1A). Subsequent immunoblot analysis revealed increased levels of full length PINK1 following 24 h CCCP treatment in myotubes (Fig. 3.1B & 3.2C). However, no observable difference in PARKIN levels was noted upon CCCP treatment (Fig. 3.1B & 3.2C). Reduced levels of the mitochondrial fusion protein Mitofusin 2 (MFN2) and enhanced levels of the autophagosomal marker LC3-II (lipidated LC3) were also observed in response to CCCP treatment of C2C12 myotube cultures (Fig. 3.1B & 3.2C & 3.2D). Despite no change in total PARKIN protein levels (Fig. 3.1C), treatment with CCCP resulted in increased levels of PARKIN protein in isolated mitochondrial fractions, which is consistent with both increased translocation of PARKIN to mitochondria and enhanced activation of PARKIN E3 ligase activity (Fig. 3.1E & 3.2F).

Figure 3.1: CCCP induces PINK1/PARKIN-mediated mitophagy in C2C12 myotubes

A) Graph showing qPCR analysis of *Pink1* and *Parkin* mRNA expression, normalized to *Gapdh*, in 24 h DMSO or 10 μ M CCCP treated myotubes (n=3). **B)** Representative images of western blot analysis of PINK1, PARKIN, MFN2, non-lipidated LC3 (LC3-I) and lipidated LC3 (LC3-II) protein levels in DMSO and 10 μ M CCCP treated myotubes. The levels of GAPDH were assessed as a loading control (n=3). **C)** Graph showing quantification of PINK1, PARKIN and MFN2 protein levels, normalized to GAPDH, in arbitrary units (n=3). **D)** Graph showing quantification of LC3-I, LC3-II and LC3-II/LC3-I protein levels, normalized to GAPDH, in arbitrary units (n=3). **E)** Representative images of western blot analysis of PARKIN and COX-IV protein levels in cytosolic (C) and mitochondrial (M) fractions isolated from myotubes treated with either DMSO or 10 μ M CCCP for 24 h. COX IV protein levels were assessed to confirm purity of mitochondrial fraction and Ponceau-S staining was performed as a loading control (n=8). **F)** Graph showing quantification of PARKIN protein levels in DMSO and 10 μ M CCCP treated cytosolic and mitochondrial fractions. Protein levels are normalized to Ponceau S, in arbitrary units (n=8) **G)** Graph showing quantification of COX IV protein levels, normalized to Ponceau S, in arbitrary units (n=8) Graph shows mean $\pm$ s.e.m. *P<0.05, ***P<0.001 (Student's t-test).

3.2 CCCP treatment leads to reduced mitochondrial mass and abnormal mitochondrial morphology *in vitro*

Next, the effect of CCCP on the levels of mitochondrial oxidative phosphorylation (OXPHOS) proteins was determined. Immunoblotting of critical mitochondrial OXPHOS proteins revealed a significant reduction in the levels of mitochondrial complex proteins necessary for oxidative phosphorylation upon CCCP treatment (Fig. 3.2A & 3.2B), with significantly lower levels of UQCRC2 (Complex III), MTCOI (Complex IV), SDHB (Complex II) and NDUFB8 (Complex I) noted upon CCCP treatment (Fig. 3.2A & 3.2B). However, no significant difference in the Complex V protein ATP5 was observed following CCCP treatment (Fig. 3.2A & 3.2B). Next, mitochondrial number was quantified through determining mitochondrial DNA (mtDNA) copy number and MitoTracker Green FM staining and FACS analysis. Results revealed a significant decrease in mtDNA copy number and MitoTracker Green FM intensity in myoblasts upon CCCP treatment (Fig. 3.2C & 3.2D), which is indicative of decreased mitochondrial number. To visualize changes in mitochondrial architecture in response to CCCP treatment, MitoTracker Red CMXRos staining and confocal microscopy were performed (Fig. 3.2E). Subsequent assessment of mitochondria morphology revealed disruption of the typical filamentous mitochondria network, resulting in a punctate mitochondria morphology, which is consistent with mitochondria swelling and fragmentation, in response to CCCP treatment (Fig. 3.2E). Collectively, these data suggest that CCCP treatment promotes PINK1/PARKIN mediated mitophagy *in vitro* model of skeletal muscle.

3.3 CCCP treatment leads to impaired mitochondrial respiratory capacity and reduced utilization of exogenous fatty acids *in vitro*

To investigate the effect of CCCP treatment on mitochondrial function, oxygen consumption rate (OCR) was assessed using the Seahorse XF^e24 extracellular flux analyzer. A dramatic reduction in real-time oxygen consumption rate was noted in myotubes treated with CCCP, when compared to vehicle (DMSO) treated controls (Fig. 3.3A). Indices of mitochondrial function were calculated and a significant reduction in basal, maximal, ATP-linked and non-mitochondrial respiration was noted upon treatment with CCCP (Fig. 3.3B). In addition, a significant reduction in spare respiratory capacity was further observed in CCCP treated myotubes, when compared to vehicle controls (Fig. 3.3B). Given that mitochondria are the major site for fatty acid oxidation, real time utilization of exogenous fatty acids (palmitate) was next measured using the Seahorse XF^e24 analyzer. Real time OCR measurements revealed a dramatic reduction in OCR in myotubes treated with CCCP, in the presence of either BSA or palmitate (Fig. 3.3C & 3.3D). Moreover, spare and maximal respiration, due to utilization of palmitate, were also significantly reduced in CCCP treated myotubes (Fig. 3.3D). Taken together, these data suggest that CCCP treatment leads to reduced mitochondrial respiration and respiration due to utilization of exogenous fatty acids in C2C12 myotubes.

3.4 CCCP treatment activates the energy sensor AMPK α *in vitro*

AMPK is key energy sensor and regulator in cells, in fact, AMPK is activated in response to reduced levels of ATP and functions to switch off anabolic (energy utilizing) processes in favor of catabolic (energy producing) processes^{270,271}. To determine whether AMPK is activated in response to CCCP treatment, the levels of active phosphorylated AMPK α (p-AMPK α) were quantified. Consistent with reduced mitochondrial respiratory capacity, western blot analysis revealed increased levels of p-AMPK α in C2C12 myotubes upon treatment with CCCP (Fig. 3.3E & 3.3F).

Figure 3.2: CCCP treatment leads to reduced mitochondrial mass and fragmented mitochondrial network

A) Representative images of western blot analysis of OXPHOS subunit (ATP5, UQCRC2, MTCOI, SDHB and NDUFB8) protein levels in C2C12 myotubes treated with either DMSO or 10 μ M CCCP for 24 h. The levels of HSP60 were assessed as a loading control (n=3). **B)** Graph showing quantification of OXPHOS protein levels, normalized to HSP60, in arbitrary units (n=3). **C)** Graph showing qPCR analysis of mitochondrial DNA (mtDNA) copy number:nuclear DNA (nuDNA) copy number ratio in myotubes treated with either DMSO or 10 μ M CCCP for 24 h. Values are expressed as fold change relative to DMSO control. The expression of the mitochondrial gene *CoI* was normalized to the nuclear gene *18S* (n=3). **D)** Graph showing results of FACS analysis of MitoTracker Green FM stained myoblasts following 24 h treatment with either DMSO or 10 μ M CCCP (MFI: Mean fluorescence intensity) (n=3). **E)** Representative confocal images of MitoTracker Red CMXRos stained myoblasts treated with either DMSO or 10 μ M CCCP for 24 h. Dapi (blue) was used to stain nuclei. Images in the top panels reflect cells captured using the 100X objective and images in the bottom panels are magnified further 1.5X and captured using the Olympus BX61 WI confocal microscope. Scale bars represent 20 μ m. All graphs show mean $\pm$ s.e.m. *P<0.05, **P<0.01 (Student's t-test).

A

B

C

D

E

F

Figure 3.3: CCCP treatment results in mitochondrial dysfunction in C2C12 myotubes.

A) Graph showing the real-time Oxygen Consumption Rate (OCR) of myotubes treated with either DMSO or 10 μ M CCCP for 24 h, as assessed by the Seahorse XF[®]24 extracellular flux analyzer (n=10). Time points where Oligomycin (Oligo), FCCP and Antimycin/Rotenone (Ant/Rot) were injected and the rate number where each OCR was measured are indicated. **B)** Graph showing quantification of basal, spare, maximal, ATP-linked and non-mitochondrial respiration, and respiration due to proton leak in myotubes treated with either DMSO or 10 μ M CCCP for 24 h (n=10). **C)** Graph showing the real-time assessment of exogenous fatty acid (palmitate) utilization in myotubes treated with either DMSO or 10 μ M CCCP in the absence (BSA) or presence of palmitate for 24 h (n=5). Time points where Oligomycin (Oligo), FCCP and Antimycin/Rotenone (Ant/Rot) were injected and the rate number where each OCR was measured are indicated. **D)** Graph showing quantification of basal, spare, maximal, ATP-linked and non-mitochondrial respiration, and respiration due to proton leak in myotubes treated with either DMSO or 10 μ M CCCP in the absence (BSA) or presence of palmitate for 24 h (n=5). **E)** Representative images of western blot analysis of phosphorylated (p-AMPK α) and total AMPK α protein levels in myotubes treated with either DMSO or 10 μ M CCCP for 24 h. The levels of GAPDH were assessed as a loading control (n=3). **F)** Graph showing quantification of p-AMPK α , total AMPK α , and the ratio of p-AMPK α /total AMPK α , normalized to GAPDH, in arbitrary units (n=3). All graphs represent mean $\pm$ s.e.m. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test with the exception of the graph in **(D)**, were performed with two-way ANOVA was performed).

3.5 C2C12 myotubes develop ER stress response upon CCCP treatment

Mitochondria and Endoplasmic Reticulum (ER) directly communicate through mitochondria associated membranes (MAMs). Due to physical and functional connection, excessive reactive oxygen species (ROS) production, unfolded protein response (UPR) or failure to buffer calcium between two organelles might trigger a stress response in either or both organelles. In fact, shift of Ca^{2+} from ER lumen to mitochondria following mitochondrial dysfunction leads to flaws in protein folding machinery thereby resulting in the accumulation of misfolded proteins²⁷². To determine whether CCCP-mediated mitophagy in C2C12 myotubes is associated with increased ER stress, the expression of ER stress indicators was examined. Subsequent qPCR analysis revealed upregulation of inositol requiring enzyme 1 (*Irel α*), C/EBP homologous protein (*Chop*) and binding immunoglobulin protein (*BiP*) following CCCP treatment, suggesting that CCCP induces ER stress by upregulating UPR genes (Fig. 3.4A). Activation of ER stress results in dimerization of PERK, a serine/threonine kinase, which phosphorylates and activates the α subunit of eukaryotic initiation factor eIF2 α , which in turn inhibits protein synthesis and activates the ER-stress mediated apoptosis pathway through CHOP. Consistent with this, an increase in the levels of phosphorylated eIF2 α (p-eIF2 α) and total CHOP protein were observed following CCCP treatment, but the levels of PERK remained unchanged (Fig. 3.4B & 3.4C), suggesting that CCCP-induced mitophagy activates the ER stress response in C2C12 myotubes. Taken together, these data suggest that CCCP treatment leads to activation of PARKIN, loss of mitochondria (mitophagy) and impaired mitochondrial function leading to ER stress in C2C12 myotubes.

Figure 3.4: CCCP-treatment results in endoplasmic reticulum (ER) stress in C2C12 myotubes.

A) Graph showing qPCR analysis of *Ire1α*, *Chop* and *Bip* ER-stress marker mRNA expression, normalized to *Gapdh*, in 24 h DMSO or 10 μ M CCCP treated myotubes (n=3). **B)** Representative images of western blot analysis of ER-Stress marker (CHOP, p-eIF2 α and PERK) protein levels in myotubes treated with either DMSO or 10 μ M CCCP for 24 h. The levels of GAPDH, Ponceau S or α -TUBULIN were assessed as loading controls (n=3). **C)** Graph showing quantification of CHOP, p-eIF2 α and PERK normalized to either GAPDH, Ponceau S or α -TUBULIN, respectively, in arbitrary units (n=3). All graphs represent mean $\pm$ s.e.m. **P<0.01, ***P<0.001 (Student's t-test).

3.6 Treatment of myotube cultures with CCCP leads to myotubular atrophy

To explore whether CCCP-induced loss of mitochondria number and function resulted in skeletal muscle atrophy, differentiated C2C12 myotubes were treated with 1, 5 or 10 μ M CCCP. Treatment with 5 μ M and 10 μ M CCCP led to an observable reduction in myotube area (Fig. 3.5A). Subsequent quantification of myotube area revealed a significant reduction in myotube area upon treatment with 5 μ M and 10 μ M CCCP (Fig. 3.5B). Consistent with this, myosin heavy chain (MyHC) and myosin light chain (MyLC) protein levels were significantly decreased upon CCCP treatment (Fig. 3.5C & 3.5D). Collectively, these data reveal that treatment with CCCP results in a notable atrophy phenotype and significant reduction in protein levels of critical myofibrillar proteins MyHC and MyLC in C2C12 myotubes.

3.7 CCCP treatment activates Akt-FoxO pathway and expression of atrogenes

Given that skeletal muscle atrophy is regulated by the Akt-FoxO pathway^{273,274}, the role of Akt-FoxO pathway in CCCP-mediated muscle wasting was assessed. Surprisingly, western blot analysis revealed increased levels of phosphorylated AKT (p-AKT) upon CCCP treatment (Fig. 3.6A & 3.6B). Despite the increase in p-AKT levels, immunoblot analysis of FOXO3a transcription factor revealed a significant reduction in the levels of phosphorylated FOXO3a (p-FOXO3a), which is indicative of increased FOXO3a nuclear translocation and thereby, increased expression of atrophy-related genes (atrogenes) (Fig. 3.6C & 3.6D). Consistent with reduced levels of p-FOXO3a, we observed a significant increase in the expression of FoxO downstream targets *Atrogin-1* and *Murf1* was observed following CCCP treatment (Fig. 3.6E). In contrast, a significant decrease in the expression of additional E3 ligases *Nedd4* and *Trim72* was noted following CCCP treatment (Fig. 3.6E). Taken together these data suggest that CCCP-mediated activation of the PINK1/PARKIN pathway results in skeletal muscle atrophy in C2C12 myotubes, through activation of muscle-specific E3 ligases *Atrogin-1* and *Murf1*.

Figure 3.5: CCCP treatment leads to myotube atrophy *in vitro*.

A) Representative images of hematoxylin and eosin (H&E) stained 72 h differentiated C2C12 myotubes treated with either DMSO or increasing concentrations (1, 5 or 10 μ M) of CCCP for 24 h. Scale bars represent 100 μ m. **B)** Graph showing quantification of average myotube area in 72 h differentiated C2C12 myotubes treated with either DMSO or increasing concentrations of CCCP for 24 h (n=2-3). **C)** Representative images of western blot analysis of MyHC and MyLC protein levels in myotubes treated with either DMSO or 10 μ M CCCP for 24 h. The levels of α -TUBULIN were assessed as a loading control. **D)** Graph showing quantification of MyHC and MyLC protein levels, normalized to α -TUBULIN, in arbitrary units (n=3). All graphs display mean $\pm$ s.e.m. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

Figure 3-6: CCCP-treatment activates the transcription factor FoxO3 and enhances expression of *Atrogin-1* and *Murfl*

A) Representative images of western blot analysis of p-AKT1/2/3 and total AKT protein levels in DMSO and 10 μ M CCCP treated myotubes. The levels of GAPDH were assessed as a loading control (n=3). **B)** Graph showing quantification of p-AKT1/2/3, total AKT, and the ratio of p-AKT1/2/3/total AKT, normalized to GAPDH, in arbitrary units (n=3). **C)** Representative images of western blot analysis of p-FOXO3a and total FOXO3a protein levels in DMSO and 10 μ M CCCP treated myotubes. The levels of GAPDH were assessed as a loading control (n=3). **D)** Graph showing quantification of p-FOXO3a, total FOXO3a, and the ratio of p-FOXO3a/total FOXO3a, normalized to GAPDH, in arbitrary units (n=3). **E)** Graph showing qPCR analysis of *Atrogin-1*, *Murfl*, *Nedd4* and *Trim72* E3 ligase mRNA expression, normalized to *Gapdh*, in 24 h DMSO or 10 μ M CCCP treated myotubes (n=3). Data represent mean $\pm$ s.e.m. **P<0.01, ***P<0.001 (Student's t-test).

3.8 Knock down of *Parkin* results in accumulation of dysfunctional mitochondria in myoblast cultures

Next, the effect of *Parkin* knock down on mitochondrial number and function in myoblasts was assessed. qPCR analysis revealed significant knock down of *Parkin* in C2C12 myoblasts transfected with *Parkin*-specific siRNA (Fig. 3.7A). In contrast to the results observed following CCCP treatment, knock down of *Parkin* in myoblasts resulted in a significant increase in mtDNA copy number and MitoTracker Green FM intensity (Fig. 3.7B & 3.7C), consistent with an increase in mitochondrial number. Despite the increase in mitochondrial number noted in response to *Parkin* knock down, the levels of critical OXPHOS proteins were not significantly altered between control and *Parkin* knock down myoblasts (Fig. 3.7D & 3.7E), and in fact, the levels of OXPHOS proteins tended to decrease upon *Parkin* knock down (Fig. 3.7D & 3.7E). In contrast, qPCR analysis revealed a significant increase in the expression of several mitochondrial complex genes in response to *Parkin* knock down, with increased expression of *Atp6*, *Ndufa9*, *Ndufs3*, *Sdha*, *Uqcrc1*, *Uqcrc2* and *Cox4* noted (Fig. 3.7F & 3.7G). Interestingly, with the exception of *Atp6*, only nuclear DNA encoded mitochondrial genes were upregulated (*Ndufa9*, *Ndufs3*, *Sdha*, *Uqcrc1*, *Uqcrc2* and *Cox4*) upon *Parkin* knock down in myoblasts (Fig. 3.7F & 3.7G). Collectively, these data revealed that knock down of *Parkin* leads to increased mitochondrial mass and mRNA expression of several mitochondrial complex genes.

Figure 3.7: *Parkin* knockdown leads to accumulation of mitochondria in vitro

A) Graph showing qPCR analysis of *Parkin* mRNA expression, normalized to *Gapdh*, in Control siRNA (Control-siRNA) and *Parkin*-specific siRNA (*Parkin*-siRNA) transfected myoblasts (n=3). **B)** Graph showing qPCR analysis of mtDNA copy number:nuDNA copy number ratio in Control-siRNA and *Parkin*-siRNA transfected myoblasts. The expression of the mitochondrial gene *Col* was normalized to the nuclear gene *18S* (n=3). **C)** Graph showing results of FACS analysis of MitoTracker Green FM stained Control-siRNA and *Parkin*-siRNA transfected myoblasts (MFI: Mean fluorescence intensity) (n=3). **D)** Representative images of western blot analysis of OXPHOS subunit (ATP5, UQCRC2, MTCOI, SDHB and NDUF8) protein levels in Control-siRNA and *Parkin*-siRNA transfected myoblasts. The levels of HSP60 were assessed as a loading control (n=3). Two different exposures of the same membrane are provided in different boxes to visualize OXPHOS immunoreactive bands. **E)** Graph showing quantification of OXPHOS protein levels, normalized to HSP60, in arbitrary units (n=3). **F)** Graph showing qPCR analysis of mitochondria DNA-encoded mitochondrial complex gene expression, normalized to *Ppia*, in Control-siRNA and *Parkin*-siRNA transfected myoblasts (n=3). **G)** Graph showing qPCR analysis of nuclear DNA-encoded mitochondrial complex gene expression, normalized to *Ppia*, in Control-siRNA and *Parkin*-siRNA transfected myoblasts (n=3). All graphs display mean±s.e.m. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

3.9 *Parkin* knock down results in impaired mitochondrial turnover

Given that *Parkin* knock down increases mitochondrial number in myoblasts, mitochondrial turnover was next analyzed. Reduced expression of mitochondrial fusion markers, Mitofusin 1 (*Mfn1*), Mitofusin 2 (*Mfn2*) and Mitochondrial dynamin-like GTPase 1 (*Opa1*), and mitochondrial fission markers, Dynamin-1-like protein (*Drp1*), Mitochondrial fission 1 protein (*Fis1*) and Mitochondrial fission factor (*Mff*), was observed in *Parkin* knock down myoblasts, although only the expression of mitochondrial fission factor (*Mff*) was significantly reduced (Fig. 3.8A). Moreover, the levels of MFN1, MFN2 and DRP1 proteins were significantly reduced in *Parkin* knock down myoblasts (Fig. 3.8B & 3.8C). Besides myoblasts, the protein levels of mitochondrial turnover markers were assessed in myotubes transfected with either non-targeting siRNA or *Parkin*-specific siRNA. A remarkable, but not significant, reduction was noted in protein levels of MFN1, whereas the levels of other mitochondrial turnover protein markers remained unchanged (Fig. 3.8D & 3.8E). These data suggest that knock down of *Parkin* in myoblasts result in reduced mitochondrial fusion and fission.

B

C

D

E

Figure 3.8: *Parkin* knockdown alters the levels of mitochondrial fusion and fission markers

A) Graph showing qPCR analysis of mitochondrial fusion (*Mfn1*, *Mfn2* and *Opa1*) and fission (*Drp1*, *Fis1* and *Mff*) marker gene expression, normalized to *Ppia*, in Control-siRNA and *Parkin*-siRNA transfected myoblasts (n=3). **B)** Representative images of western blot analysis of MFN1, MFN2 and DRP1 protein levels in Control-siRNA and *Parkin*-siRNA transfected myoblasts. The levels of GAPDH were assessed as a loading control for each respective blot (n=3). **C)** Graph showing quantification of MFN1, MFN2 and DRP1 protein levels, normalized to GAPDH, in arbitrary units (n=3). **D)** Representative images of western blot analysis of MFN1, MFN2 and DRP1 protein levels in Control-siRNA and *Parkin*-siRNA transfected myotubes. The levels of GAPDH were assessed as a loading control for each respective blot (n=3). **E)** Graph showing quantification of MFN1, MFN2 and DRP1 protein levels, normalized to GAPDH, in arbitrary units (n=3). All graphs display mean±s.e.m. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

3.10 Knock down of *Parkin* does not alter autophagy *in vitro*

To further assess mitochondrial turnover (mitophagy), the levels of the autophagosomal markers LC3-II and P62 were analyzed. As shown in Fig. 3.9A, knockdown of *Parkin* reduced P62 protein levels, whereas no significant difference in endogenous LC3-II levels were noted between control and *Parkin* knock down myoblasts (Fig. 3.9A). Moreover, no significant difference in LC3-II levels were observed between control and *Parkin*-siRNA transfected myoblasts in response to Chloroquine (CQ) treatment, suggesting that autophagic flux was not significantly altered in *Parkin* knock down myoblasts (Fig. 3.9B). Taken together, these data suggest that the knockdown of *Parkin* in myoblasts results in accumulation of mitochondria, possibly due to reduced fission and thus impaired mitochondrial turnover.

3.11 Loss of *Parkin* leads to reduced mitochondrial respiratory capacity, increased cellular ROS levels and activation of AMPK

Next, mitochondrial function was assessed using the Seahorse XF[®]24 extracellular flux analyzer. A reduction in OCR was noted in *Parkin* knock down myoblasts, when compared to control (Fig. 3.10A). A significant reduction in basal and ATP-linked respiration was noted in *Parkin* knock down myoblasts, together with a significant reduction in OCR due to proton leak (Fig. 3.10B). Although not statistically significant, a reduction in maximum respiration and spare respiratory capacity was further noted in *Parkin* knock down myoblasts, when compared to controls (Fig. 3.10B). As mitochondria play an important role in detoxifying reactive oxygen species (ROS) in the cell, ROS production was measured to further assess mitochondrial function. Consistent with impaired mitochondrial function, a moderate but significant increase in the levels of ROS was noted in *Parkin* knock down myoblasts, as measured through assessment of fluorescent H₂DCFDA (Fig. 3.10C). In addition, increased levels of active p-AMPK α were also observed in *Parkin* knock down myoblasts, when compared to controls (Fig. 3.10D & 3.10E), further supporting impaired mitochondrial function upon knock down of *Parkin*. Collectively, these data reveal that *Parkin* knockdown in myoblasts prevents overt mitophagy and results in the accumulation of dysfunctional mitochondria *in vitro*.

Figure 3.9: Autophagy remains unaltered upon knock down of Parkin

A) *Top*, Representative images of western blot analysis of P62, non-lipidated LC3 (LC3-I) and lipidated LC-3 (LC3-II) protein levels in Control-siRNA and *Parkin*-siRNA transfected myoblasts. The levels of GAPDH were assessed as a loading control. *Bottom*, Graph showing quantification of P62 protein levels and the level of LC3-II/LC3-I, normalized to GAPDH, in arbitrary units (n=3). **B)** *Top*, Representative images of western blot analysis of LC3-I and LC3-II protein levels in Control-siRNA and *Parkin*-siRNA transfected myoblasts treated with either DMSO (CQ; -) or chloroquine (CQ; +) for 24 h. Each band represents results from 3 independent samples pooled together. The levels of GAPDH were assessed as a loading control. *Bottom*, Graph showing quantification of LC3-II/LC3-I protein levels, normalized to GAPDH, in arbitrary units. With the exception of the graph in (B), All graphs display mean $\pm$ s.e.m. **P<0.01 (Student's t-test).

Figure 3.10: *Parkin* knockdown leads to mitochondrial dysfunction, increased ROS levels and AMPK α activation in vitro

A) Graph showing the real-time OCR of Control-siRNA and *Parkin*-siRNA transfected myoblasts, as assessed by the Seahorse XF^e24 extracellular flux analyzer (n=5). Time points where Oligomycin (Oligo), FCCP and Antimycin/Rotenone (Ant/Rot) were injected (arrows) and the rate number where each OCR was measured are indicated. **B)** Graph showing quantification of basal, spare, maximal, ATP-linked and non-mitochondrial respiration, and respiration due to proton leak in Control-siRNA and *Parkin*-siRNA transfected myoblasts. All OCR values were normalized to total protein (n=5). **C)** Graph showing the levels of Reactive Oxygen Species (ROS), as measured by H₂DCFDA fluorescence, in Control-siRNA and *Parkin*-siRNA transfected myoblasts (n=4). **D)** Representative images of western blot analysis of phosphorylated AMPK α (p-AMPK α) and total AMPK α protein levels in Control-siRNA and *Parkin*-siRNA transfected myoblasts. The levels of GAPDH were assessed as a loading control for each respective blot (n=3). **E)** Graph showing quantification of p-AMPK α , total AMPK α and ratio of p-AMPK α /total AMPK α , normalized to GAPDH, in arbitrary units (n=3). *P<0.05 and **P<0.01 (Student's t-test).

3.12 *Parkin* knock down results in myotubular atrophy *in vitro*

To ascertain the effect of knock down of *Parkin* and subsequent accumulation of dysfunctional mitochondria on myotube phenotype, C2C12 myotubes were transfected with either Control or *Parkin* siRNA. qPCR analysis of *Parkin* expression confirmed knockdown of *Parkin* in C2C12 myotube cultures (Fig 3.11A). Subsequent histological analysis of H&E stained myotubes revealed notable myotubular atrophy in *Parkin* knockdown myotubes (Fig. 3.11B). Subsequent quantification revealed a shift in myotube area distribution, with increased small ($<10000\ \mu\text{m}^2$) and decreased large ($>10000\ \mu\text{m}^2$) myotubes noted (Fig. 3.11C). In addition, a significant ~18% reduction in average myotube area was also observed in *Parkin* knock down myotubes, when compared to controls (Fig. 3.11D). These data reveal that knock down of *Parkin* in myoblasts not only results in accumulation of defective mitochondria, but also leads to myotubular atrophy.

Figure 3.11: *Parkin* knockdown myotubes exhibit a myotube atrophy phenotype *in vitro*

A) Graph showing qPCR analysis of *Parkin* mRNA expression, normalized to *Gapdh*, in 120 h differentiated Control-siRNA and *Parkin*-siRNA transfected myoblasts (n=3). **B)** Representative images of H&E stained 120 h differentiated Control-siRNA and *Parkin*-siRNA transfected myoblasts. Scale bars represent 100 μm . **C)** Graph showing the distribution of myotube area in 120 h differentiated Control-siRNA and *Parkin*-siRNA transfected myoblasts (n=4). **D)** Graph showing average myotube area in 120 h differentiated Control-siRNA and *Parkin*-siRNA transfected myoblasts (n=4). With exception of the frequency distribution graph (**C**), all graphs represent mean $\pm$ s.e.m. *P<0.05 and ***P<0.001(Student's t-test).

3.13 Knock down of *Parkin* increases MURF1 protein levels and 20S

proteasome activity

Next, to determine whether atrophy phenotype observed upon *Parkin* knock down is resulted from activation of Akt-FoxO pathway, the levels of p-AKT were analyzed. Western blot analysis of p-AKT and total AKT and subsequent quantification of protein levels revealed a significant increase in p-AKT and AKT levels, although p-AKT/AKT ratio remained unaltered, upon *Parkin* knock down in myotubes (Fig 3.12A & 3.12B). No significant difference in the protein levels or phosphorylation status of either FOXO1 or FOXO3a was noted in *Parkin* knock down myotubes (Fig. 3.12C & 3.12D & 3.12E & 3.12F). Loss of *Parkin* did not alter the gene expression of *Atrogin-1* and *Murf1* (Fig. 3.13A). However, subsequent western blot analysis revealed a moderate, but significant, increase in MURF1 protein levels, but not ATROGIN-1, upon knock down of *Parkin* (Fig. 3.13B & 3.13C). Hypercatabolism in skeletal muscle is commonly observed in response to muscle wasting stimuli and importantly, the ubiquitin proteasome pathway plays a critical role in degradation of myofibrillar proteins in skeletal muscle. To assess the activity of the proteasomal system in response to knock down of *Parkin*, C2C12 myotubes were transfected with either control or *Parkin*-siRNA for 24 h. Subsequent assessment of trypsin-like, chymotrypsin-like and caspase-like activity of the 20S proteasome's catalytic core using luminescence-based assay revealed increased chymotrypsin-like and caspase-like activity in *Parkin* knock down myotubes, whereas trypsin-like activity remained unaltered (Fig. 3.13D). Collectively, these data suggest that knock down of *Parkin* leads to myotubular atrophy, presumably through activation of the proteasomal system and to some extent increased MURF1.

3.14 Protein synthesis remains unaltered upon knock down of *Parkin*

Besides proteasome activity, the extend of protein synthesis in *Parkin*-siRNA transfected C2C12 myotubes was also assessed, as compared to controls. Previous studies have reported that mechanical loading, growth factors or availability of adequate nutrition modulate protein synthesis in skeletal muscle, mainly through regulation of translation initiation, which is regulated by mTOR^{275,276}. Western blot analysis and subsequent quantification of protein levels revealed no significant difference in phosphorylated mTOR (p-mTOR), total mTOR and p-mTOR/ total mTOR ratio between *Parkin* knock down and control myotubes (Fig. 3.14A & 3.14B). Also, western blot analysis revealed that protein levels of phosphorylated P70S6K (p-P70S6K) and

total P70S6K were comparable between *Parkin* knock down myotubes and control cells further suggesting that protein synthesis was not affected upon knock down of *Parkin* (Fig. 3.14C & 3.14D). To detect nascent protein synthesis, cells were incubated in methionine-free medium, supplemented with amino acid analog of methionine, homopropargylglycine (HPG). Results revealed that the levels of nascent protein synthesis were comparable between Control-siRNA and *Parkin*-siRNA transfected myotubes (Fig. 3.14E). All together, these data suggest that knockdown of *Parkin* results in increased proteasome activity, whereas protein synthesis is not altered *in vitro*.

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Figure 3.12: Loss of *Parkin* does not lead to activation of *FoxO* transcription factors

A) Representative images of western blot analysis of p-AKT1/2/3 and total AKT protein levels in Control-siRNA and *Parkin*-siRNA transfected myotubes. The levels of GAPDH were assessed as a loading control (n=3). **B)** Graph showing quantification of p-AKT1/2/3, total AKT, and the ratio of p-AKT1/2/3/total AKT, normalized to GAPDH, in arbitrary units (n=3). **C)** Representative images of western blot analysis of p-FOXO1 and total FOXO1 protein levels in Control-siRNA and *Parkin*-siRNA transfected myotubes. The levels of GAPDH were assessed as a loading control (n=3). **D)** Graph showing quantification of p-FOXO1, total FOXO1, and the ratio of p-FOXO1/total FOXO1 levels, normalized to GAPDH, in arbitrary units (n=3) **E)** Representative images of western blot analysis of p-FOXO3a and total FOXO3a protein levels in Control-siRNA and *Parkin*-siRNA transfected myotubes. The levels of GAPDH were assessed as a loading control (n=3). **F)** Graph showing quantification of p-FOXO3a, total FOXO3a, and the ratio of p-FOXO3a/total FOXO3a levels, normalized to GAPDH, in arbitrary units (n=3). Data represent mean±s.e.m. with error bars indicating s.e.m. *P<0.05 (Student's t-test).

Figure 3.13: Knock down of Parkin increases MURF1 protein levels and activity of the 20S proteasome

A) Graph showing qPCR analysis of *Atrogin-1* and *Murf1* mRNA expression in Control-siRNA and *Parkin*-siRNA transfected myotubes (n=3). **B)** Representative images of western blot analysis of MURF1 and ATROGIN-1 protein levels in Control-siRNA and *Parkin*-siRNA transfected myotubes. The levels of Ponceau S were assessed as a loading control (n=3). **C)** Graph showing quantification of MURF1 and ATROGIN-1 protein levels, normalized to Ponceau S, in arbitrary units (n=3). **D)** Graph showing the levels of trypsin-like, chymotrypsin-like and caspase-like proteasomal activity of the 20S proteasome in Control-siRNA and *Parkin*-siRNA transfected myotubes. Relative light units (RLU) were normalized to total protein and the graph represents percentage as compared to the control (n=4). Data represent mean $\pm$ s.e.m. *P<0.05, ** P<0.01 (Student's t-test).

Figure 3.14: *Parkin* knock down does not alter protein synthesis

A) Representative images of western blot analysis of phosphorylated mTOR (p-mTOR) and total mTOR in Control-siRNA and *Parkin*-siRNA transfected myotubes. The levels of GAPDH were assessed as a loading control. **B)** Graph showing quantification of protein levels of p-mTOR and total mTOR and the ratio of p-mTOR/ total mTOR, normalized to GAPDH, in arbitrary units (n=3). **C)** Representative images of western blot analysis of phosphorylated P70S6K (p-P70S6K) and total P70S6K in Control-siRNA and *Parkin*-siRNA transfected myotubes. The levels of GAPDH were assessed as a loading control. **D)** Graph showing quantification of protein levels of p-P70S6K and total P70S6K and ratio of p-P70S6K / total P70S6K, normalized to GAPDH, in arbitrary units (n=3). **E)** Graph showing nascent protein synthesis in Control-siRNA and *Parkin*-siRNA transfected myotubes. Mean fluorescence intensity (MFI) of Click-iT HPG Alexa Fluor 488 was normalized to NuclearMask Blue Stain fluorescence intensity (n=8). Data represent mean $\pm$ s.e.m. (Student's t-test).

3.15 Mitochondrial number is preserved and mitochondrial OXPHOS gene expression is reduced in *Parkin* KO primary myotube cultures

Given that knockdown of *Parkin* *in vitro* results in impaired mitochondrial function, mitochondrial number and function in skeletal muscle and primary myoblast cultures derived from *Parkin* KO mice were assessed. Although a trend towards increased mitochondrial DNA (mtDNA) copy number was noted in *Parkin* KO primary myoblast cultures (Fig. 3.15A), the increase was not statistically significant. Similarly, no significant difference in mitochondrial number, as assessed through MitoTracker Green FM staining and FACS analysis, was noted in *Parkin* KO primary myoblast cultures (Fig. 3.15B). Also, mRNA expression of mitochondrial DNA-encoded and nuclear DNA-encoded mitochondrial genes was determined through qPCR analysis. Results revealed a significant increase in the expression of the mitochondrial DNA-encoded gene *Atp6* in *Parkin* KO myotube cultures (Fig. 3.15C). A significant reduction in the mRNA expression of the nuclear-DNA encoded mitochondrial genes *Ndufa9*, *Ndufs3*, *Sdha*, *Uqcrc2*, *Uqcrcfs* and *CoxIV* was also noted (Fig. 3.15D), suggesting that OXPHOS activity is impaired in *Parkin* KO myotubes.

3.16 *Parkin* KO primary myotubes have impaired mitochondrial turnover

Next, to determine the effect of *Parkin* knock out on mitochondrial turnover, mRNA expression and protein levels of mitochondrial fission and fusion markers were assessed. The expression of mitochondrial fusion genes, *Mfn1* and *Opal*, and the mitochondrial fission gene *Drp1* were significantly reduced in *Parkin* KO primary myotubes, when compared to wild type controls (Fig. 3.15E). A reduction in the expression of *Mfn2* was also noted in *Parkin* KO primary myotubes, however the reduction was not statistically significant (Fig. 3.15E). Subsequent western blot analysis revealed a significant reduction in the levels of the mitochondrial fusion marker MFN1 in *Parkin* KO myotubes (Fig. 3.15F & 3.15G). These data suggest that knock out of *Parkin* is associated with maintenance of mitochondrial number, impaired mitochondrial turnover and reduced expression of mitochondrial OXPHOS genes in primary myoblast cultures.

Figure 3.15: Knock out of *Parkin* results in reduced OXPHOS gene expression, impaired mitochondrial turnover and preservation of the mitochondrial pool in primary myotube cultures

A) Graph showing qPCR analysis of mtDNA copy number:nuDNA copy number ratio in primary myoblasts isolated from wild type and *Parkin* KO mice. Values are expressed as fold change relative to wild type control. The expression of the mitochondrial gene *CoI* was normalized to the nuclear gene *18S* (n=3). **B)** Graph showing results of FACS analysis of MitoTracker Green FM stained primary myoblasts isolated from wild type or *Parkin* KO mice (MFI: Mean fluorescence intensity) (n=3). **C)** Graph showing qPCR analysis of mitochondria DNA-encoded mitochondrial complex gene expression, normalized to *Ppia*, in wild type and *Parkin* KO primary myotubes (n=3). **D)** Graph showing qPCR analysis of nuclear DNA-encoded mitochondrial complex gene expression, normalized to *Ppia*, in wild type and *Parkin* KO primary myotubes (n=3). **E)** Graph showing qPCR analysis of mitochondrial fusion (*Mfn1*, *Mfn2*, *Opa1*) and mitochondrial fission (*Drp1*, *Fis1*, *Mff*) marker mRNA expression in wild type and *Parkin* KO primary myotubes (n=3). **F)** Representative images of western Blot analysis of MFN1, MFN2, FIS1 and DRP1 in wild type and *Parkin* KO primary myotubes. The levels of GAPDH were assessed as a loading control. **G)** Graph showing quantification of MFN1, MFN2 and DRP1 protein levels, normalized to GAPDH, in arbitrary units (n=3). *P<0.05, **P<0.001, ***P<0.0001 (Student's t-test).

3.17 Reduced expression and protein levels of critical OXPHOS genes is observed in *Parkin* knock out Gas muscle

Despite no significant difference in mitochondrial number, a trend towards reduced levels of mitochondrial OXPHOS proteins (Complex I-V) was observed in *Parkin* KO Gas muscle, when compared to wild type controls (Fig. 3.16A & 3.16B). Notably, a significant reduction in the levels of the complex I protein NDUFB8 and the complex II protein SDHB was observed (Fig. 3.16A & 3.16B). Consistent with this, decreased expression of both mitochondrial-encoded (Fig. 3.16C) and nuclear-encoded (Fig. 3.16D) mitochondrial complex genes was also observed in Gas muscle isolated from *Parkin* KO mice, with a significant reduction in *Nd1*, *Nd6*, *Atp6* and *Ndufb6* expression noted (Fig. 3.16C & 3.16D).

3.18 Knock out of *Parkin* leads to impaired mitochondrial turnover but not autophagy in muscle

Next, to quantify the expression of genes critical for mitochondrial fusion (*Mfn1*, *Mfn2* and *Opal*) and mitochondrial fission (*Drp1*, *Fis1* and *Mff*) in skeletal muscle from wild type and *Parkin* KO mice, qPCR analysis was performed. As shown in Fig. 3.17A, no significant difference in the expression of mitochondrial fusion and fission markers was observed in Gas muscle isolated from wild type and *Parkin* KO mice (Fig. 3.17A). Subsequent immunoblot analysis revealed no significant difference in the levels of both MFN1 and MFN2 mitochondrial fusion proteins in Gas muscle isolated from wild type and *Parkin* KO mice (Fig. 3.17B & Fig. 3.17C), which is in agreement with the qPCR results. However, a significant reduction in the protein levels of the mitochondrial fission markers FIS1 and DRP1 was noted in *Parkin* KO Gas muscle tissue, when compared to wild type controls (Fig. 3.17B & Fig. 3.17C). In addition, the levels of the autophagosomal markers LC3-II and P62 were not significantly altered between muscle tissue isolated from wild type and *Parkin* KO mice (Fig. 3.17D & Fig. 3.17E), suggesting that loss of *Parkin* does not result in overt mitophagy in skeletal muscle. These data suggest that mitochondria number is preserved in *Parkin* KO mice and that loss of *Parkin* leads to changes in gene expression consistent with decreased mitochondrial fission in skeletal muscle (Fig. 3.17D & Fig. 3.17E).

Figure 3.16: *Parkin* KO mice have reduced levels of mitochondrial Complex I and Complex II proteins and reduced expression of OXPHOS genes in Gas muscle

A) Representative images of western blot analysis of OXPHOS subunit (ATP5, UQCRC2, MTCOI, SDHB and NDUFB8) protein levels in wild type and *Parkin* KO Gas muscle. The levels of HSP60 was assessed as a loading control (n=6). **B)** Graph showing quantification of OXPHOS protein levels, normalized to HSP60, in arbitrary units (n=6). **C)** Graph showing qPCR analysis of mitochondria DNA-encoded mitochondrial complex gene expression, normalized to *Ppia*, in wild type and *Parkin* KO Gas muscle (n=6). **D)** Graph showing qPCR analysis of nuclear DNA-encoded mitochondrial complex gene expression, normalized to *Ppia*, in wild type and *Parkin* KO Gas muscle (n=6). All graphs show mean±s.e.m. *P<0.05, **P<0.01, ***P<0.001 (Student's t-test).

Figure 3.17: *Parkin* KO mice display impaired mitochondrial turnover, while autophagy remains unaltered, in muscle

A) Graph showing qPCR analysis mRNA expression of mitochondrial fusion (*Mfn1*, *Mfn2*, *Opa1*) and fission (*Drp1*, *Fis1*, *Mff*) marker mRNA expression in wild type and *Parkin* KO Gas muscle (n=6). **B)** Western Blot analysis of MFN1, MFN2, FIS1 and DRP1 in wild type and *Parkin* KO Gas muscle. The levels of GAPDH were assessed as a loading control. **C)** Graph showing quantification of MFN1, MFN2, FIS1 and DRP1 protein levels, normalized to GAPDH, in arbitrary units (n=6). **D)** Western blot analysis of P62, LC3-I and LC3-II protein levels in wild type and *Parkin* KO Gas muscle. The levels of GAPDH were assessed as a loading control. **E)** Graph showing quantification of P62 protein levels and the level of LC3-II/LC3-I, normalized to GAPDH, in arbitrary units (n=6). All graphs show mean $\pm$ s.e.m. *P<0.05, ***P<0.001 (Student's t-test).

3.19 Knock out of *Parkin* results in reduced mitochondrial function in primary myotube cultures

Next, mitochondrial function in primary myotube cultures derived from wild type and *Parkin* KO mice was measured through real-time assessment of OCR using the Seahorse XF^e24 extracellular flux analyzer. An overall reduction in real-time oxygen consumption rate was apparent in *Parkin* KO myotubes (Fig. 3.18A). Moreover, a significant reduction in basal, maximal, ATP-linked and non-mitochondrial respiration was noted in *Parkin* KO myotubes (Fig. 3.18B). In addition, a significant reduction in spare respiratory capacity and OCR due to proton leak was further observed in *Parkin* KO myotubes, when compared to wild type controls (Fig. 3.18B). Consistent with impaired mitochondrial function, increased levels of ROS (Fig. 3.18C) and active p-AMPK protein (Fig. 3.18D & 3.18E) were detected in primary myoblasts harvested from *Parkin* KO mice, when compared to wild type controls, although the increase in p-AMPK was not statistically significant (Fig. 3.18E). Taken together, these data suggest that while mitochondrial number remains unchanged, mitochondrial function is impaired in skeletal muscle of *Parkin* KO mice.

Figure 3.18: Knock out of Parkin results in reduced mitochondrial function in primary myotube cultures

A) Graph showing the real-time OCR measurement of 72 h differentiated primary myotubes isolated from wild type and *Parkin* KO hind limb muscle, as assessed by the Seahorse XF[®]24 extracellular flux analyzer (n=9). Time points where Oligomycin (Oligo), FCCP and Antimycin/Rotenone (Ant/Rot) were injected (arrows) and the rate number where each OCR was measured are indicated. **B)** Graph showing quantification of basal, spare, maximal, ATP-linked and non-mitochondrial respiration, and respiration due to proton leak in 72 h differentiated primary myotubes isolated from wild type and *Parkin* KO hind limb muscle. All OCR values were normalized to total protein (n=9). **C)** Graph showing the levels of Reactive Oxygen Species (ROS), as measured by H₂DCFDA fluorescence, in 72 h differentiated primary myotubes isolated from wild type and *Parkin* KO hind limb muscle (n=4). **D)** Representative images of western blot analysis of phosphorylated (p-AMPK α) and total AMPK α protein levels in wild type and *Parkin* KO Gas muscle. The levels of GAPDH were assessed as a loading control (n=6). **E)** Graph showing quantification of p-AMPK α , total AMPK α , and the ratio of p-AMPK α /total AMPK α , normalized to GAPDH, in arbitrary units (n=6). Data represent mean $\pm$ s.e.m. *P<0.05, **P<0.01 (Student's t-test).

3.20 Absence of *Parkin* results in reduced body weight, decreased skeletal muscle weights and myofiber atrophy in mice

As knock down of *Parkin* on myotubes resulted in myotubular atrophy, phenotype of *Parkin* KO mice was next analyzed. *Parkin* KO mice have significantly reduced total body weight at 4 weeks of age, when compared to age-matched wild type mice controls (Fig. 3.19A). To confirm absence of *Parkin* in skeletal muscle tissues collected from *Parkin* KO mice we performed immunoblot analysis; no detectable levels of PARKIN protein were observed in *M. quadriceps* (Quad), *M. gastrocnemius* (Gas), *M. tibialis anterior* (TA), *M. soleus* (Sol) and *M. extensor Digitorum longus* (EDL) hind limb muscles (Fig. 3.19B). Further characterization of hind limb skeletal muscle tissue weights revealed a significant reduction in the weights of all hind limb muscles analyzed in *Parkin* KO mice, when compared to wild type controls (Fig. 3.19C & Fig. 3.19D). Consistent with this, subsequent histological analysis (Fig. 3.19E) of myofiber cross-sectional area (CSA) in OCT embedded H&E stained TA muscles, revealed a reduction in TA myofiber CSA distribution (Fig. 3.19F) and average myofiber CSA (Fig. 3.19G) in *Parkin* KO mice, when compared to wild type control. These data suggest that loss of *Parkin* results in reduced skeletal muscle mass *in vivo*.

3.21 The myofiber atrophy phenotype observed in *Parkin* KO muscle tissue may be, at least in part, due to increased MURF1 levels

Next, to determine whether the atrophy phenotype observed in *Parkin* KO mice resulted from activation of the Akt-FoxO pathway, the levels and phosphorylation status of FOXO1 and FOXO3a were assessed. Western blot analysis revealed that, although the levels of phosphorylated FOXO1 (p-FOXO1) and total FOXO1 were significantly reduced, the ratio of p-FOXO1/FOXO1 remained unaltered, in *Parkin* KO Gas muscle (Fig. 3.20A). A significant reduction in the protein levels of phosphorylated FOXO3a (p-FOXO3a) was also noted in *Parkin* KO Gas muscle tissue (Fig. 3.20B). However, the levels of total FOXO3a and the ratio of p-FOXO3a/FOXO3a were comparable between *Parkin* KO Gas muscle and wild type controls (Fig. 3.20B). Importantly, we observed increased expression of both *Murf1* and *Atrogin-1* in *Parkin* KO Gas muscle, although the increase was not statistically significant (Fig. 3.20C). In agreement with *in vitro* observations, western blot analysis revealed a trend towards increased levels of MURF1 protein in *Parkin* KO Gas muscle, albeit not statistically significant (Fig. 3.20D & 3.20E), further suggesting that MURF1 may play a role in the myofiber atrophy phenotype observed in *Parkin* KO mice.

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Figure 3.19: Four-week-old Parkin KO mice have reduced muscle weights and myofiber size

A) Graph showing average body weights of 4-week-old wild type and *Parkin* KO mice (n=6). **B)** Representative images of western blot images showing PARKIN protein levels in wild type and *Parkin* KO *M. Quadriceps* (Quad), *M. Gastrocnemius* (Gas), *M. Tibialis anterior* (TA), *M. Soleus* (Sol) and *M. Extensor Digitorum longus* (EDL) muscles. The levels of GAPDH were assessed as a loading control (n=6) **C)** Graph showing weights of Quad, Gas and TA hind limb muscles of wild type and *Parkin* KO mice. All hind limb muscle weights were normalized to tibia length (n=6). **D)** Graph showing weights of Sol and EDL hind limb muscles of wild type and *Parkin* KO mice. All hind limb muscle weights were normalized to tibia length. **E)** Representative images of hematoxylin and eosin (H&E) stained *M. Tibialis anterior* (TA) muscle sections of 4-week-old wild type and *Parkin* KO mice. Scale bars represent 100 μ m. **F)** Graph showing the distribution of myofiber cross-sectional area in 4-week-old wild type and *Parkin* KO mice TA muscles (n=6). **G)** Graph showing average myofiber cross-sectional area of 4-week-old wild type and *Parkin* KO mice TA muscles (n=6). Graphs represent mean $\pm$ s.e.m. **P<0.01 and ***P<0.001 (Student's t-test).

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Figure 3.20: Knock out of Parkin results in moderate but not significant increase in MURF1 protein levels in Gas muscle

A) *Top*, Representative images of western blot analysis of phosphorylated FOXO1 (p-FOXO1) and total FOXO1 in wild type and *Parkin* KO Gas muscle. The levels of GAPDH were assessed as a loading control (n=6). *Bottom*, Graph showing quantification of p-FOXO1, total FOXO1 and p-FOXO1/total FOXO1 levels, normalized to GAPDH, in arbitrary units (n=6). **B)** *Top*, Representative images of western blot analysis of phosphorylated FOXO3a (p-FOXO3a) and total FOXO3a in wild type and *Parkin* KO Gas muscle. The levels of Ponceau S were assessed as a loading control (n=6). *Bottom*, Graph showing quantification of p-FOXO3a, total FOXO3a and p-FOXO3a/total FOXO3a levels, normalized to Ponceau S, in arbitrary units. (n=6). **C)** Graph showing qPCR analysis of atrogenes *Murf1* and *Atrogin-1* in wild type and *Parkin* KO Gas muscle (n=6). **D)** Representative images of western blot analysis of MURF1 and ATROGIN-1 protein levels in wild type and *Parkin* KO Gas muscle. The levels of GAPDH were assessed as a loading control (n=6). **E)** Graph showing quantification of MURF1 and ATROGIN-1 protein levels, normalized to GAPDH, in arbitrary units (n=6). Data represent mean±s.e.m. *P<0.5, **P<0.01, ***P<0.001 (Student's t-test).

3.22 Genes involved in metabolic pathways are downregulated in *Parkin* KO skeletal muscle

To further understand the skeletal muscle phenotype observed in *Parkin* KO mice, microarray analysis was performed. Microarray analysis of RNA isolated from skeletal muscle of 3-month-old *Parkin* KO and age-matched wild type controls revealed several significantly ($p < 0.05$) up (≥ 1.5 fold) and down (≤ 0.667 fold) regulated genes. Subsequent KEGG pathway analysis of differentially regulated genes using DAVID pathway analysis tool revealed that nitrogen metabolism was the most significantly regulated pathway, with differential expression of 6 carbonic anhydrase genes (*Car2*, *Car3*, *Car4*, *Car12*, *Car14*, *Car15*) noted (Table 3.1). It is noteworthy that genes involved in cytoskeleton structure (p -value=0.002) and focal adhesion (p -value=0.01) are significantly differentially regulated in *Parkin* KO skeletal muscle, as compared to wild type controls, indicating that loss of *Parkin* may lead to alterations in cytoskeletal structure *in vivo* (Table 3.1). Consistent with observations made both *in vivo* and *in vitro*, genes associated with the AMPK signalling pathway tended to increase in *Parkin* KO muscle (p -value=0.015), further underscoring the identified deficiencies in mitochondrial function in *Parkin* KO mice (Table 3.1). Given that mitochondria have calcium buffering capacity in the cell, it is worth noting that genes involved in calcium signalling were predominantly decreased in *Parkin* KO muscle tissue (p -value=0.21). In addition, significant differential regulation of genes involved in fatty acid metabolism (p -value=0.004), PPAR signalling (p -value=0.01) (which regulates utilization of fatty acids) and biosynthesis of unsaturated fatty acids (p -value=0.047) underscores the importance of *Parkin* for fatty acid utilization, metabolism and biosynthesis (Table 3.1).

3.23 Expression of several ubiquitin E3 ligases and E2 ligases are increased in *Parkin* KO skeletal muscle

To further identify the molecular mechanism(s) behind the myofiber atrophy phenotype observed in *Parkin* KO mice, microarray data was scanned to identify differentially expressed genes involved in the ubiquitin proteasome system. Subsequent analysis revealed increased expression of several E3 ubiquitin ligases, notably *Trim59*, *Trim43b*, *Rnf144a*, *Zfp598*, *Traip* and *Fbxl22*, the deubiquitinating enzyme *Usp18* and the E2 conjugating enzyme *Ube2c* (Table 3.2). Next, we performed qPCR analysis to validate the changes in E2 and E3 enzyme expression in *Parkin* KO Gas muscle. Results confirmed significant upregulation of *Trim59*, *Rnf144a*, *Zfp598* and *Fbxl22* and the ubiquitin conjugating enzyme *Ube2c* in *Parkin* KO Gas muscle (Table 3.2), indicating that additional components of the ubiquitin proteasome system may play a role in the atrophy phenotype observed in *Parkin* KO skeletal muscle.

Term	Count	%	Genes Downregulated	Genes Upregulated	P-Value	Benjamini
Nitrogen metabolism	6	0.9	<i>Car14, Car15, Car2, Car4</i>	<i>Car12, Car3</i>	0.0001	0.03
Regulation of actin cytoskeleton	16	2.4	<i>Itgam, Myl10</i>	<i>Actg1, Ctnna3, Cxcr4, Ezr, Itga4, Mylpf, Myl9, Pik3cd, Thy1, Vasp</i>	0.002	0.16
Fatty acid metabolism	7	1	<i>Acadm, Acadvl, Scd3</i>	<i>Cpt1a, Fads2, Scd1, Scd2</i>	0.004	0.23
Pathways in cancer	23	3.4	<i>Adcy2, Ctnna3, Cdkn2a, Cdkn2b, Cysc, Egln3, Ednrb, Fgf1, Fgfr3, Foxo1, Gna12, Lam2c, Plcb2, Sufu, Crk</i>	<i>E2f2, Cxcr4, Fgf11, Gng7, Pik3cd, Ret, Tceb2, Wnt4</i>	0.004	0.2
Focal adhesion	14	2.1	<i>Flnc, Myl10, Crk</i>	<i>Actg1, Col5a3, Col11a1, Col11a2, Itga11, Itga4, Mylpf, Myl9, Pik3cd, Vasp</i>	0.010	0.34
PPAR signaling pathway	8	1.2	<i>Cd36, Acadm, Sorbs1, Scd3</i>	<i>Cpt1a, Fads2, Scd1, Scd2</i>	0.010	0.31
AMPK signaling pathway	10	1.5	<i>Cd36, Foxo1, Scd3</i>	<i>Pfkfb3, Cpt1a, Ccna2, Pik3cd, Ppp2r2b, Scd1, Scd2</i>	0.015	0.33
Calcium signaling pathway	12	1.8	<i>Adcy2, Cacna1h, Chrna7, Ednrb, Gna14, Plcb2, P2rx5, Slc25a5, Sphk2</i>	<i>Cd38, Itpr1, Plcd4</i>	0.021	0.35
Rap1 signaling pathway	13	1.9	<i>Adcy2, Fgf1, Fgfr3, Id1, Itgam, Plcb2, Crk</i>	<i>Tiam1, Actg1, Drd2, Fgf11, Pik3cd, Vasp</i>	0.030	0.39
Cell cycle	9	1.3	<i>Cdkn2a, Cdkn2b, Pkmyt1</i>	<i>Cdc14a, E2f2, Cdc20, Ccna2, Ccnb2, Plk1</i>	0.034	0.4
cGMP-PKG signaling pathway	11	1.6	<i>Adcy2, Ednrb, Gna12, Plcb2, Slc25a5</i>	<i>Atp1a4, Itpr1, Myl9, Pik3cd, Kcnu1, Vasp</i>	0.035	0.38
Insulin secretion	7	1	<i>Adcy2, Gck, Plcb2</i>	<i>Atp1a4, Glp1r, Pclo, Kcnu1</i>	0.046	0.42
Biosynthesis of unsaturated fatty acids	4	0.6	<i>Scd3</i>	<i>Fads2, Scd1, Scd2</i>	0.047	0.42
ECM-receptor interaction	7	1	<i>Cd36, Lamc2</i>	<i>Col5a3, Col11a1, Col11a2, Itga11, Itga4</i>	0.05	0.41

Table 3.1: KEGG pathway analysis of differentially expressed genes in *Parkin* KO Gas muscle

Table Table showing Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis of significantly upregulated (≥ 1.5) and downregulated (≤ 0.667) genes, as identified through microarray analysis, in 3-month-old *Parkin* KO Gas muscle as compared to age-matched wild type controls. Pathway names (Terms), number of genes (counts), percentage of total gene count in each term (%), gene symbols, *p*-values and Benjamini-Hochberg critical values (Benjamini) are provided.

Gene Accession Number	Gene ID	Function	Structure	Fold Change (Array)	p-value (Array)	Fold Change (qPCR)	p-value (qPCR)
NM_026785	<i>Ube2c</i>	Ubiquitin-conj. enzyme	UBCc	1.89	0.028	3.19	0.009
NM_175206	<i>Fbxl22</i>	E3 ligase	F-box, LRR	1.71	0.042	1.85	0.010
NM_080563	<i>Rnf144a</i>	E3 ligase	RING, IBR	1.59	0.007	1.66	0.030
NM_011634	<i>Traip</i>	E3 ligase	RING	1.55	0.003	1.63	0.397
NM_001170884	<i>Trim43b</i>	E3 ligase	RING	3.75	0.002	0.95	0.805
NM_025863	<i>Trim59</i>	E3 ligase	RING	2.14	0.002	1.96	0.013
NM_183149	<i>Zfp598</i>	E3 ligase	RING, Znf_C2H2	1.56	0.002	1.48	0.033
NM_011909	<i>Usp18</i>	De-ubiq. enzyme	N/A	1.67	0.008	1.35	0.229

Table 3.2: Genes involved in the ubiquitin proteasome system are upregulated in *Parkin* KO Gas muscle

Table showing fold change differences in gene expression of the ubiquitin-conjugating enzyme *Ube2c*, E3 ligases (*Fbxl22*, *Rnf144a*, *Traip*, *Trim43b*, *Trim59*, *Zfp598*) and the deubiquitinating enzyme *Usp18* in *Parkin* KO Gas muscle as compared to wild type control, as assessed by microarray (Array) and qPCR analysis. Gene accession numbers and calculated *p*-values for microarray and qPCR analysis are provided. Domains (Structure) within each protein sequence were identified using SMART (a Simple Modular Architecture Research Tool). (UBCc: Ubiquitin-conjugating enzyme E2 domain; LRR: Leucine-Rich-Repeat domain; RING: Really-Interesting-New-Gene domain; RBR: RING-Between-RING domain; Znf-C2H2: Zinc-finger Cys(2)His(2) domain; N/A: Not applicable (there are no hidden domains or features present). The terms Ubiquitin-conj. enzyme and De-ubiq. enzyme are used to represent Ubiquitin conjugating enzyme and Deubiquitinating enzyme, respectively.

4 DISCUSSION

PD is a neurodegenerative disease that is reported to affect almost 7 million people around the globe²³⁴. Affected individuals suffer from symptoms including, tremors, bradykinesia (abnormally slow movement), hypokinesia (diminished motor/muscle activity), muscle weakness, depression and dementia²⁷⁷. Clinical studies have revealed systemic loss of dopaminergic neurons in substantia nigra pars compacta (SNc), which has been associated with typical motor symptoms observed in PD²⁷⁷. In the early 1980s, the mitochondrial dysfunction theory of PD was established, following the discovery of a parkinsonian-like phenotype among drug addicts who intravenously used a mitochondrial complex I inhibitor, MPTP²³⁷. This finding was consistent with studies that have revealed reduced complex I activity, increased mtDNA mutations and reduced expression of mitochondrial genes in PD²⁷⁸. After 25 years of GWAS studies, scientists have identified candidate genes that are associated with familial PD. In particular, the identification of the E3 ligase, *PARKIN*, as a central mitochondrial quality control protein led to further studies that focused on the role of mitochondrial function in PD²⁷⁹. Studies into the function of *PARKIN* revealed that mutations in the *PARKIN* gene lead to mitochondrial dysfunction, impaired mitochondrial turnover, abnormal cristae modelling and mitochondrial morphology^{280,281,282}. Although it is widely known that mutations in *PARKIN* results in the mitochondrial dysfunction observed in PD, little is known about the role of *PARKIN* in skeletal muscle metabolism and maintenance of skeletal muscle mass. This thesis provides insight into the role of *Parkin* in skeletal muscle function using both *in vitro* and *in vivo* animal models.

4.1 CCCP induces PINK1/PARKIN mediated mitophagy in C2C12 myotubes

Mitochondrial uncoupling agents are commonly utilized in mitophagy research to recapitulate the reduced mitochondrial membrane potential observed prior to selective removal of mitochondria. Among the available mitochondrial uncoupling agents, CCCP has been shown to stimulate PINK1/PARKIN-mediated removal of mitochondria following dissipation of membrane potential *in vitro*, while other organelles remain intact^{283,284}. Although observations in HeLa¹⁹⁹, HEK-293 and HCT-116²⁸⁵ cells provide compelling evidence to support that CCCP induces PINK1/PARKIN mediated mitophagy, the effect of CCCP on mitochondrial function and turnover in skeletal muscle function and growth has not been extensively studied. Analogous to previous

studies, results presented here revealed that CCCP treatment promotes mitophagy in muscle cells through activation of the PINK1/PARKIN pathway. Importantly, we observed increased levels of active full length PINK1 protein (63kDa) upon CCCP treatment. This suggests that PINK1 is protected from processing through the action of the presilin-associated rhomboid-like protease (PARL) and mitochondrial processing peptidase (MPP), which proteolytically cleave PINK1 to a 53kDa protein²⁸⁶. We also observed increased translocation of PARKIN to the mitochondria, which is consistent with enhanced PARKIN function²⁸⁷ and induction of PINK1/PARKIN-specific mitophagy¹⁹⁹, upon CCCP treatment (Fig. 3.1C).

Moreover, the increased recruitment of PARKIN protein to mitochondria was associated with a reduction in mitochondrial number and impaired mitochondrial function as determined through mtDNA copy number analysis and real-time measurement of mitochondrial respiration (Fig 3.2C & 3.3A). Confocal analysis of DMSO and CCCP treated myoblasts revealed puncta formation upon CCCP treatment (Fig. 3.2E), suggesting that CCCP leads to disruption of mitochondrial architecture. In 1950, electron microscopy (EM) led to the discovery of autophagy. Thereafter, EM remained as the central technique utilized to visualize autophagic vesicles in the cell²⁸⁸. Although EM provides a high-resolution images of mitochondria that are engulfed by autophagosomes, it remains as a qualitative approach²⁸⁹. Therefore, EM not only need an objective expertise while interpreting data but also should be accompanied with a quantitative approach.

Moreover, CCCP treatment led to increased expression of ER stress response markers (Fig. 3.4A), underscoring crosstalk between mitochondria and the ER. Collectively, these results suggest that CCCP induces PINK1/PARKIN-mediated mitophagy in C2C12 myotubes.

4.2 Loss of *Parkin* also results in mitochondrial dysfunctional and altered mitochondrial fusion/fission in skeletal muscle

Although main function of *Parkin* is to promote mitophagy by degrading mitochondrial fusion proteins^{290,216}, recent studies have revealed that *parkin* overexpression protects against apoptosis-induced proteotoxicity in Zebrafish and improves mitochondrial function and extends lifespan in fly models, suggesting that selective elimination of damaged mitochondria enhances cellular homeostasis^{291,292}. Interestingly, results presented here suggest that loss of *Parkin* results in maintenance

of mitochondrial mass (Fig. 3.7C & 3.15B), which was associated with no overt autophagy/mitophagy (Fig. 3.9 & 3.17D & 3.17E). These data, together with the fact that loss of *Parkin* leads to impaired mitochondrial function (Fig 3.10A & 3.18A), suggest that loss of *Parkin* results in accumulation of dysfunctional mitochondria. Although Seahorse XF Analyzer provides a high-quality assessment of mitochondrial function, it has some technical limitations. First, in this study, mitochondrial function was assessed in intact cells, since isolating mitochondria may result in contamination of mitochondria with unpredictable cellular components or damage of mitochondrial integrity. Also, since PARKIN is a cytosolic protein that is recruited to mitochondria upon reduced mitochondrial membrane potential, intact cells have been preferred to obtain mitochondrial function. However, contribution of activity of cytosolic oxygen consuming enzymes to oxygen consumption rate should be considered when intact cells are used. Nevertheless, injection of Antimycin A/Rotenone shuts down oxygen consumption due to mitochondrial respiration and reveals non-mitochondrial respiration. Ultimately, subtraction of non-mitochondrial respiration from other respiratory parameters minimizes the intervention of other oxygen consuming sources. Also, another limitation of Seahorse XF Analyzer is that Seahorse XF analyzer does not provide a direct assessment of mitochondrial complex activity. Yet mitochondria are major consumers of oxygen in the cell, oxygen consumption is not a direct measurement of mitochondrial function. A direct measurement of activity of mitochondrial complexes would provide a better evidence to suggest that loss of *Parkin* leads to reduced mitochondrial complex I and complex II activities.

Results presented in the current study revealed that loss of *Parkin* does not result in a notable increase in the levels of either mitochondrial fusion or fission markers *in vitro* and *in vivo*. However, knock down of *Parkin* led to a significant reduction in both mitochondrial fusion and fission protein markers in C2C12 myoblasts (Fig. 3.8B & 3.8C). A previous study by Ishihara *et al.*, 2009 reported that knock out of *Drp1* leads to a concomitant reduction in the levels of mitochondrial fusion proteins (MFN1 and MFN2), which was thought to be a compensatory mechanism for the loss of the mitochondrial fission protein *Drp1*²⁹³. The results from this previous study are quite consistent with the reduced DRP1, MFN1 and MFN2 protein levels observed in *Parkin* knock down myoblasts (Fig 3.8B & 3.8C) and suggest that the reduced levels of MFN1 and MFN2 may be due to loss of DRP1. However, in contrast, with the exception of MFN1, knock down or loss of *Parkin* did not appear to dramatically alter the protein

levels of mitochondrial fusion or fission markers in myotube cultures. (Fig. 3.8D, 3.8E, 3.15F & 3. 15G). Recently, it has been shown that mitophagy increases and is required during myogenic differentiation of C2C12 cells²⁹⁴. Given the fact that no observable deficiencies during the early stages of myogenic differentiation were noted in either *Parkin* knock down or knock out cultures, it is interesting to surmise that the maintenance of mitochondrial fusion and fission protein levels may form the basis of an as yet unidentified compensatory mechanism to allow normal myogenic differentiation to ensue in the absence of *Parkin*. However, further work will need to be undertaken to confirm this.

In contrast to the results observed in myotube cultures, western blot analysis revealed a significant reduction in protein levels of mitochondrial fission markers in *Parkin* KO Gas muscle, as compared to wild type controls (Fig. 3.17B & 3. 17C). Given the fact that these experiments were performed in different model systems, namely cultured cells and whole muscle tissue, it is plausible that the disparity in levels of mitochondrial fission markers noted may be due to the inherent differences between cultured cells and muscle tissue *in vivo*. Previous studies have shown that parkin activates DRP1 through phosphorylation which in turn results in recruitment of DRP1 to mitochondria. In this study, DRP1 levels have been investigated in total cell lysates which may not be a direct indication of activation of DRP1. However, there are compelling evidences that phenotype observed in human cell line due to loss of *PARKIN* has been alleviated with *DRP1* expression or worsened with loss of DRP1 suggesting that decreased DRP1 levels in *Parkin*-siRNA transfected myoblasts and *Parkin* knock out Gas muscle seem to be in line with the phenotype observed in mammalian models^{295,296}. Although the mechanism that how DRP1 expression alleviate phenotype noted in *PARKIN* deficient models, results here further confirm the phenotype observed previously in different studies.

It is important to highlight that both *Mfn1* and *Mfn2* are required for development, which is underscored by the fact that knock out of both *Mfn1* and *Mfn2* is postnatally lethal in mice^{297,298}. Therefore, given the fact that the *Parkin* KO mice used in this study are healthy and survive to a similar age as wild type controls with a slightly increase mortality rate²⁹⁹, it is quite possible that mechanisms may be in place to maintain the protein levels of MFN1 and MFN2 *in vivo*, in an attempt to maintain the viability of the mice. However, further work will need to be performed to verify this.

In line with previous reports that have revealed reduced complex I and to some extent complex II activity in PD patients^{236,237,300,301}; loss of *Parkin* in skeletal muscle resulted in a trend towards reduced levels of mitochondrial complex proteins, with a significant reduction in complex I (NDUFB8) and complex II (SDHB) subunits noted in *Parkin* KO skeletal muscle (Fig. 3.16A 3.16B). However, it is important to highlight the significant increase in the mRNA expression of several nuclear DNA-encoded mitochondrial OXPHOS genes upon knock down of *Parkin* (Fig. 3.7G). Previous studies have reported increased expression of mitochondrial OXPHOS genes in diseases associated with mitochondrial complex deficiencies³⁰²; suggesting that the increased mRNA expression of OXPHOS genes noted upon loss of *Parkin* may be a compensatory response to counteract reduced OXPHOS protein levels and impaired mitochondrial respiratory capacity observed in *Parkin* knock down cells.

The generation of ATP is the ultimate product of the ETC and OXPHOS in mitochondria. However, mitochondria and the process of OXPHOS results in the formation of unwanted by-products, including ROS, which pose potential risks to nucleic acids, proteins and lipids. To counteract this, mitochondria have developed different responses to lower the levels of cellular ROS³⁰³. Consistent with mitochondrial dysfunction noted upon loss of *Parkin*, increased levels of cellular ROS in both *Parkin* knock down and knock out models was observed (Fig 3.10C & 3.18C). A recent study has revealed increased ROS levels following knock down of the mitochondrial complex I assembly factor NDUFAF1³⁰⁴, which agrees with the changes observed in the current study (Fig 3.7D & 3.7E & 3.16A & 3.16B). Therefore, it is interesting to surmise that the reduced levels of mitochondrial OXPHOS proteins observed in the absence of *Parkin* may lead to increased cellular ROS levels and mitochondrial dysfunction in muscle. It is noteworthy to mention that elevated ROS levels have previously been associated with induction of skeletal muscle atrophy³⁰⁵⁻³⁰⁷. Therefore, it is quite possible that the increased cellular levels of ROS observed upon loss of *Parkin* may also contribute to the atrophy phenotype observed. In this thesis DCFDA has been used to detect cellular ROS in *Parkin*-siRNA transfected myoblasts or *Parkin* knock out primary myoblasts. DCFDA is the most commonly used probe to detect cellular ROS. DCFDA is able to penetrate through plasma membrane and detect several ROS such as H₂O₂, •OH and O₂^{•-}³⁰⁸. Given that DCFDA does not specifically detect ROS generated by mitochondria, other fluorescent probes, mitoSOX or mitoTEMPO would have given a more precise assessment of mitochondrial ROS. However, one limitation of those dyes

that delivery of the probes are facilitated with lipophilic cation, triphenylphosphonium cation (TPP⁺), which perturbs mitochondrial membrane potential thereby ROS production³⁰⁹. To overcome such limitations due to specificity of the probes, mitochondrial scavenging enzymes should be assessed. Since superoxide ions are converted to H₂O₂ through activity of SOD2 (Reviewed in *Section 1.4.1.2*), protein levels of SOD2 would reveal whether loss of *Parkin* results in an increased ROS response in mitochondria.

AMPK has plethora of functions in the cell. However, the main function of AMPK is thought to be linked to maintaining energy homeostasis in the cell through sensing AMP:ATP and ADP:ATP ratios. Depletion of energy stores leads to activation of AMPK, which switches on catabolic pathways while inhibiting anabolic processes that consume ATP³¹⁰. Importantly, previous studies have shown that activation of the AMPK pathway alleviates atrophy of dopaminergic neurons observed in PD^{311,312}, while inhibition of AMPK has a neuroprotective role during the progression of PD³¹³. Data presented here (using both *in vitro* and *in vivo* models) reveal that loss of *Parkin* leads to increased levels of active phosphorylated AMPK α . Therefore, it is interesting to surmise that mitochondrial dysfunction in skeletal muscle, due to loss of *Parkin*, results in energy deprivation, subsequent activation of AMPK and increased catabolism, which is quite consistent with the previously described function of AMPK in maintaining energy homeostasis³¹⁴. Collectively, these data suggest that loss of *Parkin* leads to impaired mitochondrial turnover (mitophagy) and mitochondrial dysfunction in skeletal muscle, which further underscores the importance of *Parkin* function to skeletal muscle metabolism.

4.3 CCCP treatment results in atrophy phenotype *in vitro*

Previous studies have observed mitochondrial dysfunction in glucocorticoid- and disuse-induced atrophy^{167,315,316}. Consistent with this, data presented here revealed that CCCP treatment resulted in the development of myotubular atrophy in C2C12 muscle cells. Skeletal muscle atrophy has been shown to be triggered by increased expression of atrophy-related genes (atrogenes) through the activity of FoxO transcription factors²⁷³. It is widely accepted that different muscle wasting conditions, ranging from cancer cachexia to denervation, are mediated through a common Akt-FoxO-dependent mechanism. Specifically, a reduction in the phosphorylation of AKT, concomitant with

a decrease in the phosphorylation of FOXO proteins, results in sequestration of FOXO proteins to the nucleus, which in turn leads to increased expression of atrogenes²⁷³. Surprisingly, results revealed that CCCP treatment led to increased levels of p-AKT in C2C12 myotube cultures, despite the fact that a significant reduction in levels of active phosphorylated FOXO3a was observed (Fig. 3.6C & 3.6D). Importantly, a recent study by Guha *et al.*, 2010 has shown the levels of p-AKT increase in response to CCCP-induced mitochondrial dysfunction³¹⁷. Moreover, it was proposed that CCCP-mediated activation of AKT has an important role in facilitating the mitochondrial respiratory stress response³¹⁷. Taken together, these data suggest that the increased levels of p-AKT observed in the current study may be attributed to the impaired mitochondrial function noted upon CCCP treatment. Moreover, although nuclear translocation of FoxO is mainly regulated by Akt, Akt-independent regulation of FoxO through Sirtuin1 (SIRT1)³¹⁸, heat shock protein 70 (HSP70)²⁸⁷ or 14-3-3 signaling protein³¹⁹ has been reported. Thus, it is quite possible that activation of FoxO3 in response to CCCP treatment may occur independent of Akt. Nevertheless, qPCR analysis revealed significantly increased expression of the FoxO downstream target atrogenes *Murfl* and *Atrogin-1* in response to CCCP treatment (Fig. 3.6E). Collectively, these data suggest that CCCP-induced mitochondrial dysfunction, impaired mitochondrial turnover and subsequent disruption of energy homeostasis results in loss of skeletal muscle mass *in vitro* through activation of FoxO3 and downstream atrogenes.

4.4 Loss of *Parkin* Leads to skeletal muscle atrophy *in vitro* and *in vivo*

Longstanding clinical studies have reported muscle weakness, resistance to exercise and fatigue in PD patients³²⁰. Similarly, *parkin* knock out fly mutants exhibit locomotor defects¹⁷⁴. In line with the impaired skeletal muscle function described above, a notable atrophy phenotype in both *Parkin* knock down and knock out models was observed. Immunoblotting analysis revealed that p-FOXO1/FOXO1 and p-FOXO3a/FOXO3a ratios were unaltered both *in vivo* and *in vitro*. However, interestingly, protein levels of MURF1 showed a significant increase upon knock down of *Parkin* *in vitro* and this increase was reflected in *Parkin* KO Gas muscle, although the increase was not statistically significant. It is also important to note a remarkable increase in mRNA expression of *Atrogin-1* and *Murfl* in *Parkin* KO Gas muscle as compared to control. Consistent with the increased atrogene expression, *Parkin* knock down resulted in a significant increase in chymotrypsin-like and caspase-like proteolytic activities, further

indicating that loss of *Parkin* leads to increased protein degradation. Taken together, these data suggest that loss of *Parkin* results in skeletal muscle atrophy phenotype both *in vivo* and *in vitro* through activation of the muscle-specific E3 ligase MuRF1. However, it is important to highlight that loss of *Parkin* only resulted in a modest increase in MuRF1 protein levels and mRNA expression; suggesting that additional E3 ligases may contribute to the degradation of myofibrillar proteins and thus, the skeletal muscle atrophy noted in response to loss of *Parkin*.

To further identify additional mechanisms that may contribute to the atrophy phenotype observed in *Parkin* KO skeletal muscle, microarray analysis was performed. Subsequent pathway analysis (using DAVID online functional annotation tool) of the microarray data revealed that nitrogen metabolism was the most significantly regulated pathway in *Parkin* KO Gas muscle, as compared to wild type controls, with the lowest *p*-value (0.0001). A significant increase in the expression of the *Car3* gene was noted in *Parkin* KO Gas muscle, when compared to wild type controls (Table 3.1). Interestingly, muscle-specific carbonic anhydrase-3 (*Car3*) mRNA expression has been shown to be upregulated in mice and chicken skeletal muscle dystrophy models^{321,322} and in COPD-induced skeletal muscle atrophy patients³²³. Also, elevated CAR3 levels in fetal plasma has been proposed to be a marker for the early diagnosis of DMD in the developing fetus³²⁴. The function of CAR3 has been postulated to act as cellular antioxidant in skeletal muscle, which suggests that the increased *Car3* expression observed in *Parkin* KO Gas muscle may be due to elevated ROS levels³²⁵. It is important to note that genes involved in AMPK signaling are mainly upregulated in *Parkin* KO Gas muscle, which is consistent with the activation of AMPK observed in the *in vivo* and *in vitro* loss of *Parkin* models used in the current study. Interestingly, recent studies have revealed that AMPK regulates the activity of FoxO transcription factors, which are upstream regulators of atrogenes, such as *Murf1*, which further promote degradation of myofibrillar proteins and skeletal muscle atrophy^{326,327}. Therefore, it is important to speculate that the increased activation of AMPK observed in the current study may be a contributing factor towards the muscle atrophy phenotype noted in loss of *Parkin* models.

Differential regulation of pathways involved in fatty acid metabolism (*p*-value=0.004), PPAR signaling (*p*-value=0.01) and biosynthesis of unsaturated fatty acids (*p*-value=0.047) in *Parkin* KO Gas muscle underscores the role of *Parkin* in fatty acid metabolism. Indeed, previous studies have reported that *Parkin* KO mice are

resistant to fat accumulation and insulin resistance due to high fat diet (HFD), due to the fact that *Parkin* positively regulates fat uptake in mice^{328,329}.

Further analysis of protein class using PANTHER pathway analysis, revealed UPS-related genes differentially regulated in *Parkin* KO Gas muscle. Microarray analysis showed a significant increase in *Ube2c* gene expression, which is further validated by qPCR analysis. *Ube2c* is an E2-conjugating enzyme that has been associated with degradation of mitotic cyclins during cell division^{330,331}. Also, high expression of *Ube2c* has been reported in various human cancer cell lines, indicating that *Ube2c* may play a role in the proteasome-dependent cell cycle regulation in *Parkin* KO skeletal muscle³³². Anaphase promoting complex/cyclosome (APC/C) forms a complex with either cadherin-1 (CDH1) or cell division cycle 20 (CDC20) and degrades mitotic substrate through the partner E2 enzymes, UBE2C and UBE2S³³³. Lee *et al.* has revealed that PARKIN substitutes APC/C complex, hence promotes degradation of cell cycle proteins, when a serine/threonine kinase, Polo-like kinase-1 (PLK1) phosphorylates PARKIN at Ser378³³⁴. Interestingly, besides increased expression of *Ube2c*, microarray analysis has shown increased expression of other partners of PARKIN in cell cycle regulation, such as *Cdc20*, *Plk1* (Table 3.1), which underscores the role of *Parkin* in regulation of cell division through activation of ubiquitin-dependent proteolysis. Moreover, microarray analysis and qPCR analysis revealed increased expression of F-box and leucine-rich repeat protein-22 (*Fbxl22*) by 1.71-fold (*p*-value: 0.042) and 1.85-fold (*p*-value: 0.01), respectively. Importantly, a recent study showed that *Fbxl22* promotes degradation of critical sarcomeric proteins, which makes *Fbxl22* a strong E3 ligase candidate that may be responsible for the atrophy phenotype observed in *Parkin* KO skeletal muscle³³⁵. Given that PARKIN is a RBR E3 ligase, it is interesting to note increased expression of another RBR E3 ligase, *Rnf144a*, in *Parkin* KO Gas muscle, when compared to wild type controls (Table 3.2). Previous studies have uncovered a role for RNF144A in apoptosis induced upon DNA damage and concomitant activation of p53^{336,337}. The expression of two tripartite motif family proteins, *Trim43b* and *Trim59*, was also increased in *Parkin* KO Gas muscle, however, to date their function in regulation of skeletal muscle mass remains to be determined. Upregulation of the deubiquitinating enzyme, *Usp18*, has been implicated in activating the immune response upon viral infection³³⁸ and in response to exposure to interferons³³⁹ and bacterial lipopolysaccharides (LPS)³⁴⁰. Although *Usp18* function in skeletal muscle remains to be studied, Peng *et al.*, 2016 have reported upregulation of *USP18* in muscle fibers

obtained from patients suffering from dermatomyositis (DM)³⁴¹. Collectively, microarray data underscores the potential involvement of the ubiquitin-proteasome pathway in the skeletal muscle atrophy phenotype observed in *Parkin* KO mice.

Given that *Parkin* knock out mice do not show signs of nigral degeneration as reported in human PD patients, it is noteworthy that loss of *Parkin* leads to reduced synaptic excitability in nigral neurons (Reviewed in Section 1.5). Impaired mitochondrial function and reduced muscle mass in skeletal muscle of *Parkin* knockout mice can be attributed to degeneration of neurons in SNc which resides in midbrain and controls reward and movement³⁴². However, whether motor neurons controlled by those in SNc are denervated or not should be further investigated. Moreover, loss of *Parkin* may lead to an intrinsic alteration in skeletal muscle, which in turn results in impaired mitochondrial respiration and reduced muscle mass. Similarly, previous study have reported that *PARKIN* mutated fibroblasts collected from PD patients display reduced mitochondrial function and disrupted mitochondrial network further pointing out spatial effect of *PARKIN* deficiency.

4.5 Conclusion

Here I investigated the role of *Parkin* in skeletal muscle function and mass. In this study, results revealed that mitophagy is regulated through the activity of the PINK1/PARKIN pathway in skeletal muscle. In addition, results clearly show that loss of *Parkin* results in reduced mitochondrial respiration, elevated cellular ROS levels and impaired mitochondrial turnover, both *in vitro* and *in vivo*. In agreement with impaired mitochondrial function, the levels of active AMPK protein were increased, which is consistent with skeletal muscle loss observed in *Parkin* knock down myotubes. Moreover, notable myotubular atrophy was observed in both *Parkin*-siRNA transfected myotubes and *Parkin* KO skeletal muscle, suggesting a role for PARKIN in regulating skeletal muscle mass. Based on the data presented here, it is proposed that the skeletal muscle atrophy, observed upon loss of *Parkin*, is partly mediated by the muscle-specific E3 ligase, *Murf1*, which is quite consistent with the increased proteasome activity noted. Pathways that were differentially regulated in *Parkin* KO Gas muscle and candidate proteins that may promote UPS-mediated degradation of myofibrillar proteins in the absence of *Parkin* were further identified. Collectively, these data here underscore an emerging function for *Parkin* in the maintenance of skeletal muscle function and mass.

5 FUTURE PROSPECTS

Parkinson's Disease (PD) is associated with involuntary tremors, muscle weakness, abnormal gait and postural instability²⁷⁷. Although PD was first described 200 years ago³⁴³, the complex pathophysiology of PD still puzzles scientists today. To date, genetic studies have associated mutations in five genes (*PARKIN* (*PARK2*), *PINK1* (*PARK6*), *DJ-1* (*PARK7*), *SCNA*, *LRRK2*) with the familial form of PD²³⁹. Since mutations in the *PARKIN* gene account for the majority of familial PD²³⁹ and moreover, given the fact that *PARKIN* plays a central role in the mitochondrial quality control mechanisms³⁴⁴, mitochondrial dysfunction has been implicated in PD. Although it is clear that *PARKIN* has important roles in controlling mitochondrial quality, and that PD is associated with muscle weakness and loss of muscle mass, the role of *PARKIN* in regulating skeletal muscle growth and metabolism has remain unstudied. In this thesis, I suggest that loss of *Parkin* results in mitochondrial dysfunction, thereby leading to the development of skeletal muscle atrophy. Although results presented in this thesis suggests that *Murf1* may contribute to the atrophic phenotype observed upon loss of *Parkin*, microarray analysis revealed several additional E3 ligases significantly upregulated in *Parkin* KO Gas muscle, when compared to wild type controls. To further characterize the molecular mechanism behind the atrophy phenotype observed in the *in vitro* and *in vivo* loss of *Parkin* models, it would be interesting to knock down *Murf1* and additional E3 ligases, such as *Fbxl22*, in *Parkin* KO skeletal muscle using AAV6-mediated intramuscular administration, which has been shown to effectively target skeletal muscle tissue³⁴⁵. Subsequently, I would perform skeletal muscle histology and assess for changes in myofiber size, measure the protein and mRNA levels of muscle wasting markers and determine proteasomal activity in muscle tissue. In addition, it would be interesting to assess mitochondrial function and number in *Parkin* KO mice.

Previous studies have shown that the indirect fly muscle (IFM) of *pink1* and *parkin* loss-of-function mutant fly display fragmented mitochondria, which is rescued upon overexpression of the mitochondrial fission marker *drp1*³⁴⁶⁻³⁴⁸. Consistent with previous reports, in this thesis, I found that loss of *Parkin* results in reduced levels of the mitochondrial fission markers DRP1 and FIS1 *in vivo* and that this was associated with impaired mitochondrial turnover and mitochondrial dysfunction. Therefore, I would like to perform rescue experiments to investigate whether overexpression of *Drp1* and *Fis1*

can improve mitochondrial turnover and function and whether this has any impact on the muscle atrophy phenotype noted in *Parkin* KO skeletal muscle.

A recent study has suggested *Parkin* KO mice are resistant to denervation-induced muscle wasting²⁴⁷. However, to date very few studies have investigated the effect of *Parkin* in additional muscle wasting models. Importantly, chronic inflammation and increased inflammatory response have been demonstrated previously in PD patients³⁴⁹. Given that glucocorticoids (GC) are commonly used anti-inflammatory agents that have been shown previously to induce skeletal muscle atrophy³⁵⁰, it would be interesting to study GC-induced skeletal muscle atrophy in *Parkin* KO mice. Dexamethasone is a synthetic glucocorticoid that is widely used in medicine³⁵¹. As such, I would propose to investigate the effect of loss of *Parkin* on the progression of muscle atrophy associated with excess dexamethasone treatment. To do so, mice will be administered dexamethasone in drinking water for up to 15 days. Subsequent analysis of the resulting skeletal muscle phenotype and the levels of muscle wasting markers will be assessed in control and *Parkin* KO mice. This study will allow us to understand if loss of *Parkin* is able to impart protection against muscle wasting stimuli, in addition to denervation, and would also provide us with an opportunity to further understand, at the molecular level, the role of *Parkin* in GC-induced skeletal muscle atrophy.

PARKIN is a RING-between-RING type E3 ubiquitin ligase that has been shown to direct specific protein substrates to ubiquitin and facilitate their degradation³⁵². PARKIN translocation to mitochondria is regulated by a serine/threonine kinase, PINK1, in a phosphorylation dependent manner. Also, PARKIN has other interacting partners that have been shown to play critical roles in regulating PARKIN function, thereby mitophagy³⁵³. Hence, it would be interesting to investigate the potential interacting partners of PARKIN in muscle wasting conditions. A recent study has revealed increased levels of *Parkin* mRNA expression and PARKIN protein levels in denervated muscle³⁵⁴, suggesting that *Parkin* is involved in denervation-induced muscle wasting phenotype in mice. However, the potential partners of PARKIN in denervation model remains to be unstudied. To elucidate the PARKIN-interacting proteins in denervation model, I would cut the sciatic nerve of mice and collect the protein from hind limb muscle 7 days and 14 days post-denervation. Subsequent protein isolation and utilization of a commercial protein-protein interaction microarray chip would provide compelling evidences to portray mechanism behind *Parkin*-mediated denervation in mouse model.

Given that: 1) PD is the second most common neurodegenerative disease associated with aging around the world and 2) mitochondrial function is impaired during aging, it would be interesting to study *Parkin* function during sarcopenia, which is defined as aging-related skeletal muscle wasting. Aging has been shown to impair PARKIN translocation to mitochondria upon stress stimuli, which has been shown to impair mitophagy³⁵⁵. Moreover, a recent study has suggested that *parkin* overexpression protects against proteotoxicity observed in fly muscle due to aging and extends lifespan²⁹². As such, I would like to investigate the role of *Parkin* in aging mice to explore whether *Parkin* overexpression can protect mice from the detrimental effects of aging-mediated muscle wasting. To investigate the role of *Parkin* during aging, I would compare the expression of *Parkin* mRNA and PARKIN protein levels in skeletal muscle of aged (2-year-old) and young mice (6-month-old). To further elucidate a protective role for *Parkin* during aging, it would be important to investigate the skeletal muscle phenotype in *Parkin* overexpressing muscle as compared to control. In addition, I would further analyse muscle wasting markers in skeletal muscle tissue of *Parkin* overexpressing muscle, such as the levels of key atrogenes, to investigate the role of *Parkin* in the progression of muscle wasting associated with aging.

A recent report has revealed that mitophagy is required for successful myogenic differentiation of C2C12 myoblasts *in vitro*²⁹⁴. In addition, *Parkin* has been shown to be differentially expressed during developmental stages. Therefore, it would be interesting to study the role of *Parkin* in myogenesis. First, the levels of *Parkin* mRNA expression and protein in differentiating C2C12 muscle cells will be analysed to elucidate a potential role for *Parkin* during differentiation. Next, the levels of critical myogenic differentiation markers will be assessed, such as MyoD and myogenin, and the levels of these myogenic markers will be correlated to changes in *Parkin* levels. In addition, histological staining will be performed and myotube number and myotube fusion index will be further quantified in *Parkin* loss-of-function and gain-of-function models. These data will provide insight into the direct role of *Parkin* in controlling myogenic differentiation.

Besides mammalian studies, a glance to skeletal muscle and PD from human perspective would reveal mechanisms behind PD and possible therapeutic targets to cure PD. As such, investigation of skeletal muscle from PD patients would provide more realistic and precise evidences to treat the second most common neurodegenerative disease among aging population. To achieve this goal, mitochondrial respiration of

primary myotubes collected from PD patients need to be assessed using Seahorse XF analyser. Protein levels and activity of mitochondrial OXPHOS complexes need to be assessed to further validate altered mitochondrial respiratory capacity in skeletal muscle of PD patients. Also, mitochondrial fusion and fission proteins should be investigated in protein lysates collected from skeletal muscle of PD patients. Moreover, mitochondrial ROS levels should be measured to understand mechanism further.

Overall, the results presented in this thesis identify an emerging role for *Parkin* in the regulation of skeletal muscle mass and function. In addition, results from this thesis further suggest that methodologies aimed at maintaining balanced *Parkin* expression and function may have therapeutic potential for individuals suffering from PD.

6 APPENDICES

Appendix 6.1: Copyright clearance obtained for Figure 1.3

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Title: Transforming growth factor- β and myostatin signaling in skeletal muscle

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Publication: Journal of Applied Physiology

Publisher: The American Physiological Society

Date: Mar 1, 2008

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Title: Oxidative stress induces mitochondrial dysfunction in a subset of autistic lymphoblastoid cell lines

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Publication: Translational Psychiatry

Publisher: Nature Publishing Group

Date: Apr 1, 2014

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