CONTRACTILE ACTIVITY-INDUCED SIGNALING MECHANISMS IN AGED SKELETAL MUSCLE: INFLUENCE ON MITOCHONDRIA

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ABSTRACT

The process of aging has an influential impact on the quality of skeletal muscle. With advancing age, even in the absence of disease, skeletal muscle has been acknowledged to decline in quantity and quality, a phenomenon referred to as sarcopenia. Currently, the mechanisms which instigate this process remain incompletely defined. However, considerable research has occurred surrounding the role of the mitochondria and their potential to contribute to sarcopenia. Mitochondria are cellular powerhouses supplying the majority of biochemical energy through ATP generation. Mitochondria require proper maintenance within the cellular milieu and this occurs through a balance between the biogenesis and mitophagic degradation of the organelles. Interestingly, exercise is a potent stimulus for both of these cellular pathways. In this work, we sought to examine aspects of mitochondrial biogenesis and mitophagy in aged skeletal muscle to determine how they may change compared to young subjects and whether these processes remain responsive to exercise, in the face of aging.

We examined the transcription of PGC-1α, a key player in the synthesis of mitochondria. PGC-1α has been acknowledged to decline in aged skeletal muscle, however whether this is due to impaired transcription, was previously unexplored. We identified that PGC-1α transcription was reduced basally in aged muscle compared to their younger counterparts. Interestingly, the gene remained responsive to an acute bout of contractile activity. We next assessed the degree of autophagy and mitophagy flux in aged skeletal muscle, at rest and following adaptation to chronic contractile activity (CCA). Aged muscle exhibited increased mitophagic turnover compared to young muscle, in contrast to prevalent notions in the literature. Following CCA, evaluation of mitophagic flux revealed a decrease in organelle turnover, likely due to an improvement in organelle quality.

Taken together, the significance of this research is that we have uncovered the molecular basis for the decline in mitochondrial content in aging muscle. Furthermore, exercise is capable of restoring a healthy mitochondrial pool through restoration of the balance between biogenesis and mitophagy.

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LIST OF ABBREVIATIONS

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
A.U.	Arbitrary Units
AC	Aged Control
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AIF	Apoptosis-Inducing Factor
AMBRA1	activating molecule in beclin-1-regulated autophagy
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ATF2	Activating Transcription Factor 2
ATG	Autophagy-related
ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma 2
BM	Body mass
BNIP3	BCL2/adenovirus E1B 19-kDa-interacting protein 3
BNIP3L/NIX	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like
C/CON	control
CA	contractile activity
Ca^{2+}	calcium
CAMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic AMP
CCA	chronic contractile activity
CCCP / FCCP	Carbonyl cyanide m-chlorophenyl hydrazone/ Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone / Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
cDNA	complementary DNA
CGIs	CpG islands
CLEAR	coordinated lysosomal expression and regulation
CMA	chaperone-mediated autophagy
COL	colchicine
COX	cytochrome oxidase
COX IV	cytochrome oxidase subunit IV
CpG	cytosine phosphate guanine
CRE	cAMP response element
CREB	cAMP response element binding protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSA	cross-sectional area
CTSD	cathepsin D

CUGBP1	CUG binding protein 1
DHPR	dihydropyridine receptors
DNA	deoxyribonucleic acid
DNMTs	DNA methyltransferase
DRP1	dynamin-realted protein 1
E-box	enhancer box
ECC	excitation contraction coupling
EDL	extensor digitorum longus
ENDO G	endonuclease G
Epi. Fat	epididymal fat
ER	endoplasmic reticulum
ERRs	estrogen-related receptors
ETC	electron transport chain
F344BNxF1	Fisher 344 Brown Norway F1 hybrid
FIP200	FAK family-interacting protein of 200 kDa
FIS1	Mitochondrial fission 1 protein
FOXO3	forkhead box O3
FUNDC1	FUN14 Domain Containing 1
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GABP	GA-binding protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA-4	GATA Binding Protein 4
GATE 16	Golgi-associated ATPase enhancer of 16 kDa
gDNA	genomic DNA
GFP	green fluorescent protein
H^+	hydrogen ion
H2O2	hydrogen peroxide
HDAC5	histone deacetylase 5
HIF-1α	Hypoxia-inducible factor 1-alpha
HSC70	Heat shock cognate 70 kDa protein
HuR	human antigen r
i.p.	intraperitoneal
IGF-1	insulin-like growth factor 1
IMF	intermyofibrillar
IMM	inner mitochondrial membrane
IMS	intermembrane space
LAMP	lysosomal-associated membrane protein
LC3-I	Microtubule-associated proteins 1A/1B light chain 3-I
LC3-II	Microtubule-associated proteins 1A/1B light chain 3-II
LIR	LC3-interacting region

MCOLN1	mucolipin 1
MDV	mitochondria derived vesicle
MEF2	myocyte-enhancer factor 2
MFF	mitochondrial fission factor
MFN1/2	mitofusin-1/2
MID49	Mitochondrial dynamics protein of 49 kDa
MID51	Mitochondrial dynamics protein of 51 kDa
MKK6	mitogen-activated protein kinase kinase 6
MPP	mitochondrial processing peptidase
MRF4	myogenic regulatory factor 4
mRNAs	messenger RNA
mtDNA	mitochondrial DNA
mTORC1	mechanistic target of rapamycin complex 1
mtPTP	mitochondrial permeability transition pore
mtUPR	mitochondrial unfolded protein response
MyoD	Myogenic Differentiation 1
n.s.	not significant
Na^+	sodium
NBR1	neighbour of BRCA-1
NDP52	nuclear dot protein 52
NFE2L2/Nrf2	Nuclear factor, erythroid 2 like 2
NMJ	neuromuscular junction
NRF-1	nuclear respiratory factor-1
NRF-2	nuclear respiratory factory-2
NT-PGC1α	N-terminal PGC-1α
NuGEMPs	Nuclear genes encoding mitochondrial proteins
O_2	oxygen
$O_2^{\bullet-}$	superoxide
OCT	optimal cutting temperature
OH•	hydroxyl
OMM	outer mitochondrial membrane
OPA1	optic atrophy 1
OPTN	optineurin
P	phosphate
p38	p38 mitogen activated kinase
P62/SQSTM1	sequestosome 1
P-AMPK	phospho-AMPK
PARL	Presenilins-associated rhomboid-like protein
PAS	phagophore assembly site
PCR	polymerase chain reaction

PDK4	Pyruvate dehydrogenase kinase 4
PE	phosphatidylethanolamine
PGC-1α	peroxisome proliferator activated receptor gamma coactivator 1 alpha
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3KC3-C1	class III phosphatidylinositol 3-kinase complex I
PI(3)P	Phosphatidylinositol 3-phosphate
PINK1	PTEN-induced putative kinase 1
PKA	protein kinase A
P-p38	phospho-p38
PPARy	Peroxisome proliferator-activated receptor y
qPCR	quantitative polymerase chain reaction
R/REC	recovery
RAB7	Ras-related protein 7
RHEB	Ras homolog enriched in brain
RLU	relative light units
ROS	reactive oxygen species
rPGC-1α	rat PGC-1α promoter construct
RyR	ryanodine receptor
S/STIM	stimulation
SAM	S-Adenosyl methionine
SDH	succinate dehydrogenase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SirT1	Sirtuin 1
Sp1	specificity protein 1
SR	stimulation and recovery
SS	subsarcolemmal
T	total
TA	tibialis anterior
TAX1BP1	tax-1 binding protein 1
TBK1	tank binding kinase 1
TCA	tricarboxylic acid cycle
Tet1	Ten-Eleven Translocation 1
Tfam	mitochondrial transcription factor A
TFEB	transcription factor EB
TIM	translocase of the inner membrane
TOLLIP	Toll interacting protein
TOM	translocase of the outer membrane
TORCs	Transducer of regulated CREB-binding proteins
TPS	trains per second

UB	ubiquitin
UBD	ubiquitin binding domain
ULK1	unc-51 like kinase 1
USF-1	upstream stimulatory factor 1
v-ATPase	Vacuolar-type H^+-ATPase
VDAC	voltage dependent anion channel
VEH	vehicle
YC	young control
YUACC	York University animal care committee
YY1	yin yang 1
$\Delta\Psi_m$	mitochondrial membrane potential

CHAPTER ONE: REVIEW OF LITERATURE

1.0 SKELETAL MUSCLE, MITOCHONDRIA AND AGING

Skeletal muscle is a specialized tissue comprising approximately 30-40% of total body mass in young, healthy individuals (119). This tissue is highly labile to changes in physical activity with beneficial adaptations occurring with increased periods of contractile activity. This notably includes the proliferation of mitochondria (2, 101, 104, 140, 234, 274). However, with extended periods of inactivity or aging, deleterious manifestations, such as reductions in overall muscle mass and depletion of mitochondria can occur (41, 208, 266). Central to the performance of skeletal muscle are the mitochondria, intracellular organelles that provide the bulk of cellular energy and regulate a host of molecular pathways. To understand how skeletal muscle and mitochondria change with aging or exercise, a description of the architecture of this tissue as well as the organelles in the healthy state will first be provided.

1.1 ARCHITECTURE OF SKELETAL MUSCLE

Skeletal muscle allows the body to locomote, maintain posture, engage in respiration and communicate with the environment. At the cellular level, these gross functions are supported by the requirement of this tissue to regulate metabolism, energy homeostasis, heat regulation, insulin sensitivity and amino acid metabolism. Thus, alterations in the quality and/or quantity of this tissue can influence the wellbeing of the complete organism.

Skeletal muscle is a multi-nucleated tissue that is composed in layers of wrapped bundles starting with the myofibrils (77). Myofibrils are the smallest cylindrical units that house the thick and thin filaments, myosin and actin, respectively, that comprise the sarcomere. The sarcomere is the functional unit which allows for shortening of the muscle, upon an appropriate stimulus and give muscle the striped, or banded appearance (Fig. 1). Many myofibrils are ensconced in a

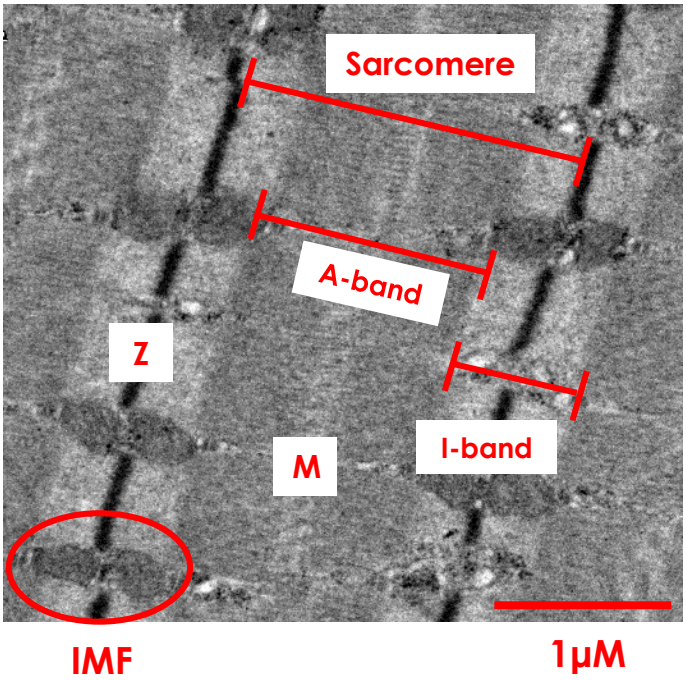

Fig. 1. Electron Micrograph of Skeletal Muscle. Longitudinal section of skeletal muscle from the extensor digitorum longus of *rattus norvegicus*. Sarcomeres are depicted with the A-band, darker portion, and I-band, lighter portion, highlighted. Additionally, pairs of IMF mitochondria are circled at the Z-line. Z; Z-line; M; M-line; IMF; intermyofibrillar mitochondria. Magnification 19000x. Unpublished image by H.N. Carter.

membrane to make up the myofibre. Numerous bundles of myofibres are surrounded by layers of connective tissue and within these bundles, blood vessels are interspersed for the transportation of oxygen, nutrients and removal of waste products. Groups of muscle fibres receive innervation from a common motor neuron and together these are referred to as the motor unit. The innervation to a group of myofibres will result in the same pattern of nerve impulses and the muscle fibres will exhibit similar contractile characteristics (199, 221).

Two special properties of skeletal muscle are that is excitable and capable of contraction. The process of muscle shortening elicited by neural impulses is referred to as excitation-contraction coupling (ECC). Action potentials generated from the motor cortex will travel to the muscle to elicit shortening of the muscle fibres. At the synapse, the action potential causes the release of the neurotransmitter, acetylcholine, which will bind to receptors located on the motor endplate of the neuromuscular junction. The binding of acetylcholine to its target receptors will propagate the action potential along the plasma membrane by voltage-sensitive Na^+ channels that then spreads down the transverse tubules. The transverse tubules are intrusions of the sarcolemmal membrane that align between the A- and I-bands of the sarcomere. The transverse tubules are flanked by the sarcoplasmic reticulum, forming what is referred to as the triad. As the action potential progresses through the transverse tubules, dihydropyridine receptors (DHPR) located within are sensitive to the change in voltage and will undergo a conformational change. The result is that the DHPR will interact with the ryanodine receptors (RyR) located on the adjacent sarcoplasmic reticulum. The DHPR interacting with the RyR will "pull the plug" opening the floodgates on the sarcoplasmic reticulum for calcium transients into the intramyocellular milieu. This released calcium will bind to troponin, changing its configuration to facilitate removal of tropomyosin from blocking the myosin-binding site on actin filaments. In this configuration, actin

and myosin are able to interact in an adenosine triphosphate (ATP)-dependent manner to generate shortening of the sarcomeres and muscle contraction. Upon cessation of the nerve impulse, calcium is quickly removed from the intracellular space to facilitate relaxation of the muscle. Quenching of calcium in skeletal muscle is mediated largely by the pumping action of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (283).

1.1.1 FIBRE TYPES

In humans, three distinct fibre types have been categorized in relation to the innervation they receive, the isoforms of contractile proteins they express and their oxidative capacity. These differences result in variations in size, contractile properties, ATP economy, mitochondrial volume and fatigue resistance (239). There are various terms used to classify fibres relating to the contractile characteristics or mitochondrial volume found within. Visualization of muscle fibre types can be readily observed through myosin ATPase histochemistry (65, 90, 231).

Type I (slow-twitch, oxidative) fibres contain high mitochondrial volume, slow shortening velocity and develop the least amount of specific tension. Type I fibres are smallest in diameter and are often postural muscles, that are regularly recruited. In contrast, the remaining classes of fibres, IIa (fast-twitch, oxidative) and IIx (fast-twitch, glycolytic), are larger and capable of producing greater force. Type IIx fibres produce the greatest force but are the least resistant to fatigue, housing the least amount of mitochondria. Corresponding to the large fibre size, the neural cells are also large, requiring a greater stimulus for depolarization. Type IIa fibres are often considered the intermediate between these two extremes, exhibiting a middle ground with features of both Type I and Type IIx fibres (105). In rodents, three groupings of type II fibres have been noted, including IIa, IIx and IIb.

1.2 CHANGES IN MUSCLE ARCHITECTURE WITH AGING

Aging is a foreseeable occurrence with the progressive decline of body tissues that ultimately results in a failure of the organism to survive. While seemingly bleak as a definition, maintenance of quality of life throughout the aging process is key to the retention of independence and to avoid the onset of comorbidities (29, 279). As skeletal muscle provides the platform for independent movement, as well as its interaction and regulation of other corporeal systems, it is important to consider the changes that result in this tissue.

While definitions continue to evolve for applications to human research trials and the medical community (54, 176, 193, 194), sarcopenia is the term used to describe the progressive age-related loss of skeletal muscle. Indeed, numerous studies have documented declines in the bulk and strength of this tissue across global populations (119, 186, 265). In North America, the aging population will experience a surge in numbers over the next few decades (14, 40, 61, 271). This places greater reliance on healthcare and financial infrastructures. While the consequences of sarcopenia are often the current targets of interventions and treatment, sourcing the root cellular cause(s) and defining preventative strategies to spare or mitigate this phenomenon is imperative to the individual's, as well as socioeconomic wellbeing. However, this is not an easy feat as sarcopenia has presented as a complex and multifactorial occurrence, being influenced by a host of intrinsic and extrinsic factors (7, 100, 121, 171).

1.2.1 DENERVATION

The loss of muscle mass with age does not fully explain all the features that accompany aging muscle. For example, strength losses are disproportionate and larger than the decline in muscle bulk (83, 109), suggesting other defects beyond myofibre size are responsible. Other notable changes may include, but are not limited to, loss of satellite cells (160, 286), excitation-

contraction coupling defects (235), myonuclear decay (8, 43, 85), mitochondrial insufficiency (7, 37), adipose infiltration (53) and fibrosis (275). In particular, rekindled interest and debate has arisen surrounding denervation and reinnervation that may transpire during aging.

The cycle of excitation-contraction coupling is central to the ability to generate force. In the aging muscle context, various aspects of the ECC pathway have been examined to determine if the functional decrease in force development is attributable to deficits in this pathway. Evidence has pointed to defects in the neuromuscular junction (NMJ) with aging, which may affect the receptivity of incoming neural signals. For example, aged muscle does not possess the characteristic pretzel-shaped NMJ, but rather it is less compact and disorganized (160). Further down the pathway, deficits in the quantity of DHPR receptors have been noted (235), suggesting a defect may exist in the coupling between the DHPR and RyR leading to aberrations in calcium release. This may ultimately affect the ability to generate muscle contraction and force. Further changes with aging that have been documented are reduced elasticity (95), shifts in myosin expression (i.e. fast to slow) (76, 206) and infiltration of adipocyte droplets in inter- and intramyocellular regions (53). Together, these changes would impair the performance of muscle fibers.

The prevailing theory for many years has been that type II fibres exhibit the most robust atrophy and type I fibres are less susceptible (203). This atrophy of type II fibres supports the loss in muscle size and would partly explain the declines in strength-producing capabilities. However, recent evidence has challenged this long-held belief. It appears that type I fibres may be classified incorrectly in aging subjects due to denervation/reinnervation or cross-innervation events from type II input (231, 232, 270). This results in the presence of hybrid fibres that express more than

one isoform of myosin heavy chain. Therefore, myosin histochemistry may not be sensitive enough to accurately identify and classify fibres expressing more than one isoform in the aging context.

1.3 STRUCTURAL FEATURES OF MITOCHONDRIA

Mitochondria within skeletal muscles exist in two discrete locations. These distinct areas of mitochondrial subcellular location have been found to exhibit divergent biochemical properties (2, 50). The first mitochondrial compartment is the subsarcolemmal (SS) found beneath the sarcolemmal membrane surrounding the myofibrils and are adjacent to the myonuclei. The second area of organelles is the intermyofibrillar (IMF) mitochondria which are found interspersed between the myofibrils. Through electron micrographs, SS mitochondria often appear as discrete circular structures while IMF mitochondria are visualized as elongated interconnected structures (114). In a longitudinal view of myofibrils, electron micrographs reveal the presence of IMF mitochondria often in pairs at the Z-line (Fig. 2).

Mitochondria are composed of two lipid membranes, which separate the organelle into two distinct compartments. The inner most compartment is the matrix which is surrounded by the highly convoluted inner mitochondrial membrane (IMM) which generates the cristae folds (Fig. 3). The space between the IMM and outer mitochondrial membrane (OMM) is aptly termed the intermembrane space (IMS).

The OMM is highly permeable enabling the passage of substrates and ions. Specialized protein import complexes, translocase of the outer membrane (TOM), facilitate the entry of nuclear-derived proteins that can be destined for the OMM, intermembrane space or will be passed to the translocase on the inner membrane (TIM) complex to reach the IMM or matrix. The intermembrane space is a highly acidic environment due to the accretion of H^+ ions pumped by three holoenzyme complexes of the electron transport chain (ETC). The elegantly folded structure

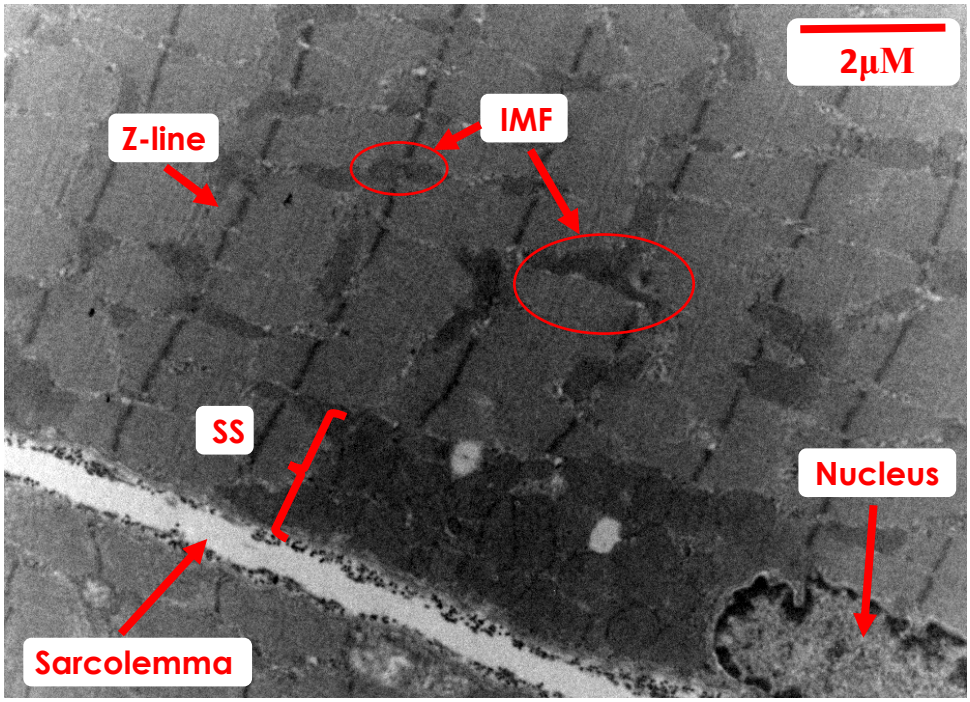

Fig. 2. Localization of SS and IMF mitochondria. Longitudinal section of extensor digitorum longus muscle from *rattus norvegicus*. SS mitochondria are evident below the sarcolemma membrane and adjacent to the nucleus. IMF mitochondria are observed in reticular structures or as pairs flanking the Z-line of the sarcomere. SS; subsarcolemmal; IMF; intermyofibrillar. Magnification 9600x. Unpublished image captured by H.N. Carter.

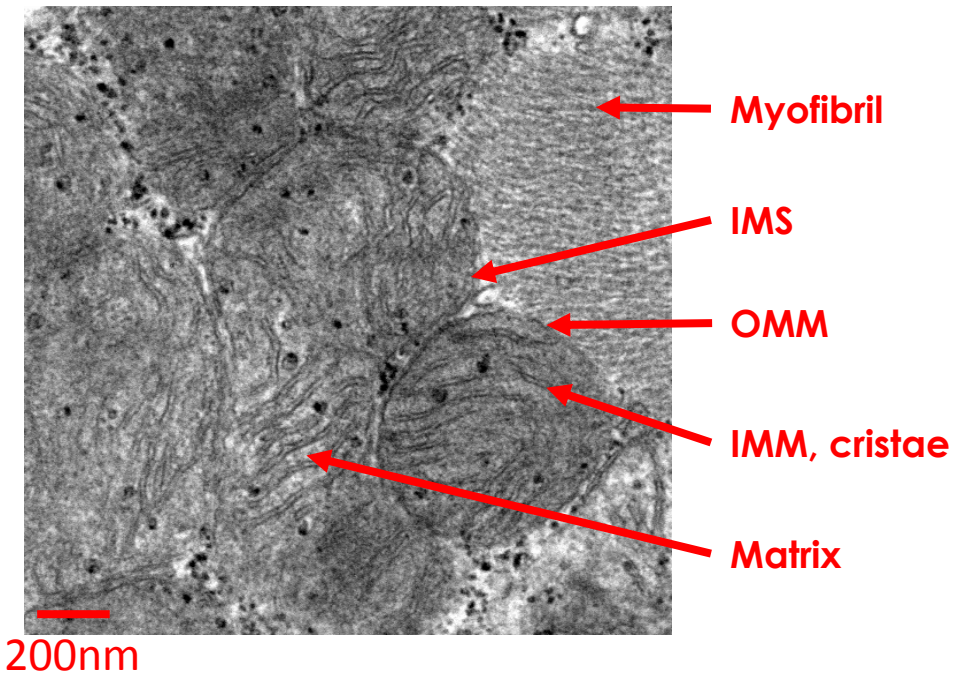

Fig. 3. Mitochondrial Compartments. Electron micrograph of SS mitochondria adjacent to a myofibril from skeletal muscle of *rattus norvegicus*. The elaborately folded inner membrane generating the cristae folds can be readily observed. OMM; outer mitochondrial membrane; IMM; inner mitochondrial membrane; IMS; intermembrane space. Magnification 62000x. Unpublished image by H.N. Carter.

of the IMM (Fig. 3) enhances the surface area for the placement of ETC protein complexes which are held in place by cardiolipin molecules. The IMM is highly impermeable and passage of any substrates requires the appropriate channel, transporter or carrier. The matrix is densely packed with proteins and enzymes for the tricarboxylic (TCA) cycle, ATP synthesis, reactive oxygen species detoxification, protein quality control and synthesis and contains multiple copies of mtDNA.

1.3.1 OXIDATIVE PHOSPHORYLATION

The process of mitochondrial ATP synthesis starts on the IMM when electrons are donated from metabolized nutrients to complexes I and II. The electrons are shuttled from complex I to IV, facilitating the pumping of protons into the intermembrane space by complexes I, III and IV. This creates an electrochemical gradient, called the mitochondrial membrane potential ($\Delta\Psi_m$), and a source of potential energy. In the presence of adenosine diphosphate (ADP), these protons can flow through complex V (ATP synthase) into the matrix, providing the chemical energy to phosphorylate ADP to make ATP. Oxygen will also be consumed in proportion to ATP synthesis by complex IV, and measurement of oxygen flux is often used to determine energetic efficiency of the organelles (167, 168). Of note, electrons do not always pass smoothly between the ETC complexes. At complexes I and III, it is possible that electrons can be prematurely donated to oxygen and generate volatile free radicals also known as reactive oxygen species (ROS) (82, 120). Mitochondrially-situated antioxidants, such as manganese superoxide dismutase and glutathione peroxidase, are in place to help cope with this stress. However, if the organelle is unable to manage the detoxification of ROS, damage can ensue to proteins, membranes or DNA. If the organelles are beyond the state of repair, their clearance and replacement must occur in order to continue to meet the metabolic demands of the muscle.

1.3.2 REGULATION OF APOPTOSIS

Mitochondria are peculiar in that while they largely provide life-sustaining energy, they are also blatant regulators of programmed cell death, or apoptosis. Within the mitochondria there are numerous protein factors that assist in the proper function of the organelle. This includes proteins such as cytochrome c, apoptosis-inducing factor (AIF) and endonuclease G (ENDO G). Under homeostasis, these factors reside in various organelle locales, however upon a stimulus which alters the configuration of the mitochondrial permeability transition pore (mtPTP) in the OMM, these proteins can be released. Once in the cytosol, they can trigger caspase-dependent or –independent protein cascades that result in DNA fragmentation and forfeiture of the myonucleus. Notable stimuli which can lead to the opening of the mtPTP and release of pro-apoptotic proteins include the loss of membrane potential, impaired ATP synthesis and excessive generation of ROS.

In aging muscle, there is a favorable environment to instigate mitochondrially-mediated apoptosis and experience DNA fragmentation (163). The mtPTP exhibits greater sensitivity to stimuli to open and release pro-apoptotic proteins (85). Indeed, studies on aging muscle have found pro-apoptotic release as a common event (41, 85). Elevations in ROS generated from the organelles in addition to impaired calcium handling are part of the signals that mediate opening of the mtPTP. Combined, these events facilitate higher myonuclear degradation. With the loss of the myonucleus, the genetic derivations that support the surrounding structures are now absent and regional atrophy of the myofibre may ensue (31).

Notably, chronic contractile activity (CCA), a model of exercise, is successful to ameliorate the degree mitochondrially-mediated apoptosis in aging muscle (2, 163). With adaptation to the exercise, increases in the anti-apoptotic protein B-cell lymphoma 2 (BCL-2), which inhibits

opening of the mtPTP are observed (2). Reductions in the release of proteins from the organelle are accompanied by lower levels of nuclear DNA fragmentation, the hallmark of apoptosis (163).

1.3.3 REACTIVE OXYGEN SPECIES

As oxidative phosphorylation progresses by the actions of the ETC, there may be occurrences where electrons are inappropriately donated to oxygen before reaching Complex IV (82, 120). When the electrons combine with oxygen, together they generate highly volatile substances such as superoxide ($O_2^{\bullet -}$) or hydroxyl ($OH^{\bullet}$) radicals or H_2O_2, collectively referred to as reactive oxygen species (ROS). Defensive mechanisms, in the form of antioxidants, are locally positioned to guard the mitochondria from the potential havoc these radicals could cause. However, if the formation of ROS exceeds the capabilities of the detoxification enzymes, damage to lipid membranes, proteins (such as ETC components) and mtDNA could transpire. If the damaged mitochondria are not readily removed or repaired, perpetuation of further damage may result.

Basally, SS mitochondrial produce more ROS compared to IMF mitochondria, and it has been observed in aged muscle that ROS produced from both fractions is heightened (41, 163). Production of ROS can be modulated through muscle activity. With chronic contractile activity of the muscle, quantities of ROS are reduced and antioxidant enzyme content is enhanced (163, 217). Indeed, aged muscle exposed to CCA exhibits declines in ROS production from both mitochondrial subfractions (163).

1.4 AGING, MITOCHONDRIA AND EXERCISE

Whether mitochondrial changes occur in skeletal muscle with aging is still highly controversial in the literature and continues to be studied (240). While numerous studies have documented deleterious changes of the organelles through a plethora of markers and functional

measures (28, 41, 81, 97, 123, 130, 150, 163, 172, 243, 263, 264, 280, 291), a competing body of evidence has found no differences often with similar measurements (60, 134, 150, 222). Moving forward, it remains imperative to match young and aged groups for physical activity levels when making comparisons as this may be a source of potential bias.

Equally debatable is the capacity of aged muscle and mitochondria to adapt to exercise training paradigms. In recent reviews we have compared and discussed a number of studies that either used the same relative or absolute training intensity between young and aged groups (37, 103). We concluded that mitochondrial adaptations can occur with a sufficient duration and intensity in aged muscle, albeit the magnitude of adaptation can vary. It is possible that for observation of mitochondrial adaptations a longer time frame for training may be required in aged muscle (220, 288). This extension of time may be necessary when the muscle milieu possesses decreased signaling (162) and transcriptional drive towards biogenesis factors (see section 2.0), requires large renovation due to the starting presence of dysfunctional organelles, or needs to improve the capacity for turnover through mitophagy (discussed in section 3.0).

2.0 MITOCHONDRIAL BIOGENESIS

Mitochondria are semi-autonomous organelles that are found in nearly all body tissues. They are enriched in tissues that have high energy demands, such as skeletal muscle, heart, brain, kidneys and brown adipose tissue. Mitochondria are composed of greater than 1100 proteins (34), and the vast majority of gene products need to be derived from nuclear DNA. However, 13 critical subunits for the electron transport chain are encoded by the maternally-inherited mtDNA. Possession of its own genetic material is a highly unique feature to mitochondria, likely a remnant of its endosymbiotic origins (173), and makes the study of this organelle fascinating.

In order to expand the population of organelles in skeletal muscle, numerous cellular events must transpire. This includes upregulation of gene expression, translation of mRNAs, import of proteins into the mitochondria, transcription of mtDNA, fusion of existing organelles to create a larger reticular network and fission for recycling of organelles that are no longer suitable to support muscular demands. Mitochondrial biogenesis is considered to occur in response to a stimulus, such as exercise, when 1) there is an enhanced requirement to generate ATP and/or 2) mitochondrial mass/volume has increased through fusion and/or enlargement of the reticulum through the import/generation of new mitochondrial constituents. Physiological enhancement of mitochondrial mass in skeletal muscle is advantageous as it will bolster endurance capacity, spare carbohydrates, reduce acidosis and increase lipid utilization.

With aging, while controversial, numerous reports have found that organelle number, quality and morphology exhibit defects in skeletal muscle, potentially pointing to a shortcoming in the maintenance of these organelles (37, 103). Additionally, with the instigation of training, aged muscle may respond with a lower capacity, or require an extended time period to reach a similar magnitude as young subjects, suggesting an attenuation of the signaling mechanisms governing

organelle biogenesis (37, 103). The following review will cover notable aspects of mitochondrial biogenesis and provide commentary on organelle changes in aging muscle with an exercise perspective.

2.1 NUCLEAR GENE TRANSCRIPTION

Exploration of the transcriptional regulation of nuclear genes encoding mitochondrial proteins (NuGEMPs) has yielded interesting insight into how these organelles are maintained and fortified with exercise. For nuclear gene expression to occur, the integration between cellular signals, chromatin modifications, transcription factor recruitment to *cis*-acting elements, binding of coactivator proteins, and assembly of the RNA polymerase machinery are all necessary molecular events. This makes elucidating the regulation of nuclear-encoded mitochondrial genes a seemingly overwhelming challenge. Despite this complexity in the regulation of nuclear gene expression, numerous factors have been successfully identified which regulate NuGEMPs, and as molecular techniques continue to advance, more factors will certainly be defined.

Identification of the regulatory transcription factors that govern NuGEMP expression largely began after the characterization of the cytochrome c gene (254), an essential component of the electron transport chain. In the promoter region of cytochrome c, regulatory sequences were identified which bound a transcription factor that was termed nuclear respiratory factor-1 (NRF-1) (72). Thereafter, NRF-1 was found to have binding sites in the promoters of many other nuclear encoded mitochondrial genes, including subunits of the respiratory chain, ETC assembly factors, transcriptional regulators of mitochondrial DNA and components of the mitochondrial protein import machinery (253, 256). In addition to NRF-1, the family member NRF-2 (also known as GA-binding protein [GABP]), was recognized to control the expression of all nuclear-derived subunits of complex IV of the electron transport chain (212). A key discovery surrounding NRF-

1/2 NuGEMP regulation was that both of these transcription factors had binding elements in the promoter region of mitochondrial transcription factor A (Tfam), the principal transcription factor of mtDNA (287). The highlight of this discovery was that it provided a molecular link between nuclear gene expression and mitochondrial gene expression.

While NRF-1 and 2 are major contributors to the expression of NuGEMPs, many other transcription factors have also been demonstrated to participate in the process. Of note is the transcription factor family, estrogen-related receptors (ERRs). In particular, ERRα has been well studied to define its role in regulating mitochondrial genes (192). Indeed, ERRα can regulate the expression of components of the electron transport chain as well as mitofusin-2 (MFN-2) which regulates the fusion of the organelles (39), and this has often been found to be in a PGC-1α-dependent manner (discussed below).

The ubiquitously expressed transcription factors Sp1 and Yin Yang 1 (YY1) also exert functions to regulate NuGEMP expression. Interestingly, YY1 is not exclusively associated with upregulation of NuGEMPs, but rather can exert either stimulatory or inhibitory effects on transcription (254). This type of regulation likely arises from intracellular cues that are received and protein interactions on target promoters. Loss of YY1 specifically from skeletal muscle results in abnormal mitochondrial morphology and impaired function of the organelles (26), highlighting the important of this transcription factor to mitochondrial integrity.

A large breakthrough surrounding the expression of NuGEMPs, was the discovery of a transcriptional coactivator protein that could bind a variety of transcription factors to upregulate numerous genes involved in mitochondrial biogenesis (229). This coactivator protein is referred to as peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α). A vast amount of research has been devoted to PGC-1α since this coactivator can mediate enhancement

of mitochondrial biogenesis with exercise and has been found to be dysregulated in a variety of conditions, including sarcopenia.

2.2 PGC-1α AND MITOCHONDRIAL BIOGENESIS

PGC-1α was discovered through a yeast two-hybrid screen for cofactors of PPARγ in brown adipose tissue (229). PGC-1α was demonstrated to increase mitochondrial mass in response to numerous stimuli including cold, thyroid hormone administration as well as exercise (229). Investigation into the mechanisms through which PGC-1α mediates these effects on mitochondria yielded the discovery that it binds and coactivates almost all nuclear receptors and many other transcription factors (255). Indeed, PGC-1α possesses multiple LXXLL amino acid motifs to facilitate binding with nuclear receptors and other domains of the protein serve to interact with other transcription factors (55, 229). PGC-1α possesses no direct DNA binding activity, but when activated and associated with factors occupying promoters, it can potently upregulate the expression of a vast array of NuGEMPs (Fig. 4). Following binding of PGC-1α to transcription factors, chromatin modifying enzymes, such as histone acetyl transferases, are recruited to facilitate opening of the DNA followed by recruitment of the transcriptional initiation machinery (227). In skeletal muscle, NRF-1 and 2, ERRs, PPARs, and YY1 are among the nuclear receptors/transcription factors that are bound and coactivated by PGC-1α to promote expression of genes for mitochondrial biogenesis.

2.2.1 PGC-1α AND SKELETAL MUSCLE

PGC-1α exhibits enriched expression in tissues with high metabolic demand. In skeletal muscle, PGC-1α protein levels are greatest in type I fibres in comparison to type II, which correlates with the content of mitochondria found in these respective fibre types (156). Additionally, PGC-1α skeletal muscle overexpression elicits a shift in fibre types toward slower,

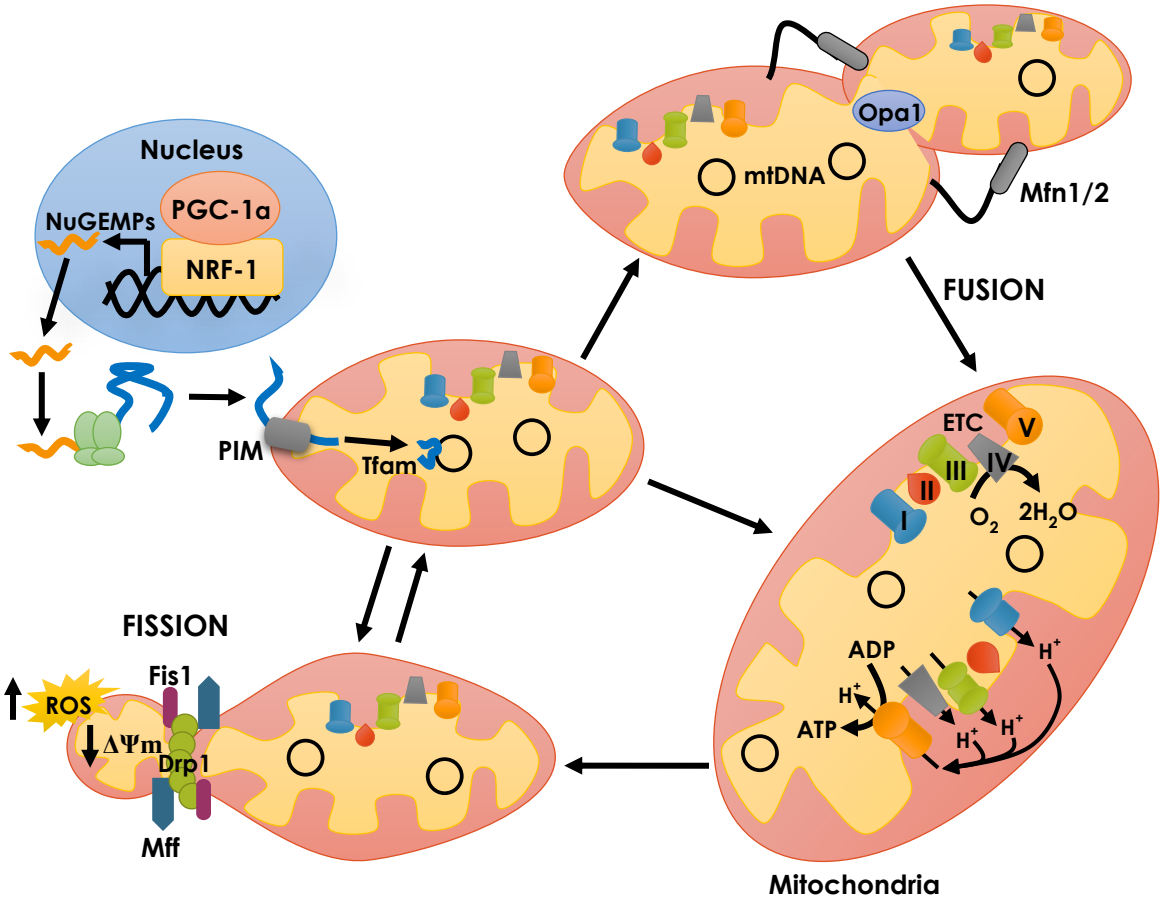

Fig. 4. Mitochondrial Biogenesis. The transcriptional coactivator PGC-1α can bind and upregulate the activity of a host of receptors/transcription factors. This increases the mRNA expression of a plethora of NuGEMPs which are transcribed then exported into the cytosol. These gene products will be translated by ribosomal machinery followed by import into the organelle through the PIM. For instance, entry of Tfam will permit transcription of mtDNA. Accretion of proteins inside the organelle along with fusion of neighboring mitochondria will increase the reticulum, creating a greater capacity for energy provision. The ETC passes electrons and pumps H^+ ions generating the $\Delta\Psi_m$, a source of potential energy. Oxygen will be consumed at complex IV and when H^+ ions pass down the concentration gradient through Complex V, free ADP can be phosphorylated to generate the energy rich molecule, ATP. In the event a portion of the organelle loses its $\Delta\Psi_m$ or emits greater ROS through inappropriate donation of electrons, this segment can be divided from the reticulum by fission proteins. PGC-1α; peroxisome proliferator-activated receptor γ coactivator-1α; Tfam; mitochondrial transcription factor A; ETC; electron transport chain; PIM; protein import machinery; NRF-1; nuclear respiratory factor-1; NuGEMPS; Nuclear Genes Encoding Mitochondrial Proteins; OPA1; optic atrophy 1; MFN-1/2; mitofusin-1/2; MFF; mitochondrial fission factor; DRP1; dynamin-related protein 1; FIS1; mitochondrial fission protein 1; $\Delta\Psi_m$; mitochondrial membrane potential; ROS; reactive oxygen species. Adapted from (37).

oxidative fibres, favoring greater fatigue resistance, as animals have improved endurance capacity and higher mitochondrial content (156). This suggests that PGC-1α governs a skeletal muscle program including the regulation of fibre type and mitochondria found within. Global knockout of PGC-1α does not result in a remarkable skeletal muscle fibre phenotype, likely due to hyperactivity brought about through altered neural function in these animals (157). Skeletal muscle-specific PGC-1α-null animals, do however exhibit a shift in muscle fibre phenotype towards more type II fibres (91). Furthermore, animals lacking PGC-1α in skeletal muscle present with reduced ability to exercise, lower mitochondrial content, impaired respiration and greater potential to release apoptotic proteins (3, 91, 285).

PGC-1α expression and activity is regulated at the transcriptional, subcellular and post-translational level in skeletal muscle. Transcriptional control of its gene expression is discussed in detail in section 2.1.2. PGC-1α protein surprisingly has been found to localize mainly in the cytosol (296). Upon receipt of an exercise stimulus, the protein can be phosphorylated by 5' adenosine monophosphate-activated protein kinase (AMPK), and translocate to the nucleus (118, 296). Additionally, deacetylation of PGC-1α by Sirtuin 1 (SirT1) assists with maximal transcriptional coactivation capabilities of the coactivator (35, 149, 200).

Some studies have challenged the requirement for PGC-1α in exercise-induced mitochondrial biogenesis (154, 244, 245). Often using knockout models, some studies have reported that mitochondrial improvements transpire with exercise even in the absence of PGC-1α. However, the presence of PGC-1α is necessary to confer the beneficial effects of training in middle-aged mice (153). A potential confounding variable for these interpretations may be how knockouts are generated. Disruption of certain exons may not obscure all forms of PGC-1α. Residual splice variants of PGC-1α may be left intact to perform auxiliary functions. A more

detailed discussion of PGC-1α isoforms can be found in section 2.2.4. With the advent of gene editing technology, it may now be possible to tailor specific deletions to PGC-1α, its isoforms and family members to uncover their specific roles or possible redundancies that may influence these contradictory observations.

2.2.2 TRANSCRIPTIONAL REGULATION OF THE PGC-1α GENE

With the importance of PGC-1α for the expression of a plethora of mitochondrial genes, it becomes important to consider what transcription factors and upstream signals regulate the expression of this key coactivator. The cell exerts many levels of control of the expression of cellular factors. Initially, whether or not a gene may be transcribed influences the mRNA abundance and ultimately the amount of protein in the cellular milieu.

Often investigators use mRNA levels to suggest changes in transcription. However, increased or decreased mRNA levels are not a direct indication of altered gene transcription and should be interpreted with caution. While mRNA levels are partly regulated by transcription, mRNA is also subject to the influence of stability or decay factors that can ultimately change the total amount of mRNA independent of changes in gene transcription (98). Thus, to conclusively determine if transcription is altered, a method which evaluates nuclear activity is warranted, such as a nuclear run-on assay or a promoter-reporter assay (e.g. luciferase constructs).

Acute exercise results in the turnover of many metabolites and molecules, the production of reactive oxygen species, mechanical muscle stretch and the activation of numerous signaling pathways. In an effort to determine if any of these cellular changes elicit effects on PGC-1α transcription, researchers have harnessed molecular biology tools to gain deeper insight into the regulation of PGC-1α with acute exercise. In the 2000s, many studies were designed to examine the influence of acute exercise or exercise signals, on the regulation of the PGC-1α promoter.

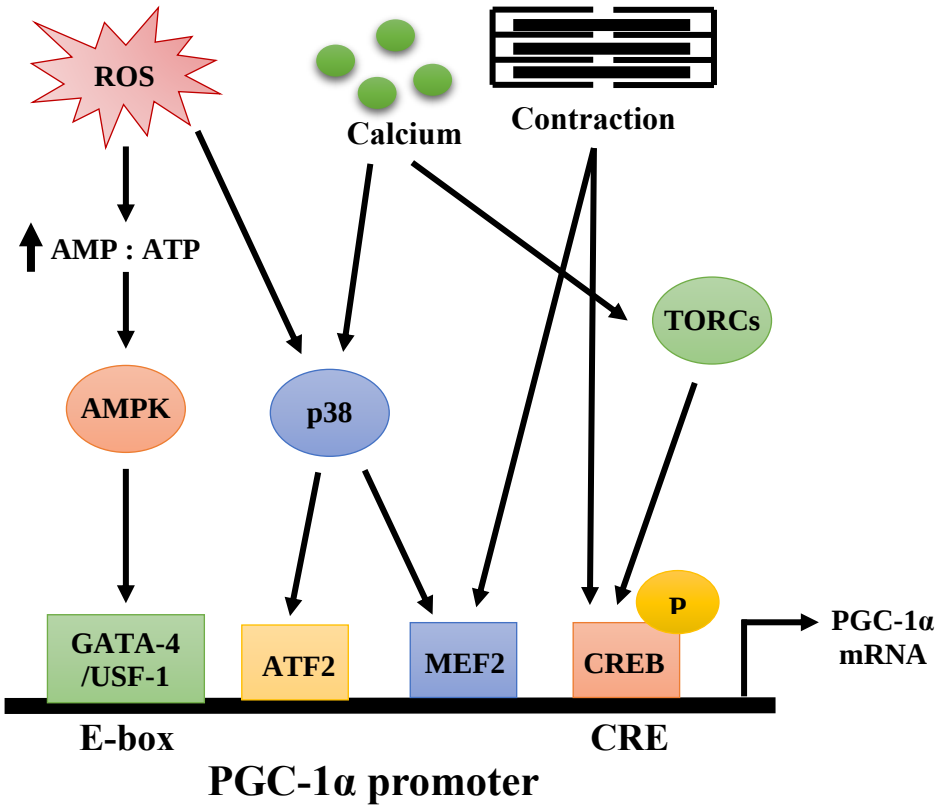

Fig. 5. Schematic of factors that regulate the PGC-1α promoter. Numerous have been identified that promote expression of the PGC-1α gene. In response to contractile activity, cycling of ATP, calcium and ROS production are characterized events. Enhancement in ROS production, triggering a shift in the ratio AMP:ATP leads to action of AMPK. AMPK will stimulate the binding of factors GATA-4 and USF-1 to an E-box motif to stimulate PGC-1α expression. ROS ad calcium have been identified to elicit activation of the kinase p38, which promotes the activity of the transcription factors ATF2 and MEF2. Additionally, calcium regulates the action of TORCs which fortify the binding of CREB to CRE motifs. Contractile activity of muscle has also been shown to induce MEF2 and CREB binding to the promoter area as mutation of these sites blunts the effects of contraction-induced upregulation of PGC-1α. PGC-1α; peroxisome proliferator activated receptor gamma coactivator 1 alpha; ROS; reactive oxygen species; AMPK; 5' adenosine monophosphate-activated protein kinase; GATA-4; GATA binding protein 4; USF-1; upstream stimulatory factor 1; ATF2; activating transcription factor 2; MEF2; myocyte-enhancer factor 2; CREB; cAMP response element binding protein; E-box; enhancer box; CRE; cAMP response element; TORC; transducer of regulated CREB-binding proteins.

In 2003, Pilegaard, Neufer and Saltin demonstrated in humans that PGC-1α transcription was stimulated following an acute bout of exercise and peaked at 2 hours during the recovery period (225). The subjects had performed one-legged training exercise for four weeks prior to the acute bout and interestingly, greater activation of PGC-1α transcription was noted in the trained leg compared to the untrained leg. This is notable because the training-induced adaptation resulted in a lower workload placed on the trained leg, versus the untrained leg.

In the same year, Spiegelman's group reported that PGC-1α gene expression is subject to an autoregulatory loop, with calcium being a strong signaling candidate of this process (92). This work highlighted the fact that Ca^{2+}/calmodulin-dependent protein kinase (CAMK) IV, calcineurin (CnA) and myocyte-enhancer factor 2 (MEF2) were all factors involved in the transcriptional regulation of the PGC-1α promoter. Furthermore, PGC-1α protein could coactivate the MEF2 family of transcription factors, including when they resided on the PGC-1α promoter, creating a self-regulatory loop. These observations also fortified findings by Wu et al. describing that CAMK IV, an effector of Ca^{2+} signaling, was able to positively regulate the PGC-1α promoter in myotubes (297). An unbiased cDNA screen further confirmed a role for calcium signaling in the regulation of PGC-1α gene expression (300). Binding sites for cAMP response element binding (CREB) are located on the promoter of PGC-1α and it was discovered that CREB can be coactivated by the family of transducers of regulated CREB (TORCs) in muscle cells (300). This coactivation of CREB likely occurs through calcium and/or AMP-mediated activation of TORCs.

Corroborating previous findings, bioluminescence real-time imaging of mouse TA muscle, demonstrated that low-frequency nerve stimulation produced a 3-fold increase in PGC-1α promoter activity (6). This effect was eliminated when MEF2 or CRE sites (which bind MEF2 and CREB transcription factors, respectively) were mutated on the PGC-1α promoter. Subsequent

work by the same group also identified that activating transcription factor 2 (ATF2) or histone deacetylase 5 (HDAC5) mutation blocked the effect on contractile activity-induced PGC-1α promoter activity, suggesting these factors are also involved in PGC-1α gene expression (4, 5).

The mitogen-activated kinase p38 (p38) has long been noted to become phosphorylated with acute exercise (66, 161). Interestingly, p38 is capable of increasing PGC-1α promoter activity through targeting transcription factors for phosphorylation, such as ATF2 and MEF2 (5). Furthermore, p38 appears to be regulated in response to calcium by the upstream factor CAMK II (295). p38 is also capable of targeting PGC-1α protein directly for phosphorylation in the presence of inflammatory cytokines (228). This post-translational modification appears to increase the stability of PGC-1α protein which would assist in coactivation of its own gene as well as other targets. However, whether p38-mediated PGC-1α protein phosphorylation with muscle contraction exerts a relevant role in the transcription of PGC-1α, has yet to be elucidated.

Adding to the body of evidence surrounding PGC-1α promoter regulation with exercise, work by Irrcher et al. demonstrated *in vitro* that exercise-induced signals, such as ROS or AMPK, increased PGC-1α promoter activity (115, 116). It was identified that through an enhancer box (E-box) motif, GATA binding protein 4 (GATA-4) or upstream stimulatory factor-1 (USF-1) were transcription factors that could respond to these signals to increase human PGC-1α promoter activity, providing more molecular details on the regulation of this key coactivator in the context of contractile activity-induced signaling towards mitochondrial biogenesis. Elaboration of the influence of ROS, AMPK, p38 and Ca^{2+} has come from work which has examined these signaling factors in the presence and absence of inhibitors using a PGC-1α GAL4-DNA binding assay (305). Indeed, many of these pathways appear to rapidly converge on PGC-1α promoter activity and form redundant pathways in an effort to preserve the increase in PGC-1α during muscle contraction.

Taken together, this evidence largely points to calcium as a key regulator of PGC-1α gene expression, particularly in skeletal muscle with contractile activity. Calcium is necessary for muscle contraction and performance as well as maintenance of mitochondria. Thus, for calcium to be a regulatory molecule of the pivotal coactivator for mitochondrial, metabolic and skeletal muscle genes, it provides a common link between contraction-induced signals and maintenance or adaptation of metabolic muscle components, including mitochondria.

2.2.3 EFFECTS OF AGING ON PGC-1α

Numerous studies have correlated a decline in PGC-1α expression (mRNA or protein) with poor muscle and mitochondrial factors in aging skeletal muscle (41, 58, 112, 124, 142, 163, 249). Indeed, in elderly subjects stratified according to function based on a battery of measures, those classified as high-functioning demonstrated greater levels of PGC-1α compared to the low-functioning group (124). However, both high- and low-functioning aged groups had lower quantities of PGC-1α when compared to younger counterparts.

Integral to the expression of PGC-1α are the upstream signaling inputs that drive its transcriptional and post-translational regulation. Signaling factors towards PGC-1α, such as AMPK, p38 and CAMK have been found to be lower basally in aged muscle (162, 236). Acute exercise is known to potently evoke enhanced phosphorylation of these factors triggering their downstream cascades. However, the response of these kinases in aged muscle to acute exercise is blunted, suggesting an age-related deterioration of signaling towards PGC-1α expression (162). It would be interesting to determine whether chronic exercise could rectify the signaling towards and expression of PGC-1α in aged muscle. Should this occur, would contraction-mediated induction of PGC-1α in aged muscle be able to evoke a turnaround in the function and quality of the mitochondria?

As documented in chapter four, we examined PGC-1α promoter activity in young and aged skeletal with a single bout of *in situ* contractile activity (38). Basally, aged muscle harbored lower PGC-1α promoter activity compared to young resting muscle. However, with acute contractile activity, both age groups were able to upregulate PGC-1α promoter activity, suggesting that aged muscle retains the capacity to drive PGC-1α expression in a contraction-dependent manner. Furthermore, chronic contractile activity, a model of exercise training, enhanced PGC-1α mRNA levels in aged muscle so they were similar in expression to that of young control muscle. This suggests that chronic exercise may restore transcription of the PGC-1α gene, however further experiments are required to determine this supposition.

2.2.4 SPLICE VARIANTS OF PGC-1α

A larger picture surrounding PGC-1α has begun to emerge through the identification of an alternative upstream promoter as well as alternative splicing of mRNA transcripts which generate distinct, functional protein products. Even early on in the investigation of PGC-1α there were reports of smaller variants (16, 70) which have only recently begun to be described in more detail.

Initial studies from independent laboratories described two variants for PGC-1α, denoted as PGC-1α-b and PGC-1α-c in murine skeletal muscle which were synthesized from an alternative upstream promoter and the use of an alternative first exon (48, 187, 188, 303). The original PGC-1α was labelled as PGC-1α-a or PGC-1α1, depending on the scientific group. These variants of PGC-1α were found to make functional protein that could transcriptionally coactivate gene expression *in vitro* and *in vivo* (187, 188). Furthermore, these two identified variants were found most abundantly in metabolic tissues such as skeletal and cardiac muscle, while seemingly undetectable in other tissues. The alternative promoter which gave rise to these variants was found to be located ~14kb upstream from the canonical PGC-1α promoter (now referred to as the

proximal promoter) (303). Gene expression from this new upstream alternative promoter was coupled to the alternative exon1, known as exon1b, while the rest of the transcript was identical to the original PGC-1α-a. This alternative exon1b shortened the transcript and the translated protein at the N-terminal which gave rise to PGC-1α-b (51, 175). Alternative transcript splicing from the upstream promoter also occurred producing PGC-1α-c, which was further shortened at the N-terminus, however only by a few amino acids. These initially described variants were found to positively respond to aerobic exercise and to β-adrenergic stimulation in skeletal muscle (187). Furthermore, PGC-1α-b and PGC-1α-c exhibited a greater response to either of these stimuli than the traditional PGC-1α-a and accounted for the majority of the increase when total transcript change was calculated.

Examination of regulatory factors that controlled the alternative promoter revealed similarity to the proximal promoter. The transcription factors myogenic differentiation 1 (MyoD) and myogenic regulatory factor 4 (MRF4) were able to increase alternative promoter activity through an E-box motif (303). Additionally, overexpression of CAMK IV, CnA or mitogen-activated protein kinase 6 (MKK6) recruited CREB to bind to a CRE-response element and increase promoter activity. Thus, it appears that similar signals, such as calcium, may regulate the activity of both promoters.

Early reports of a very short protein variant of PGC-1α that was induced with exercise, has been now identified as the N-terminal (NT) variants of PGC-1α (NT-PGC1α) (16). These variants are approximately 30-37kDa and are generated due to a premature stop codon between exons 6 and 7, excluding the central and C-terminal portions. There are three described variants of the NTs with two arising from the alternative promoter (NT-PGC-1α-b and NT-PGC-1α-c) and NT-PGC-1α-a transcribed from the proximal promoter (175). The generation of these smaller proteins

excludes many motifs including the nuclear localization signal. Indeed, imaging has revealed a vastly cytosolic presence of the truncated variant in skeletal muscle (261). However, NT-PGC-1α-a appears to translocate to the nucleus upon activation of protein kinase A (PKA), a kinase known to phosphorylate the PGC-1α (42). Neither contraction of muscle fibres nor AMPK activation by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) did not induce any alterations in the cellular localization of these shortened forms of PGC-1α (261).

A recent study from the laboratory which discovered the original PGC-1α described a truncated variant of the protein, PGC-1α4 (identical to NT-PGC-1α-b) which was responsible for the adaptations associated with resistance exercise (246). Indeed, the authors described muscle hypertrophy through the ability of PGC-1α4 to induce insulin-like growth factor 1 (IGF-1) and repress myostatin. However, this result has not been entirely reproduced. Further work will therefore be required to accurately determine if the variants of PGC-1α are able to confer specific exercise-induced adaptations. Moreover, whether there is an age-related alteration in the expression or activity of the variants remains to be examined.

2.3 PROTEIN IMPORT INTO MITOCHONDRIA

Most of the gene products for mitochondria are derived from nuclear DNA and the resulting protein products are synthesized in the cytoplasm. This necessitates the import of these proteins into the appropriate compartment or membrane of the organelle. To achieve this feat, membrane import channels exist on the outer and inner mitochondrial membranes, termed translocases of the outer membrane (TOM) and translocases of the inner membrane (TIM), respectively. Proteins that require entry to the organelle typically contain a positively charged N-terminal mitochondrial targeting sequence that guides them to the organelle with the aid of chaperone proteins. Incorporation of nuclear-derived mitochondrial proteins is considered a

valuable indicator of mitochondrial biogenesis (102). With dysfunctional organelles arising in aged muscle, it suggests that perhaps protein import has become defective. Examination of protein import capacity from aged skeletal muscle as well as heart tissue, have noted no defects in the movement of proteins into the organelle (52, 108, 163).

2.4 FUSION AND FISSION

The form of a biological entity is often suggestive of the function it is able to perform. In the case of mitochondria, they are constantly changing shape through fusion events, which connect areas, while fission processes serve to sever contact between organelles (68, 242). These changes in form influence the ability of the mitochondria to supply ATP, use substrates, release pro-apoptotic factors and determine whether they may be removed through mitophagy (155, 284).

Mitochondrial fusion is regulated through the factors mitofusin-1/2 (MFN1/2) to tether the outer membranes together (17, 46, 71) and optic atrophy 1 (OPA1) is in charge of mediating linkage of the inner mitochondrial membrane and cristae structure (49, 211). Pruning of the mitochondrial reticulum occurs through the regulation of fission. Dynamin-related protein 1 (DRP1) will encircle the area of the organelle through binding to the fission factors, mitochondrial fission 1 protein (FIS1), mitochondrial dynamics protein of 49 kDa (MID49), mitochondrial dynamics protein of 51 kDa (MID51) and mitochondrial fission factor (MFF) (166, 214, 267). This wrapping of DRP1 around the organelle will constrict and pinch the organelle from the rest of the reticulum, creating a smaller, unconnected piece.

Maintaining equilibrium between fusion and fission is necessary, as an imbalance creates an unwelcome environment in skeletal muscle. For example, skeletal muscle-specific loss of OPA1 (278), overexpression of the fission factors FIS1 or DRP1 (242) or loss of MFN2 (257) all exhibit features of precocious aging including fragmented mitochondria, dysfunction of remaining

organelles and muscle atrophy. With aging, either decreases in the expression of both groups of mitochondrial morphology factors or a skewed balance toward the favored expression of fission regulators have been documented (112, 114, 124, 207, 306). These data harmonize with the observation that mitochondria appear more fragmented in aged muscle (114, 163). Thus, engaging in strategies, like exercise, that promote a balanced expression of these regulators is an attractive option. In young muscle, exercise increases fusion regulators, through PGC-1α (17, 268), and elongated organelle structures are a notable feature following training paradigms (114). These observations have been extended to also occur in aging muscle, as elevations in the expression of factors promoting fusion have also been noted following training coincident with reduced mitochondrial fragmentation (12). These changes in mitochondrial morphology with aging have been described to be dependent on the expression of PGC-1α (89).

2.5 EPIGENETICS

Epigenetics refers to changes in gene expression that occur without alteration to the inherent sequence of the DNA. It is widely recognized that epigenetic variations influence development and that epigenetic marks have been demonstrated to exist in tissue-specific patterns. Evidence has detailed that epigenetic modifications are dynamic in somatic tissues in response to varying stimuli, such as exercise or composition of the diet (139). DNA methylation and histone modifications are well-described to alter gene expression through either condensing or unwrapping DNA. Epigenetic modifications have mainly been attributed to the nuclear genome, yet recent evidence has demonstrated that methylation may also influence mitochondrial DNA, albeit this remains quite controversial. Mitochondrial DNA lacks protective histone proteins, however it has been described that Tfam may bind and protect mtDNA forming the nucleoid (147, 148). It

therefore may conceivable that post-translational modifications to Tfam may be considered an epigenetic modification.

2.5.1 METHYLATION OF GENOMIC DNA

Methylation of DNA is a covalent modification that occurs on the fifth carbon of cytosines that are part of CpG dinucleotides (5mC). Methylation of the DNA is catalyzed by DNA methyltransferases (DNMTs) using the universal methyl donor *S*-adenosyl-$_{L}$-methionine (SAM). The 5mC modification of DNA is typically associated with gene silencing, particularly if the methylation occurs in gene promoter regions (Figure 6). Recently, it has been described that 5mC can be hydroxylated by ten-eleven translocase 1 (Tet1) to form 5-hydroxymethylcytosine (5hmC) (88). Like 5mC, the 5hmC epigenetic mark is a stable modification. 5hmC has been correlated with transcriptional activation and also as a precursor step in the demethylation of the DNA in conjunction with the base-excision repair pathway (88). The differences on the effects of gene transcription by 5mC and 5hmC may be related to where in the gene segment the modifications occur (i.e. promoter vs. gene body). Currently, studies are now attempting quantification of both DNA methylation events for further insight into transcriptional regulation.

In the approximately 3 billion base pair human genome, 56 million CpG sites have been identified and are obviously an underrepresented sequence. Interestingly, these underrepresented CpG sites have been found in clusters referred to as CpG islands (CGIs). An area of at least 550bp is considered a CGI if the ratio of observed CpG/expected CpG is greater than 0.65. CGIs have been found in approximately 70% of human promoter regions, however, interestingly, these CGIs are largely unmethylated (57).

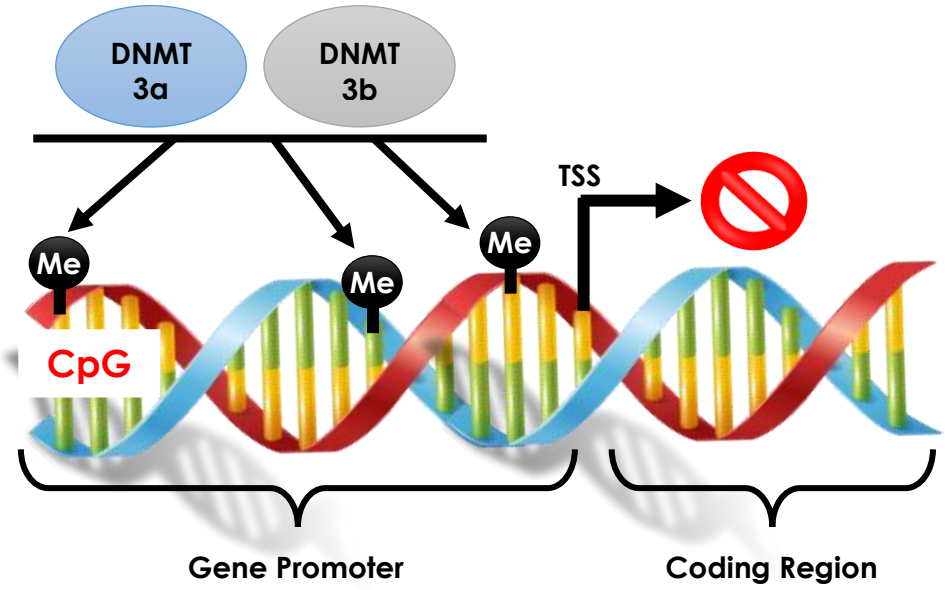

Fig. 6. Schematic of DNA methylation. Cytosine residues that precede guanine nucleotides can be methylated at the 5' position. DNA methyltransferases facilitate this epigenetic modification using the methyl group from the universal donor, S-Adenosyl methionine. Often when promoter regions are methylated, transcription silencing of the target gene results. This modification to DNA is reversible. DNMT; DNA methyltransferase; TSS; transcription start site; Me; Methyl group.

2.5.2 DNA METHYLATION, SKELETAL MUSCLE AND EXERCISE

In skeletal muscle, DNA methylation is involved in the development of the tissue by regulating the expression of myogenic genes (15). In mature skeletal muscle, recent work has begun to highlight a role for exercise in altering methylation patterns which correlates with changes in gene expression. For example, immediately following an acute bout of aerobic exercise, methylation of the promoter regions of key metabolic genes, such as PGC-1α, Tfam, Glut4 and pyruvate dehydrogenase kinase 4 (PDK4), were reduced (20). This decrease in methylation is negatively correlated with an increase in mRNA expression in the subsequent recovery period. A recent study went a step further and examined 5mC and 5hmC modifications on the proximal and alternative PGC-1α promoters after approximately 1 hour of intensifying rotorod exercise in mice (164). The authors observed that their exercise protocol elicited significant increases in the PGC-1α-b and PGC-1α-c mRNA variants, but not the traditional PGC-1α-a variant. Examination of CpG sites up to -1000bp from the transcription start site of each promoter revealed no alterations in 5mC or 5hmC following exercise. However, basally there was greater 5hmC modification associated with the traditional PGC-1α promoter. This observation correlated to higher mRNA levels of PGC-1α-a in sedentary animals compared to the levels of PGC-1α-b or PGC-1α-c. The lack of changes noted with exercise in this study may be due to the mode and duration of exercise or that samples were taken one hour following the exercise and transient changes in methylation were missed. Taken together, these findings highlight a dynamic, transient role for methylation to control gene expression in skeletal muscle immediately following acute exercise and the subsequent upregulation of mRNA expression (Fig. 7).

With regular exercise training, adaptations in muscle occur due to the accumulation of new proteins facilitated, in part, by consistent upregulation of gene expression. Furthermore, gene

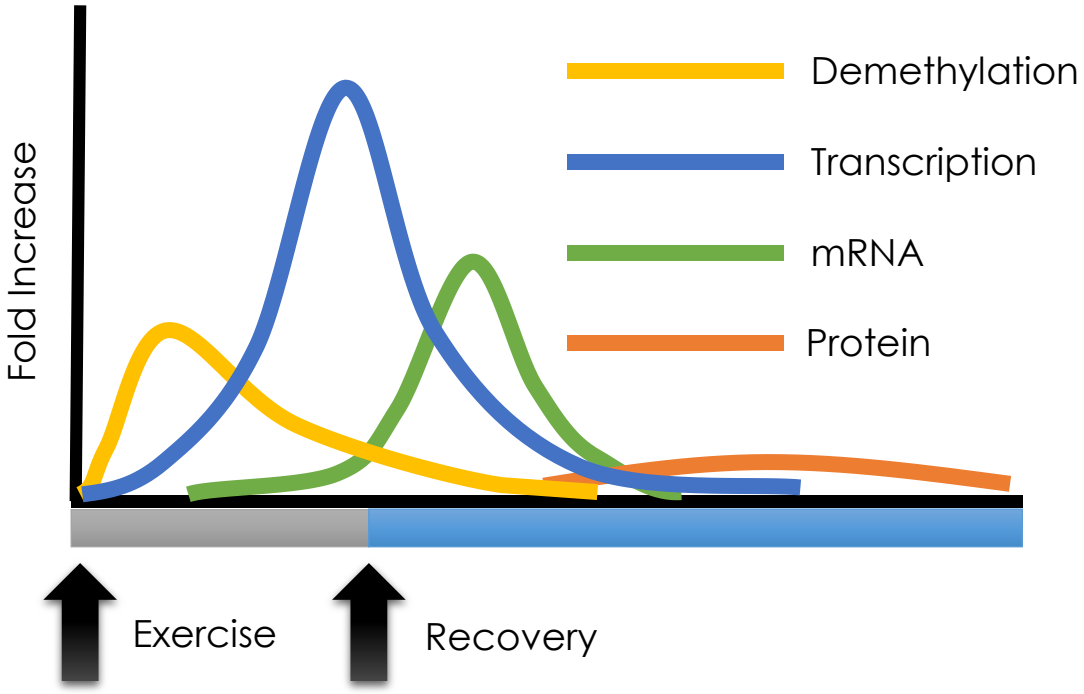

Fig. 7. Responses to an acute bout of exercise and recovery. An acute bout of exercise stimulates the removal of methyl groups from promoters of metabolic and mitochondrial genes. This event precedes and is permissive for a large increase in the transcriptional response. With enhanced transcription, gene products in the form of mRNA will be generated. mRNA will be subject to the influence of stability and decay factors, thus the magnitude of transcript is often lower than the transcriptional response. Stabilized mRNA can be translated into protein in the cytosol. With a single bout of exercise, this change in protein will be small. However, with repeated sessions of exercise, the amount of protein can start to accumulate to elicit measureable changes in mitochondrial content and quality.

expression profiles have been shown to shift after exercise training, potentially indicating a role for alterations in the methylation profile of DNA. Indeed, examination of genome-wide promoter methylation after 4 weeks of treadmill training in mice revealed differential methylation patterns compared to sedentary controls (131). Notably, genes that corresponded with muscle growth and differentiation as well as metabolism exhibited decreased methylation and enriched mRNA expression. In another study, a six-month aerobic exercise intervention was implemented in subjects with or without a family history of type 2 diabetes to determine if exercise could alter methylation patterns in these cohorts (204). In both groups, exercise training was positively correlated with many metabolic genes that exhibited reduced methylation patterns. In another study, 3 months of exercise training was undertaken in previously sedentary individuals to examine changes in methylation patterns of skeletal muscle (158). An important advantage of this study was that supervised one-legged knee extension exercise was used, providing an intra-individual control of the non-exercised leg. This assisted in eliminating the confounding variables of diet and environment which can affect methylation profiles. The researchers identified that differences in methylation occurred predominantly in the enhancer, gene body and intragenic regions as opposed to CGIs and promoter regions. Regions that had altered methylation profiles were consistent with genes involved in muscle remodeling and energetics. Furthermore, MEF2 motifs were consistently found in the regions that harbored alterations in methylation status which is interesting as MEF2 is a *bonafide* regulator of PGC-1α transcription. Altogether, these studies suggests that engagement in exercise training remodels the methylation profile of the DNA to facilitate gene expression associated with muscle adaptation to exercise. It would be interesting in the future to differentiate between the 5mC and 5hmC patterns after exercise training to gain more specific insight into the epigenetic changes with training. It will also be interesting to determine what signals (i.e. energy

turnover, calcium, metabolites or reactive oxygen species) mediate the changes in DNA methylation and by what molecular mechanisms DNA methylation/demethylation is achieved with exercise (i.e. DNMTs).

2.6 SUMMARY

Mitochondrial biogenesis is essential to support skeletal muscle basally and with exercise-induced metabolic demands. Organelle content and quality are regulated with fastidious care in a multistep pathway (Fig. 4). At the heart of the proper execution of mitochondrial biogenesis is the transcriptional coactivator, PGC-1α. In young, healthy muscle PGC-1α is an important player to mediate the beneficial adaptations wrought by repeated bouts of contractile stimuli. Unfortunately with aging, declines in this coactivator are evident, accompanied by mitochondrial derangements and muscle atrophy. Therefore, restoration of the expression of this coactivator presents as an attractive opportunity to assist in rejuvenation of aging muscle and mitochondria.

3.0 AUTOPHAGY AND MITOPHAGY IN SKELETAL MUSCLE

The maintenance of a healthy pool of mitochondria in skeletal muscle is not only dictated by the degree of biogenesis, but also by the pruning of the mitochondrial reticulum to degrade old, superfluous or damaged organelles. Indeed, a fine-tuned balance between the generation and removal of mitochondria are key events in determining mitochondrial homeostasis (223). The process of specifically removing mitochondria from the cellular milieu is termed mitophagy. Mitophagy is a selective form of the broader process of the evolutionarily-conserved pathway, autophagy. In brief, a stimulus for mitochondrial removal, such as diminished membrane potential or increased ROS emission, will trigger the identification and molecular labelling of this portion of the organelle for removal. Concurrently, a cascade of autophagy-related (ATG) proteins work to generate a *de novo* structure called the autophagosome. This double membrane vesicle will be guided to enwrap the defective organelle and once fully engulfed, will then be transported to the lysosome. At the lysosome, the autophagosome, with its mitochondrial cargo inside, will fuse with the lysosome and release the contents into the acidic lumen of the lysosome. Herein, the resident pH-dependent hydrolases can breakdown the components of the mitochondria, including proteins, DNA and lipid membranes to basic cellular building materials. Now liberated, these resources can then be ejected from the lysosome for their use as cellular building blocks or energy substrates and also to serve as negative signaling feedback for the suppression of autophagy/mitophagy.

When this pathway of intracellular recycling is functioning correctly, alongside mitochondrial biogenesis, organelle homeostasis can be achieved. However, interruption of the normal function of autophagy/mitophagy in skeletal muscle can lead to features of premature aging and muscle disease (36, 177, 233, 299). Furthermore, continuing debate surrounds how autophagy/mitophagy may change with aging. Prominent notions in the literature express that autophagy is

downregulated with aging (93, 181, 198, 247), however lack of conclusive evidence has been available for skeletal muscle tissue to firmly support this concept. Indeed, these conclusions are often based on data extrapolated from lower organisms (181), non-muscle tissue (277), genetic models or *in vitro* work that lacks a physiological context. Thus, investigation into the mechanisms which govern mitochondrial turnover through mitophagy in the context of exercise and aging within skeletal muscle are important to enhance our understanding of mitochondrial homeostasis and muscle health.

Three types of autophagy have been described to occur. The most commonly studied is referred to as macroautophagy, and this process targets intracellular organelles, bulk portions of the cytosol and protein aggregates through encapsulation of material in the newly generated autophagosome. Microautophagy has been observed when small cellular components that require degradation directly indent through the lysosomal membrane with no carriers (185). The last form, which is exclusive to mammals, is chaperone-mediated autophagy (CMA). This constitutes the selective lysosomal degradation of proteins that harbor a KFERQ-motif that targets them to the lysosome through the chaperone protein HSC70 and binding to the LAMP-2A receptor on the lysosomal surface (132). Macroautophagy rules the majority of cellular components in their fate for lysosomal degradation, and hereafter will be referred to as autophagy and will remain the focus of this discussion.

3.1 PATHWAY FOR AUTOPHAGY

To understand the removal of mitochondria, a review of upstream events that govern the formation and degradation of the double-membrane autophagosome are key. Core autophagy components have been demonstrated to be necessary for selective forms of autophagy. Indeed, the presence of the autophagosome is often considered the hallmark of autophagy. Autophagosomes

are created in the cell through the action of numerous protein complexes and the donation of a portion of membrane. In an effort to characterize the pathway, autophagy has been divided into stages including 1) induction 2) nucleation, 3) elongation, 4) closure/maturation, 5) fusion and 6) degradation. While the actions of these steps are known to occur in the process of autophagy, the temporal order of the events does garner some debate and often has been determined through lower organisms, mitotic tissue, knockout models and/or cell culture systems. Thus, exact extrapolation of all events to post-mitotic mammalian skeletal muscle has not been fully documented, yet the stages detailed in other tissues and organisms provide a framework of the events that likely govern autophagy/mitophagy in this tissue.

3.1.1 INDUCTION

Often considered a master regulator of autophagy, the serine/threonine unc-51-like kinase (ULK1) has been positioned as the most upstream regulator (281). ULK1 exists in a complex with FIP200 (94), ATG13 (75, 106) and ATG101 (107, 184) for protein complex stabilization, maintenance of kinase activity and subcellular localization. Each of the proteins in the induction complex is subject to post-translational modifications, such as phosphorylation, that can either advance or constrain autophagy in the cell. Activation of the ULK1 complex is largely regulated by two potent kinases that sense changes in nutrient and energy status, mechanistic target of rapamycin complex 1 (mTORC1) and AMPK, respectively. These three kinases, mTORC1, AMPK and ULK1, have all been documented to be capable of phosphorylating the others to regulate autophagy (18, 63, 67, 136, 152, 165, 260). For example, under conditions with ample nutrients, mTORC1 is active and localized on the cytosolic side of the lysosomal membrane (143, 250) where it can phosphorylate target residues of ULK1, thus suppressing autophagy. Conversely, when energy levels (ATP) are low in the cell, AMPK becomes active. AMPK will phosphorylate

and inhibit mTORC1, thus releasing mTORC1 inhibition on ULK1. Additionally, AMPK can directly couple with ULK1 and phosphorylate this protein to activate the induction complex, leading to the initiation of autophagy. This entwined relationship suggests that the cell has multiple regulators to interpret and fine-tune cues to either promote or repress autophagy initiation through the ULK1 complex. This regulation may serve to discriminate between bulk autophagy that typically occurs during starvation or fasting periods, versus selective autophagy, like mitophagy. However, more investigation is necessary to understand the role of ULK1 and downstream autophagy events.

3.1.2 NUCLEATION

Nucleation is a critical step in the formation of the autophagosome (1). Nucleation succeeds the induction step and involves the activated ULK1 complex acting on the downstream protein complex, class III PI3K Complex I (PI3KC3-C1) (248). Activation of this nucleation complex generates an autophagy-specific pool of phosphatidylinositol-3-phosphate [PI(3)P] at the site of phagophore assembly (13). The phagophore or isolation membrane, is the precursor structure that ultimately develops into the autophagosomal membrane. The nucleation of the phagophore begins with a donated portion of membrane whose origin remains under current debate. The generated pool of PI(3)P will recruit necessary factors that will translocate to the nascent membrane and assist in further nucleation (13, 226). The PI3KC3-C1 protein complex consists of many subunits including the lipid kinase VPS34, VPS15/p150, Beclin 1, ATG14 (135) and activating molecule in beclin-1-regulated autophagy (AMBRA1).

The activated ULK1 complex is capable of phosphorylating VPS34 (248), Beclin 1 (218, 248) as well as AMBRA1 (21). These events will increased the lipid kinase activity of VPS34 to generate PI(3)P (248), stimulate BECLIN 1 (218) and liberate the nucleation complex from the

cytoskeletal network (21), respectively. In yeast, the spatial location for nucleation is well characterized as the pre-autophagosomal structure (PAS), however in mammals a defined spatial region for autophagosome biogenesis is less clear and appears to transpire at many sites in cells. It has been considered that nucleation may take part around the endoplasmic reticulum (ER) at areas called the omegasome (13, 226), which are often enriched for PI(3)P. Further studies suggest that junction points between two organelles, such as ER-mitochondria (27), ER-Golgi (78) or possibly ER-plasma membrane (197) contact sites, may also serve as areas for phagophore nucleation. Very recently, work has demonstrated that nascent phagophores are generated from recycling endosomes (230).

Also integral to the nucleation of the phagophore is the sole transmembrane protein in autophagy, ATG9. ATG9 appears to reside in vesicles and upon stimulation of induction and nucleation events, these vesicles may donate lipids for phagophore expansion (174, 213).

3.1.3 ELONGATION

Once the stage is prepared with the nascent phagophore membrane and the milieu of PI(3)P, expansion of the cup-shaped membrane must occur to form a comprehensive structure. To achieve this, two ubiquitin-like conjugation events occur that serve to grow the lipid membrane. The first series of conjugation involves ATG5, ATG12 and ATG16L1 (190) and this event has been described to participate in the second conjugation event. Briefly, ATG12 is conjugated through a covalent bond to ATG5 due to the actions of ATG7 and ATG10, which act in a similar fashion to ubiquitin E1-activating and E2-conjugating enzymes, respectively. Upon the pairing of ATG12 to ATG5, this is permissive for the reversible binding of ATG16L1. The ATG12–ATG5-ATG16L1 complex can then dimerize with itself to exert its role for the second conjugation system involved

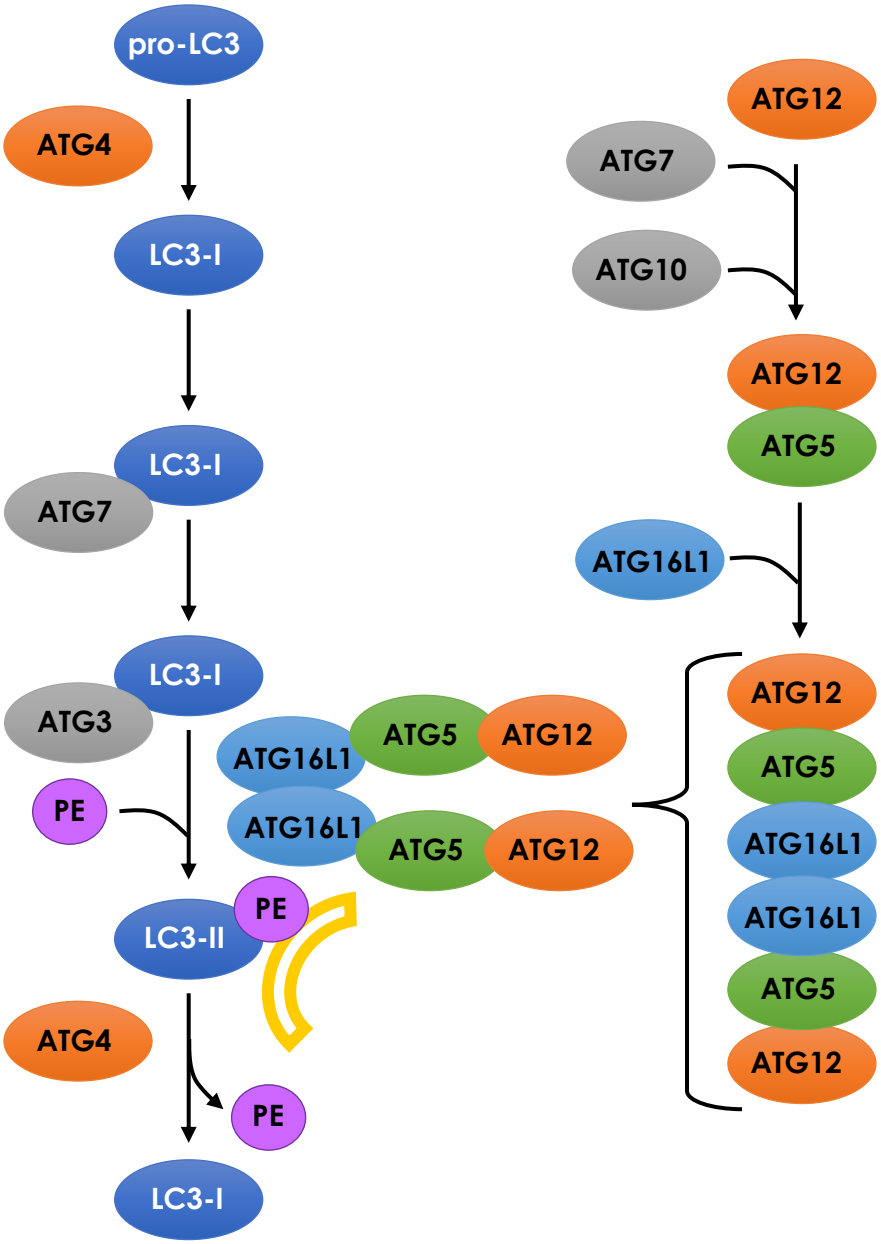

Fig. 8. Autophagy conjugation events for membrane elongation. ATG12 will be exposed to the actions of ATG7 and ATG10 leading to the irreversible binding to ATG5. In this configuration, ATG16L1 can now bind, then the complex can dimerize with itself. In another conjugation event, the newly synthesized pro-LC3 will be cleaved by ATG4 to generate LC3-I, which localizes in the cytosol. LC3-I can be processed by ATG7 and ATG3 preparing for lipidation with PE by the ATG12–ATG5-ATG16L1 complex. With lipidation of LC3-I it now converts to LC3-II and can associate with the autophagosomal membrane assisting with the elongation around selected cargo for degradation. LC3-II can be returned to the LC3-I conformation by removal of the PE group through the action of ATG4. ATG; autophagy-related; LC3; Microtubule-associated proteins 1A/1B light chain 3; PE; phosphatidylethanolamine.

in membrane elongation (74). It is notable that this complex can be found on the outside of the immature autophagosomal membrane, but is removed upon maturation.

The second ubiquitin-like conjugation system involves the conversion of pro-LC3 to the lipidated form, microtubule-associated proteins 1A/1B light chain 3-II (LC3-II), which becomes anchored in the membrane of the autophagosome (113, 128, 298). This generation begins with the cleavage at the C-terminal of newly synthesized pro-LC3 by the protease ATG4 to form a cytosolic pool of microtubule-associated proteins 1A/1B light chain 3-I (LC3-I), which has a molecular mass around 16-18kDa (252). From here, ATG7 serves again as an E1-like activating enzyme preparing LC3-I for the E2-like actions of ATG3. Subsequently, the ATG12–ATG5-ATG16L1 dimeric complex poses as the E3-enzyme and will confer attachment of phosphatidylethanolamine (PE) generating LC3-II. LC3-II migrates to around 14-16kDa on an SDS-PAGE gel and is found associated with autophagosomal membranes, in contrast to exclusively cytosolic pool of LC3-I. Of note, while in yeast only one protein exists for this lipidation event (Atg8), mammals possess seven orthologues. The LC3 family has three members, A, B and C, with LC3B being the most studied. The other forms are referred to as the gamma-aminobutyric acid receptor-associated protein (GABARAP) family and also come in three distinct isoforms as well as golgi-associated ATPase enhancer of 16 kDa (GATE-16) (290). While less is known regarding all of these mammalian versions, it has been discussed that the LC3s exert greater action in the expansion of the phagophore, while GABARAPs participate in the maturation of the autophagosome. Once generated, LC3-II is embedded in the autophagosomal membrane and remains there until the degradation step. For simplicity, these related proteins, whether GABARAPs or LC3 are all referred to as LC3-I/II.

Together, these conjugation events assist in the expansion of the phagophore membrane, growing the cup-shaped structure through the addition of lipid molecules, towards a mature, spherical structure.

Recently the requirement of the LC3/GABARAP families in the generation of the autophagosome has been challenged. Using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) generated knockouts of each or all of the LC3 isoforms, it was found that they appear not to be essential for autophagosome biogenesis (202), contrary to previous work (290). However, in the absence of six variants, autophagosomes took longer to generate and were smaller in size. Furthermore, engulfment of mitochondria was still able to occur in their absence. However, a requisite function of these proteins was facilitating fusion of autophagosomes with lysosomes. Whether this is a direct or indirect effect of these proteins is still not clear. Furthermore, whether these observations extend beyond a mammalian *in vitro* system remains to be determined.

3.1.4 CLOSURE, MATURATION AND FUSION

Once the autophagosome has formed, it will be sealed and proteins that aided in the process of its biogenesis and expansion need to be removed from the outer membrane. As mentioned, LC3-II is retained in both the inner and outer membrane and this feature has been harnessed for visualizing autophagosomes (138, 189). Creation of mice that harbor N-terminal labelled green fluorescent protein (GFP)-LC3-II have been highly useful for visualizing autophagy *in vivo* in a variety of tissues and under varying stimuli. Only fully formed and closed, autophagosomes will be able to progress to fusing with the lysosome. Of note, autophagosomes can intersect with the endosomal pathway and can fuse with endosomes creating a structure called the amphisome. This amphisome will still go on to fuse with the lysosome.

Figure 9.

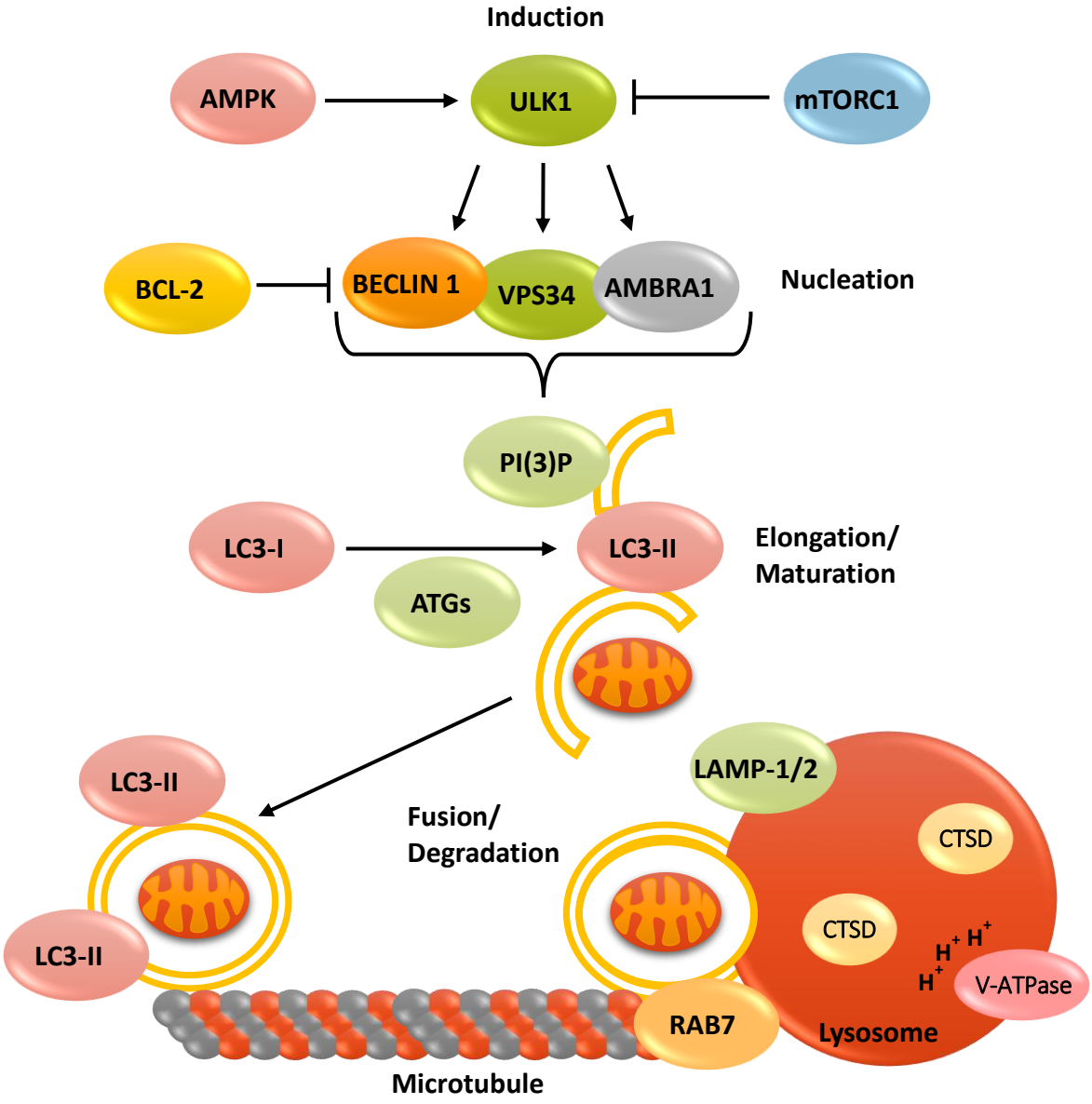
Induction
AMPK
ULK1
mTORC1
BCL-2
BECLIN 1
VPS34
AMBRA1
Nucleation
PI(3)P
LC3-I
LC3-II
Elongation/
Maturation
ATGs
LC3-II
LAMP-1/2
Fusion/
Degradation
CTSD
CTSD
LC3-II
H+ H+
H+
V-ATPase
RAB7
Lysosome
Microtubule

Fig. 9. Major steps in the autophagy pathway. The process of autophagy progresses through a series of steps that generate the *de novo* structure, the autophagosome around cellular cargo to be sequestered and removed. Induction requires ULK1 and associated proteins which is counter regulated by AMPK and mTORC1. Following induction, nucleation of the precursor membrane can occur through the action of the PI3KC3-C1 complex, containing Beclin 1, VPS34 and AMBRA1. Beclin 1 is negatively regulated by BCL-2. With the nucleation complex active, a pool of PI(3)P is generated around the endoplasmic reticulum, recruiting various factors including the processed form of LC3, LC3-II. LC3-II will decorate the autophagosomal membrane. Fully closed autophagosomes can travel via microtubules for fusion with the lysosome. Fusion occurs with the aid of RAB7. Lysosomes have numerous membrane proteins including the LAMPs and v-ATPase, which maintains the acidic environment. Inside the lysosome, substrates can be broken down through the actions of numerous hydrolases, such as CTSD. AMPK; 5' adenosine monophosphate-activated protein kinase; ULK1; unc-51 like kinase 1; mTORC1; mechanistic target of rapamycin complex 1; BCL-2; B-cell lymphoma 2; AMBRA1; Activating Molecule in Beclin-1-Regulated Autophagy; LC3; Microtubule-associated proteins 1A/1B light chain 3; ATG; autophagy-related; LAMP; lysosomal-associated membrane protein; RAB7; Ras-related protein 7; CTSD; cathepsin D; v-ATPase; Vacuolar-type H^+-ATPase.

Autophagosomes traverse the cell on the molecular roads formed by microtubules (10, 289). Since autophagosomes can be formed in various locales, they require the ability to move towards lysosomes, which are often found in the perinuclear region. Indeed, destabilization of these filaments, such as by colchicine treatment, prevents autophagic vessels from reaching the lysosome and consequently the cargo-loaded vesicles accumulate in the cell (127). While a host of proteins have been shown to be involved for fusion events in yeast, less is conclusively known regarding the mechanisms responsible for mammalian cells. Ras-related protein 7 (RAB7) has been demonstrated to be likely responsible for attaching autophagosomes to microtubule motors for their movement, as well as tethering of the autophagosomes to lysosomes (138). Additionally, the lysosomal associated membrane proteins 1/2 (LAMP-1/2) have been documented to assist with fusion (73, 111).

3.1.5 DEGRADATION

The outer autophagosomal membrane is fused with the lysosome leaving the inner membrane free for release along with the sequestered contents into the lysosome. The LC3-II that decorated the outer surface of the autophagosome will be cleaved off by ATG4 returning it to a LC3-I configuration. Since the inner membrane is released into the lysosome for digestion, proteins involved in autophagosome generation as well as adaptors or receptors that link cargo to the inner autophagosome membrane are also subject to digestion. LC3-II and p62 are two such proteins that succumb to a degradative fate, and this makes them highly useful for measures of autophagic flux (discussed below).

The pH within the lysosome is maintained at an acidic level around pH 4.5-5.0 for proper function of the occupant hydrolases. This acidification is due to the action of the proton pump, the vacuolar-type H^+-ATPase (v-ATPase) found in the lysosomal membrane (11). Over 50 enzymes

can be found inside lysosome that facilitate the breakdown of delivered cargo. The cathepsin family of proteases are abundantly located within the lysosome and CATHEPSIN D (CTSD) is a major player in the breakdown of delivered substrates (23, 292). Mutations in many of the hydrolases or lysosomal proteins have been documented to result in disease broadly classified as lysosomal storage diseases. These can include Gaucher, Danon, Neimann-Pick and Pompe diseases, to name a select few (216). Once macromolecules are spliced into basic constituents, they will be discharged from the lysosome through appropriate channels.

The identification of a master regulator for lysosomal biogenesis, transcription factor EB (TFEB), has recently evolved our understanding of lysosomes and their coordination with autophagy (215, 258, 259). Normally, TFEB is repressed adjacent to lysosome through phosphorylation by mTORC1, but in response to cues for autophagy/mitophagy induction, TFEB will be dephosphorylated and translocate to the nucleus. Once there TFEB can occupy promoters of target genes containing the known coordinated lysosomal expression and regulation (CLEAR) sequence (5'-GTCACGTGAC-3') or and E-box motif, which includes numerous lysosomal and autophagic factors such as LC3B, p62, mucolipin 1 (MCOLN1), BECLIN1, LAMP1 among many others (179, 259). Indeed, overexpression of TFEB increases lysosomal abundance and enhanced autophagic degradation activity. A highlight surrounding the discovery of TFEB is that there exists a reciprocal relationship with the master mitochondrial transcriptional regulator, PGC-1α, whereby each of these factors can regulate the others promoter (69, 282, 285). TFEB binds directly to the promoter of PGC-1α, while PGC-1α is capable of regulating TFEB expression. What factors PGC-1α may coactivate on the TFEB promoter, remain to be identified. This sets the stage for a mechanism where mitochondrial turnover is regulated through potent, interconnected factors likely to ensure a balance between the effective removal and generation of mitochondria.

Interestingly, TFEB also induces mitochondrial adaptations with exercise independent of its interactions with the PGC-1α pathway (170).

3.2 MITOPHAGY MECHANISMS

Initially, autophagy was considered a non-selective process that would recycle any cellular constituents in order to maintain homeostasis. However, autophagy has now been well characterized to operate in a highly specific fashion targeting organelles, protein aggregates and even infectious material. In the case of organelles, selectively exists for a wide range of components including, but not limited to, mitochondria, ribosomes, peroxisomes and ER (241). In the case of mitochondria two pathways have largely been described that mediate clearance of this organelle through mitophagy. While not mutually exclusive, ubiquitin-dependent and -independent mechanisms are the most discussed.

The ubiquitin-dependent mechanism is largely controlled through two proteins, PTEN-induced putative kinase 1 (PINK1) and PARKIN. These two proteins act in a common pathway to label mitochondria with polyubiquitin chains that serve to connect the organelle to LC3-II in the autophagosome through adaptor proteins. Ubiquitin-independent clearance of mitochondria is mediated through receptors which can attach to both the outer mitochondrial membrane and then attach to LC3-II in the autophagosomal membrane.

3.2.1 PINK1, PARKIN AND UBIQUITIN-MEDIATED MITOPHAGY

The identification of PINK1 and PARKIN as mediators for mitophagy has spurned a burgeoning field of research. PINK1 and PARKIN mutations are correlated with the onset of recessive familial Parkinson's disease and have been intensely studied in neural tissue (224). Initial studies from the Youle lab demonstrated the link between PARKIN and mitophagy in mammalian cells. In the face of impaired mitochondria, induced through the ETC uncoupler CCCP/FCCP,

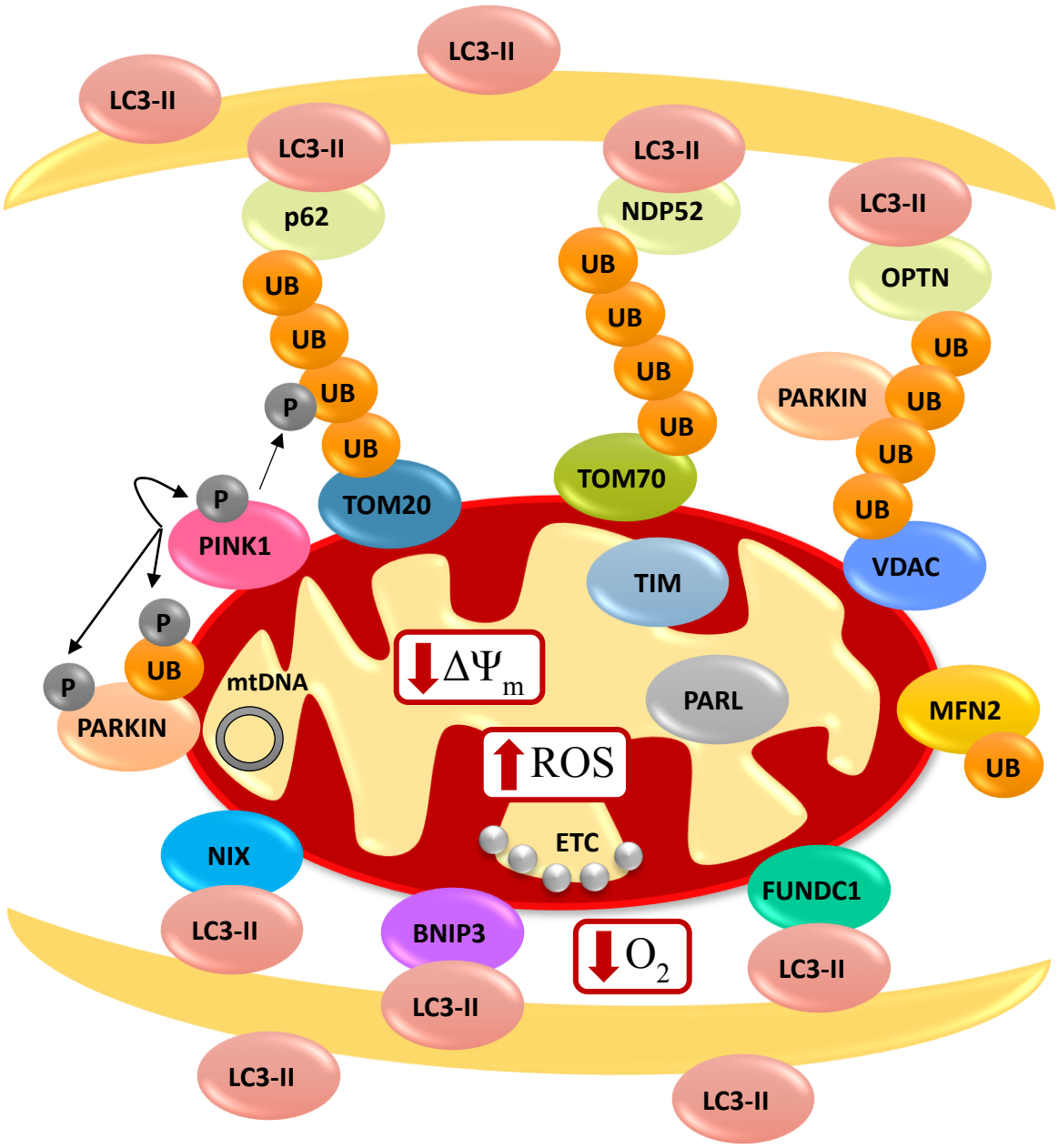

Fig. 10. Mitophagy mechanisms.

Figure 10. Mitophagy mechanisms. Clearance of mitochondria often occurs through ubiquitin-dependent and –independent events. 1) Ub-dependent mitophagy. With the loss of $\Delta\Psi_m$ and increased ROS, PINK1 is no longer imported through TOM and TIM channels. Instead, PINK1 is stabilized on the OMM where it can autophosphorylate, target the E3 ligase, PARKIN as well as molecules of ubiquitin. This leads to full PARKIN activity which is then capable of adding Ub molecules to outer membrane proteins such as, TOM 20/70, VDAC or MFN2. Expansion of ubiquitin chains and phosphorylation of the molecules serves to amplify the mitophagy signal. Adaptor proteins will then link the cargo to the autophagosome. Proteins such as p62, NDP52 and OPTN will act as a scaffold between the targeted cargo and the autophagosomal membrane through binding to both ubiquitin and LC3-II. 2) Ub-independent mitophagy. Receptors, such as NIX, BNIP3 and FUNDC1 are located in the OMM and possess motifs that enable them to bind with LC3-II in the autophagosome. Clearance of mitochondria through these receptors is often triggered by hypoxia (low O_2) events or ROS. Ub; ubiquitin; ROS; reactive oxygen species; $\Delta\Psi_m$; mitochondrial membrane potential; OMM; outer mitochondrial membrane; LC3; Microtubule-associated proteins 1A/1B light chain 3; PINK1; PTEN-induced putative kinase 1; TOM; translocase of the outer membrane; TIM; translocase of the inner membrane; PARL; Presenilins-associated rhomboid-like protein; VDAC; voltage-dependent anion channel; MFN-2; mitofusin-2; OPTN; optineurin; NDP52; nuclear dot protein 52; ETC; electron transport chain; NIX; BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like; BNIP3; BCL2/adenovirus E1B 19-kDa-interacting protein 3; FUNDC1; FUN14 Domain Containing 1; P; phosphate group.

PARKIN would be recruited to these organelles to facilitate their mitophagic degradation (195). Further work has identified PARKIN as an integral component to the degradation of mitochondria in numerous tissues. Loss of PARKIN results in the suppression of stimulus-induced mitochondrial turnover (44, 45), decreased mitochondrial respiration (45, 84) and enhanced oxidative stress (45). PINK1 has also been documented to have a major role in mitochondrial clearance as the absence of PINK1 results in mitochondrial defects, including shifts in mitochondrial morphology, impaired ETC respiration, declines in membrane potential, increased ROS and reductions in Ca^{2+} buffering capacity (9, 99). Through these observations, PINK1 and PARKIN have been clearly demonstrated to exert critical functions to maintain mitochondrial homeostasis.

Recent evidence has elaborated on the sequence of events through which PINK1 and PARKIN act to facilitate mitophagy. In the beginning, there were discrepancies about the sequence of PINK1 and PARKIN actions and also whether they were necessary or simply sufficient for mitophagy. For instance, in heart tissue, ablation of PINK1 did not prevent the recruitment of PARKIN to mitochondria (146). In the drosophila model, PINK1 and PARKIN mutants exhibited similar phenotypes and it was shown that overexpression of PARKIN could compensate for the absence of PINK1. However, PINK1 was unable to compensate for the lack of PARKIN (219). While many studies have highlighted that PINK1 and PARKIN work in a common pathway for mitochondrial removal, recent advances have given greater scope to the sequence of events and roles each of these proteins in mitophagy.

PINK1 is a serine/threonine kinase and normally exists at low levels within the cell in the absence of mitochondrial stress. Under basal conditions, PINK1 is rapidly imported into the organelle through the TOM and TIM import machinery, which is highly dependent on mitochondrial membrane potential (178, 196). Once inside the mitochondria, PINK1 is cleaved by

two proteases. Mitochondrial processing peptidase (MPP) will scission the N-terminal mitochondrial targeting sequence from PINK1 (86), while presenilins-associated rhomboid-like (PARL) protease will cleave PINK1 to a smaller 52kDa fragment from its full length of 64kDa (56, 122, 180). This shortened version of PINK1 can be extruded from the mitochondria and degraded by the proteasome in the cytosol through the N-end rule proteasome pathway (301).

This regulation of PINK1 drastically changes under conditions when mitochondria exhibit stress, such as dissipation of membrane potential. Under these circumstances, full-length PINK1 becomes stabilized on the OMM, likely near the TOM proteins (178, 209). From this position, PINK1 is able to exert its inherent kinase activity to instigate the selection and labelling of the damaged mitochondria for degradation (Fig. 10). Three key phosphorylation events occur from PINK1 that serve to amplify the signal for mitophagy. These include PINK1 autophosphorylation (210), phosphorylation of the E3 ligase PARKIN (262) and phosphorylation of the 76 amino acid protein, ubiquitin (129, 133, 145) (Fig. 10). Interestingly, PINK1 phosphorylates both ubiquitin and PARKIN on the Ser65 residue.

Recent experiments have detailed that phosphorylated ubiquitin attracts PARKIN with high affinity. Upon phospho-ubiquitin binding to PARKIN, an allosteric modification occurs which ultimately stimulates the E3 ligase activity of PARKIN. In this state, PARKIN is now capable of efficiently adding ubiquitin molecules to substrates on the OMM and/or expanding polyubiquitin chains (Fig. 10). These polyubiquitin chains can then serve as substrates for PINK1 to phosphorylate more ubiquitin molecules, creating a feedforward amplification of the mitophagic signal (64, 144) . Many proteins residing on the OMM have been identified as PARKIN substrates. These include the outer translocase proteins, TOM20 and TOM70 (251), the outer channel protein,

voltage dependent anion channel (VDAC) (80, 273), and regulators of mitochondrial morphology, MFN1/2 (79) (Fig. 10).

As described earlier, mitochondria are reticular in form and undergo changes in morphology through fusion and fission processes to expand or fragment the network, respectively. Evidence has shown that fission of mitochondria is a prerequisite for mitophagy, as the impaired segment of the organelle must be segregated (32). Disassociation from the larger network likely aids in the engulfment of the organelle and ensures that only the damaged portion will be degraded. Targeted ubiquitination and degradation of the mitochondrial fusion proteins MFN1/2 prevents the damaged segment of mitochondria from reconnecting with the healthy portion of the network (276). However, if a portion of mitochondria that has been fragmented from the network is capable of regaining its membrane potential, there is the potential for this segment to reconnect with the larger reticulum and be spared from mitophagic degradation (155).

3.2.2 ADAPTOR PROTEINS

While the addition of ubiquitin/ubiquitin chains to OMM proteins amplifies the mitophagic signal, the organelle still requires being encircled by the autophagosome. This guidance is achieved through adaptor proteins that act as a scaffold between the ubiquitin molecules emanating from the organelle and LC3-II in the autophagosomal membrane. To date, mammalian cells have been described to express six adaptor proteins that possess the requisite motifs to bind both ubiquitin and LC3-II. Binding of ubiquitin occurs through an ubiquitin-binding domain (UBD) and LC3-II interactions transpire through a LC3-interacting region (LIR) (Fig. 10). These adaptor proteins include p62/sequestosome-1, Optineurin (OPTN), nuclear-dot protein 52 (NDP52), neighbor of BRCA-1 (NBR1), Tax-1 binding protein 1 (TAX1BP1) and toll-interacting protein (TOLLIP).

The best studied of these is p62 and initially was considered the main adaptor protein to link mitochondria with autophagosomal membranes (25, 80, 117). However, recent work has shed light on adaptors beyond p62 and their role in mitophagy. Lazarou and colleagues demonstrated the role for other adaptors through genome editing that targeted NDP52, OPTN, TAX1BP1, NBR1 and p62 (151). NDP52 and OPTN were found to be potent adaptors for PINK1/PARKIN-mediated mitophagy and also capable of recruiting upstream autophagic factors like ULK1 to proximal mitochondrial locations. Adding to these observations, research has shown that tank-binding kinase 1 (TBK1) is capable of phosphorylating the adaptor proteins OPTN, NDP52, TAX1BP1 and p62 enhancing their ability to bind with ubiquitin-labelled mitochondria for mitophagy (237).

3.2.3 RECEPTOR-MEDIATED MITOPHAGY

Elimination of mitochondria from the cell has also been described to occur independently or possibly in conjunction with the PINK1-PARKIN-ubiquitin axis. Protein receptors that can insert into the OMM through their transmembrane domains and connect to LC3 via a LIR have also been demonstrated to facilitate mitophagic degradation (Fig. 10). These receptors include BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like (NIX/BNIP3L) (59), BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) (238) and FUN14 Domain Containing 1 (FUNDC1) (47). NIX was identified to readily participate in mitophagy through the eradication of mitochondria during the maturation of reticulocytes to red blood cells (205). BNIP3 and NIX have pro-autophagic functions through their ability to bind BCL-2, thus preventing its ability to inhibit BECLIN 1 (304). Additionally, they are capable of binding the upstream mTORC1 effector ras homolog enriched in brain (RHEB), suppressing mTORC1 activity and promoting autophagy (182). Both BNIP3 and NIX mitophagic functions are regulated through phosphorylation events adjacent to their innate LIR region. All three of these receptors are also

involved in mitophagic removal in response to a hypoxic stimulus. Indeed, NIX and BNIP3 are transcriptionally upregulated by hypoxia-inducible factor 1-alpha (HIF-1α) and forkhead box O3 (FOXO3) (22, 169). Periods of hypoxia also favor the production of ROS from the mitochondria, which also stimulates mitophagy through BNIP3 and NIX.

3.3 MITOCHONDRIA-DERIVED VESICLES

Mitophagy removes a complete segment of organelle, eliminating membranes, mtDNA, ETC complexes and inherent quality control factors, such as proteases and regulators of the mitochondrial unfolded protein response (mtUPR). Complete organelle removal may not always be required for rejuvenation and this complete demolition may be ill-advised depending on the energy requirements of the cell or the magnitude of the mitochondrial defect. First documented in 2008, mitochondria have been demonstrated to form mitochondrial-derived vesicles (MDVs) (201). These are small membrane-bound vesicles released by the organelle. The MDVs carry oxidized cargo and their formation is often stimulated through increased ROS production (269) in contrast to membrane depolarization, which kindles mitophagic removal. Interestingly, the MDVs can be delivered to peroxisomes or lysosomes independent of upstream autophagic machinery components and may serve as a quality control mechanism that precedes mitophagy (33).

3.4 AUTOPHAGY AND MITOPHAGY FLUX

Autophagy and mitophagy are ongoing dynamic processes, which makes the assessment of these pathways *in vivo* quite challenging. Indeed, low levels of basal autophagy continually transpire and stimulus-induced autophagy/mitophagy may occur within minutes to hours. In an effort to standardize the assessment of autophagy, numerous guidelines, methodological resources and commentaries have been published to assist researchers in the appropriate approaches to quantify and interpret changes in autophagy and/or mitophagy (127, 141, 191, 272, 293, 302).

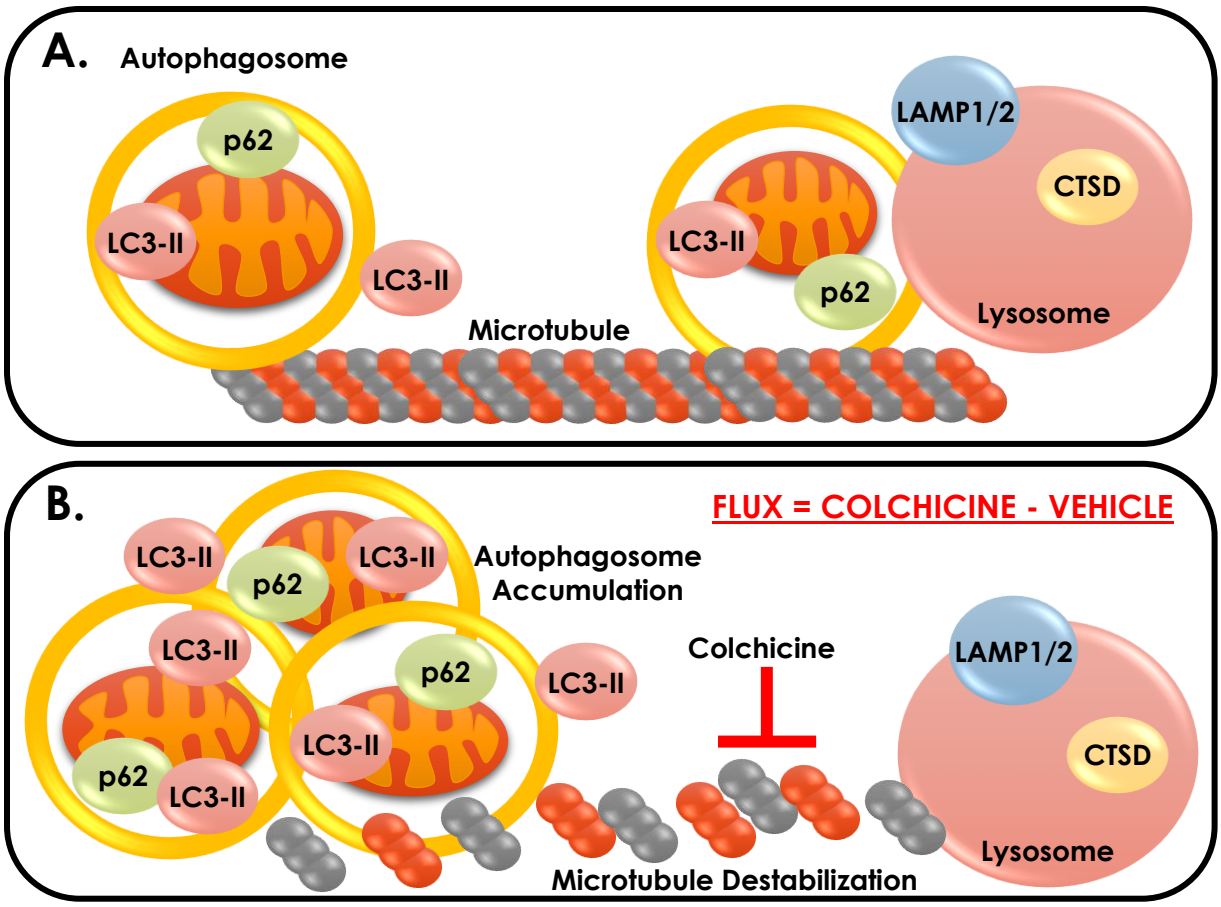

Figure 11. Autophagy flux.

Fig. 11. Autophagy flux. Autophagy is a dynamic process and requires specialized methods to estimate the degree of ongoing degradation. A) Under physiological conditions, autophagy proceeds without impairment. Autophagosomes that are labelled with LC3-II and contain cargo anchored by adaptor proteins, like p62, will travel on microtubules to the lysosome. Upon fusion with the lysosome, the contents can be degraded by resident pH-sensitive enzymes. B) With the administration of a microtubule destabilizer, such as colchicine, autophagosomes will accumulate in the cellular milieu. The vesicles accrue along with the cargo and proteins found on the autophagosome membrane and inside, such the adaptor molecules. Evaluation of the amount of LC3-II or p62 in the colchicine condition, by western blotting or fluorescence, is then subtracted from the uninhibited condition. This method will reveal an estimated amount of autophagic turnover, otherwise known as flux. LC3; Microtubule-associated proteins 1A/1B light chain 3 LAMP; lysosomal-associated membrane protein; CTSD; cathepsin D.

While the rules for assessing autophagy are more finite in cell culture systems (141), *in vivo* measurements, particularly in skeletal muscle, continue to evolve. Assessment of autophagy flux is the highest standard for describing changes in autophagy/mitophagy and considers the amount of degradation that would have transpired.

Often static measurements of protein markers or mRNAs taken at one set time point can lead to misinterpretation and misunderstanding of the robustness of autophagy. For example, since p62 (or other adaptors) and LC3-II can be degraded during autophagy/mitophagy, it is difficult to solely rely on their total protein abundance to indicate increased or decreased autophagy. Furthermore, many post-translational modifications occur during autophagy/mitophagy, which may also need to be monitored beyond total protein abundance. Many studies have interpreted increased autophagy through an enhanced ratio of LC3-II:LC3-I and decreases in p62 levels (159, 299). However, these proteins may also be transcriptionally and translationally upregulated during stressful stimuli when turnover of substrates is occurring, altering the profile of protein abundance due to enhanced synthesis. Moreover, should a defect exist along the autophagy pathway it may further modify the accumulation or degradation of these markers.

This complex situation in measurement of autophagy markers has led to the adoption of pharmacological strategies that purposefully block the pathway in the short-term and then make comparisons to an experimentally identical condition without the blockade (Fig. 11). With acute inhibition of autophagy, accumulation of autophagosomes and/or prevention of lysosomal degradation can occur (127). This blockade will induce an accretion of proteins associated with the autophagosome or substrate recognition, such as LC3-II or p62 (Fig. 11). Comparison of the difference in protein expression between the blocked and the uninhibited conditions permits the assessment of the degree of autophagy that would have occurred. To draw conclusions regarding

changes in mitophagy, isolation of the organelles followed by western blotting for autophagosome markers would infer the degree of organelles targeted for degradation. Additionally, imaging techniques that would permit visualization of markers for mitochondria, autophagosomes and lysosomes would be useful for colocalization assessment to infer mitophagy. This method of autophagy inhibition has been validated in skeletal muscle of model organisms such as mice and rats, however remains contraindicated for the assessment of autophagy in humans. The drugs colchicine, bafilomycin A1, leupeptin and chloroquine are most commonly used to disrupt either lysosomal degradative functions or fusion of autophagosomes with lysosomes.

3.5 AUTOPHAGY AND MITOPHAGY WITH EXERCISE

Autophagy and mitophagy have begun to be well characterized in response to exercise. With an acute bout of exercise, numerous studies have established an induction of this recycling process, in young, healthy muscle (44, 87, 96, 285). A landmark study in the elucidation of acute exercise and autophagy regulation came forth from Beth Levine's lab in 2012 (96). This work demonstrated enhanced autophagy following acute treadmill exercise in mice. Additionally, through mutation of BCL-2 to inhibit stimulus-induced autophagy via BECLIN 1, they described that with impaired autophagy endurance capacity decreased and glucose homeostasis was lost. Numerous studies since have shown that acute exercise is potent stimulus for autophagy and mitophagy. Indeed, autophagic and mitophagic flux assessment following acute exercise solidified our understanding with data from our laboratory (285). However, what befalls mitochondrial turnover within aging muscle following either an acute session of exercise or in response to chronic training paradigms remains relatively unexplored.

Two studies have suggested that autophagy flux increases following chronic exercise training in young model organisms (126, 159). Furthermore, only inferences towards mitophagy were made

as isolation of the organelles was not performed. It is a long standing observation that mitochondrial volume and quality increases following a suitable training regimen. This enhancement in organelle number and composition may arguably lead to an enhanced need for autophagy. However, given that the stimulus for biogenesis is great, the quality of the organelles is high and the muscle is environment is favorable, it is possible that the requirement for mitophagic turnover may actually be relatively low (137). Potential evidence for this points to mitochondrial structure which tends to become more elongated following training along with the increased expression of fusion factors. Since fission is a prerequisite for mitophagy and expression of fission proteins are often lower in trained muscle, there may be fewer organelles that require a complete mitophagic makeover. Furthermore, mitophagy-inducing stimuli, such as ROS and dissipation of membrane potential, are much less prevalent in the trained state suggesting the presence of fewer triggers for mitophagy turnover. It becomes tempting to speculate that with improved organelle quality induced by exercise training, that inherent quality control mechanisms of the mitochondria improve, such as mtUPR (183), and perhaps MDVs become the primary source for exclusion of small organelle portions for renewal.

3.6 AUTOPHAGY AND MITOPHAGY IN AGED SKELETAL MUSCLE

While controversial, the aging muscle milieu loses mitochondria and the remaining organelles are often defective in nature. As described previously, damaged organelles can result from enhanced emission of ROS, increased mtDNA mutations or greater sensitivity to opening of the mtPTP. This seeming preference for aging muscle to harbor poor quality organelles likely arises from perturbations to both biogenesis and mitophagic pathways. This leads to the concept that improper mitochondrial quality control mechanisms must be evident in skeletal muscle.

Whether the mitochondria are the initiators their own demise or are collateral damage remains to be determined.

Initial investigations into mitophagy in aging skeletal muscle have used proteins markers to infer whether this pathway experiences alterations. Assessment of upstream and downstream effectors have documented changes in autophagy and mitophagy markers in aging muscle (19, 62, 125, 207, 294). However, most often the observations are not in agreement. These varying findings impact the interpretations that are generated and leave the field without a consensus. Additionally, we now understand that due to the dynamic nature of autophagy and mitophagy, upstream markers are not solely valid to deduce the changes in autophagy/mitophagy flux. Furthermore, few studies have attempted to isolate mitochondria from aging muscle to examine the translocation or expression levels of mitophagic factors, or assess mitophagic flux. Assessment of isolated organelles from our laboratory has demonstrated that increased expression of PARKIN and p62 are present on mitochondria from aged muscle (207). These data suggest that either the organelles are targeted for degradation at a higher rate or possibly that they are not being removed from the system effectively by the lysosome. These data generate the need to assess mitophagic flux in aged muscle to compose a larger picture of mitochondrial regulation.

The lysosome is the terminal step in the recycling of mitochondria, thus proper function of this depot will maintain the capacity for cellular turnover. Aged tissues, including skeletal muscle, have consistently shown evidence of a substance termed lipofuscin, or the "age pigment" (24, 110, 207, 277). Lipofuscin granules are localized within lysosomes and are indigestible material. The lysosomal theory of aging suggests that due to the accumulation of garbage in the lysosome, this puts the brakes on cellular turnover of structures including mitochondria causing an accumulation of defective components (30). This buildup of harmful substances, like mitochondria can

perpetuate the loss skeletal muscle through their ability to release pro-apoptotic factors. Whether the presence of lipofuscin negatively impacts the degradation of autophagosomes and their cargo in skeletal muscle is currently unknown.

3.7 SUMMARY

Mitochondrial homeostasis is achieved through the continuous formation and removal of organelles. Impairments in the autophagy or mitophagy pathways are noted to cause decrements in skeletal muscle form and function. Aging muscle often presents with poor quality mitochondria, suggesting that the removal of organelles may be reduced, however little evidence has been gathered to support this concept. In particular, the hallmark measurements of autophagy and mitophagy flux are lacking from aged muscle. Exercise is a confirmed stimulus to instigate mitochondrial turnover in young, healthy subjects allowing for remodeling of the organelle network. Additional questions also remain whether exercise can elicit the same effects on autophagy/mitophagy in aged muscle for mitochondrial renovation.

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CHAPTER TWO: PhD Objectives and Hypotheses

Mitochondria are fundamental organelles to the function of skeletal muscle. Aging skeletal muscle accrues poor quality mitochondria which may be related to deficiencies in biogenesis and/or mitophagy. Understanding the mechanisms that keep these organelles in a state of homeostasis is imperative. Exercise is a known stimulus to elicit beneficial changes in skeletal muscle. The effects of exercise, whether acute or chronic, are mediated largely through the transcriptional coactivator PGC-1α. Indeed, upregulation of the PGC-1α gene is a well-documented event with acute exercise. However, aged muscle is at a disadvantage as PGC-1α levels are reduced in aged muscle and numerous upstream signaling modifiers, including those that drive its gene expression, are dampened.

The process of mitochondrial biogenesis is counterbalanced through the removal of old or damaged organelles via mitophagic degradation in the lysosome. Considerable evidence exists that autophagy and mitophagy are upregulated in skeletal muscle in response to an acute session of aerobic exercise. However, whether this upregulation in mitophagic turnover remains following a training paradigm remains relatively unexplored. Given that aged muscle contains a population of dysfunctional mitochondrial it may suggest that mitophagy would be impaired in the aged milieu, yet studies that examine flux are lacking. Additionally, it is important to consider whether aged muscle would respond with alterations in mitophagic flux following a training paradigm, to potentially improve the quality of organelles therein. These observations have led to our following objectives.

OBJECTIVE #1:

To assess the transcriptional activation of the PGC-1α gene in young and aged muscle following an acute bout of exercise and recovery period.

HYPOTHESES #1

1) PGC-1α transcription will be reduced in aging muscle basally compared to young muscle;
2) Young muscle will increase PGC-1α transcription following an acute bout of contractile activity;
3) Aged muscle will exhibit an impaired elevation in PGC-1α transcription following acute contractile activity.

OBJECTIVE #2

To determine whether basal differences exist in autophagy and mitophagy flux in young and aged muscle. Additionally, to explore how autophagy and mitophagy flux might change in both young and aged muscle following adaptations to chronic contractile activity.

HYPOTHESES #2

1) Autophagy and mitophagy flux will be reduced in aged muscle;
2) Following CCA adaptations, young muscle will exhibit reduced mitophagy flux;
3) Aged muscle will experience enhanced flux after the CCA period.

CHAPTER THREE

Effect of contractile activity on PGC-1α transcription in young and aged skeletal muscle

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Abstract

Mitochondrial impairments are often noted in aged skeletal muscle. The transcriptional coactivator PGC-1α is integral to maintaining mitochondria, and its expression declines in aged muscle. It remains unknown whether this is due to a transcriptional deficit during aging. Our study examined PGC-1α transcription in muscle from young and old F344BN rats. Using a rat PGC-1α promoter-reporter construct, we found that PGC-1α transcription was reduced by ~65% in aged TA muscle, accompanied by decreases in PGC-1α mRNA and transcript stability. Altered expression patterns in PGC-1α transcription regulatory factors, including Nrf2, USF1, ATF2 and YY1, were noted in aged muscle. Acute contractile activity (CA) followed by recovery was employed to examine whether PGC-1α transcription could be activated in aged muscle similar to that observed in young muscle. AMPK and p38 signaling was attenuated in aged muscle. CA evoked an upregulation of PGC-1α transcription in both young and aged groups, while mRNAs encoding PGC-1α and COX IV were induced during the recovery period. Global DNA methylation, an inhibitory event for transcription, was enhanced in aged muscle, likely a result of elevated methyltransferase enzyme Dnmt3b in aged muscle. Successive bouts of CA for 7 days to evaluate longer-term consequences resulted in a rescue of PGC-1α and downstream mRNAs in aged muscle. Our data indicate that diminished mitochondria in aged muscle is partly due to a deficit in PGC-1α transcription, a result of attenuated upstream signaling. Contractile activity is an appropriate countermeasure to restore PGC-1α expression and mitochondrial content in aged muscle.

New and Noteworthy

PGC-1α is a regulator of mitochondrial biogenesis in muscle. We demonstrate that PGC-1α expression is reduced in aging muscle due to decreases in transcriptional and post-transcriptional mechanisms. The transcriptional deficit is due to alterations in transcription factor expression, reduced signaling and DNA methylation. Acute exercise can initiate signaling to reverse the transcriptional defect, restoring PGC-1α expression toward young values, suggesting a mechanism whereby aged muscle can respond to exercise for the promotion of mitochondrial biogenesis.

INTRODUCTION

Deficits in mitochondrial content and quality are often observed in aging muscle, although this topic remains under considerable debate (9, 22). While some studies have not noted changes in mitochondria with aging (reviewed in (9)), others have documented reductions in mitochondrial content (10, 38), enhanced ROS emission (38), decreased calcium retention capacity (18), increased apoptotic susceptibility (10, 18) and an impaired signaling for biogenesis (36, 47). Instrumental to the quality of mitochondria is the nuclear transcriptional coactivator, peroxisome proliferator-activated receptor γ, coactivator 1α (PGC-1α) (50). PGC-1α binds and coactivates numerous transcription factors/nuclear receptors that occupy promoters of nuclear genes encoding mitochondrial proteins (NuGEMPs) (21). Indeed, mice deficient for PGC-1α in skeletal muscle exhibit reduced mitochondrial content and function and lower endurance capacity (2, 35). In contrast, overexpression of the coactivator in skeletal muscle enriches mitochondrial content and exercise performance (34, 53). In aging muscle, PGC-1α transcript and protein content are reduced (10, 13, 25, 31, 32, 38), yet whether this deficit occurs through transcriptional defects has yet to be determined. Indeed, the limitation in solely measuring mRNA is that transcripts are subjected to the presence of stability as well as decay factors, which can alter the accumulation of specific transcripts (12). Thus, mRNA concentrations are not a direct reflection of the transcriptional machinery, and assessing promoter activity directly is beneficial to determine whether transcriptional differences exist.

An acute bout of exercise is a stimulus which is known to activate transcription of the PGC-1α gene in young, healthy muscle (45, 56). Previous work has documented that contractile activity of skeletal muscle activates divergent signaling pathways and kinases that impinge upon both PGC-1α protein (28) and factors that regulate the PGC-1α gene promoter (3, 4, 15, 26, 27, 56).

Interestingly, PGC-1α also exists in a self-regulatory loop whereby it coactivates its own transcription (20). Thus, in scenarios with diminished PGC-1α protein content, this likely also impacts the transcription of its own gene. Whether basal or exercise-induced transcription of PGC-1α remains intact in aged muscle has not been examined.

Transcription is a highly regulated process that can be affected by epigenetic modifications (30). The methylation status of the DNA is one such epigenetic marker, and increases in the methylation of promoter regions are widely regarded as inhibitory for transcription. Results indicating global hypermethylation of DNA from aging human muscle (57), as well as increased NuGEMP-specific methylation (49) suggest an altered genomic landscape for gene expression with aging. This may partly explain the vast changes in transcriptional profiles that occur between young and aged muscle (17, 25, 29, 39, 42). DNA methylation/demethylation is an active process, and recent evidence details dynamic changes after exercise in young, healthy muscle, which permits the upregulation of mRNAs vital for mitochondrial function, including PGC-1α (7). The addition of methyl groups to DNA is mediated by a group of proteins known as the DNA methyltransferases (DNMTs), and the examination of PGC-1α transcription concomitant with markers of epigenetic regulation may provide insight into the regulation of mitochondrial content and quality within aging muscle.

Thus, the purpose of our study was to examine the transcriptional activity of the PGC-1α promoter in young and aged muscle basally, and following an acute exercise stimulus. We hypothesized that PGC-1α promoter activity would be reduced in aged muscle, and that a single bout of contractile activity would elicit a diminished response compared to young muscle.

MATERIALS AND METHODS

Animals. Young (6-7mo) and aged (34-35mo) male Fisher 344 Brown Norway F1 hybrid rats (F344BNxF1) were obtained from the National Institute of Aging (Bethesda, MD, USA), and were maintained and used in accordance with the York University Animal Care Committee and Canadian Council of Animal Care guidelines. The ages of the animals employed correspond approximately to the human years of adult (18-20) and octogenarian (80-85). Upon arrival, animals were housed 2-3/cage and acclimatized to the environment for a minimum of one week prior to experimentation. Animals were fed and watered *ad libitum* and maintained on a 12h/12h light/dark cycle in a temperature-controlled environment.

PGC-1α promoter generation. Using rat genomic DNA, a 1.5kb region upstream of the transcription start site for PGC-1α was amplified by PCR using the following primers: Forward: 5'- GGA CCC GGG ACT AAT GTT TTC CTT CTA AG -3' Reverse: 5'- TCC CTC GAG ACT CCA ATC CAC TCT -3'. The resultant product was cloned into a PGL3 basic vector (Promega, Madison, WI, USA) upstream of the luciferase coding region using KpnI and HindIII restriction enzymes. Plasmid DNA was grown and isolated with a Maxi Prep kit (Qiagen, Toronto, ON, CAN) for subsequent electrotransfection experiments. Correct plasmid amplification was confirmed using restriction enzyme digest and visualized on an agarose gel with ethidium bromide. The construct was tested *in vitro* and *in vivo* versus an empty PGL3 vector to confirm endogenous transcriptional activation of the promoter sequence in skeletal muscle (data not shown). The PGC-1α vector was co-transfected with PRL-CMV to serve as a loading control to correct for transfection efficiency.

PGC-1α Promoter and Electroporation. Similar to previous experimentation (12, 52), animals were anesthetized with isoflurane and 50μg of the rPGC-1α construct, concomitant with 1μg of

PRL-CMV DNA, was injected bilaterally with an ultra-fine 29-gauge syringe (BD Canada) into the TA muscles under aseptic conditions. Twenty 100V/cm^2 pulses were applied to the muscle with forcep-style electrodes using an ECM 380 BTX electroporation system (Harvard Apparatus, Saint-Laurent, QC, CAN) to facilitate uptake of the construct into the muscle. Conductive gel was applied to the electrodes to assist with transfection. The animals were given 7 days for gene amplification (14) prior to *in situ* contractile activity and muscle removal.

mRNA stability. The mRNA stability assay was performed as described previously (12, 33, 52). Briefly, total RNA was isolated from the tibialis anterior (TA) of Sprague-Dawley rats and was subsequently incubated with isolated cytosolic extracts from the TA muscles of either young or aged animals. Incubations lasted for 0, 15 or 30 mins and after each time point, total RNA was reisolated and reverse transcribed to cDNA. Sequence specific primers for PGC-1α [n=3;(12)] were used to quantify cDNA using semi-quantitative PCR. The PCR products were subjected to electrophoresis on ethidium bromide-stained 1.8% agarose gels. mRNA content was quantified as a percentage of t=0.

In Situ Contractile Activity. Animals were anesthetized with an i.p. injection of ketamine/xylazine cocktail (0.2 ml/100g body mass) and supplemented as required for the duration of the experiment. The left and right TA muscles were exposed and trimmed of constraining connective tissue. Additionally, the left sciatic nerve was gently uncovered and doused with warmed 0.9% saline. The distal tendon of the left TA was tied to a hooked pin and the animal was placed perpendicular to a force gauge and the tendon was attached to the force transducer (Grass FT 10: Grass Instruments, Quincy, MA, USA) via the pin. Data was amplified using a PowerLab/4SP and recorded on Chart5 software (ADInstruments, Colorado Springs, CO, USA). Initially, a length-tension curve was performed to determine optimal resting muscle length to elicit maximal

contraction. Following this, maximal (tetanic) and submaximal (twitch) contractions were determined by first stimulating the sciatic nerve, followed by a brief rest period, and then these measures were repeated via direct muscle stimulation. Maximal tetanic and twitch force were calculated for nerve and muscle stimulation and compared as a means to examine whether age-related denervation or neuromuscular transmission defects were present in the TA muscle. Subsequent to these tests, direct muscle stimulation was performed to induce contractile activity. The intramuscular electrodes were inserted parallel to muscle fibres and accompanied by a temperature probe. Muscle temperature was maintained at 37ºC throughout by the use of adjustable heat lamps. Muscle dehydration was prevented by flushing the muscles with saline and wrapping them in plastic.

To induce contractile activity and the activation of intracellular signaling pathways, 40 minutes of stimulation was performed in 10 minute bouts of increasing intensity (0.25, 0.5, 1, 2 trains/s). Muscles were either excised immediately, or the animal was maintained under anesthesia to allow for 2 hours of quiet muscle recovery followed by muscle extraction. During the stimulation and the recovery periods, oral body temperature was monitored using the CODA monitor (Kent Scientific, Torrington, CT, USA) and muscle temperature and hydration were also continually maintained.

Chronic Contractile Activity. To evaluate long-term chronic consequences of repeated contractile activity, young and aged animals were implanted with stimulators to activate the left TA and EDL muscles as previously described (1, 38). Animals were subjected to 3 hours of stimulation per day, for 7 consecutive days. TA and EDL muscles were harvested 21 hours following the last stimulation bout and flash frozen in liquid nitrogen.

Luciferase Activity. The TA muscle was pulverized to a fine powder at the temperature of liquid nitrogen. Approximately 30mg of powder was diluted in 1X Passive Lysis Buffer (Promega, Madison, WI, USA), by 5-fold on ice. The sample was sonicated on ice 3 x 3s and subsequently spun in a microfuge at 4°C for 10min at 16.1x*g* and the supernate was collected. Using a luminometer, (Lumat LB 9507, Berthold Technologies, Oak Ridge, TN, USA) initial background readings of the passive lysis solution to confirm buffer integrity were followed by addition of 20µl of sample into a test tube that was mixed with 100µl of luciferase substrate followed by 100µl of renilla substrate (Promega, Madison, WI, USA). Samples were measured in triplicate. Luciferase values were corrected for renilla values to control for transfection efficiency and the triplicate readings were averaged.

COX Activity. Extracts and enzyme activities were prepared and measured as previously described (40, 41). Briefly, tissue was pulverized to a fine powder and extracts prepared in enzyme extraction buffer (100mM Na-K-Phosphate, 2mM EDTA, pH 7.2). Extracts were added to a solution containing fully reduced cytochrome c (Sigma, Mississauga, ON, CAN) and the rate of oxidation was measured as the change in absorbance over time at 550nm using a plate reader (Synergy HT, Bio-tek, Thorold, ON, CAN).

High Resolution Respirometry. The red gastrocnemius muscle was freshly isolated for examination of respirometry using the Oroboros O2k, Innsbruck, Austria. Samples were extracted and immediately placed in ice-cold BIOPS buffer (10 mM Ca-EGTA, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1). Small muscle fibre bundles were separated on ice in BIOPS buffer with fine forceps under a dissection microscope. The isolated bundles were incubated at 4ºC for 30 mins in saponin (50µg/mL) with gentle rotation. Fibre bundles were washed and weighed then

added to the oxygen chamber in Miro5 buffer (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1, and 0.1% BSA). The chambers were hyperoxygenated with 100% O_2 followed by stabilization for a background reading. Glutamate (10mM) and malate (2mM) were added followed by ADP (2.5mM) to assess State IV and State III respiration at 37°C (43, 44). Oxygen flux values were corrected for background readings and fibre mass. Respiratory control ratios were assessed to ensure quality of the fibres and mitochondrial respiration.

Genomic DNA Isolation. gDNA was isolated from 20-25mg of pulverized tissue powder using the DNeasy Blood and Tissue Kit (Qiagen, Toronto, ON, CAN). Purity and concentration were determined using a NanoDrop 2000 (ThermoFisher, Mississauga, ON, CAN).

Global Methylation. gDNA was prepared for the 5-methylcytosine DNA ELISA kit (Zymo Research, Irvine CA, USA) according to manufacturer's instructions. Briefly, samples were loaded into the wells and incubated with an antibody highly specific for 5-methylcytosine. A secondary antibody was added for detection and absorbance measured on a plate reader at 450nm. A standard curve was prepared and read simultaneously with the samples of interest.

qPCR. Tissue was pounded to a fine powder on liquid nitrogen and mRNA was isolated using TRIzol reagent according to manufacturer's instructions. Quality and concentration of the mRNA was assessed using the NanoDrop 2000. Subsequently, mRNA was converted to cDNA using Superscript III (ThermoFisher, Mississauga, ON, CAN). For mRNA evaluation of basal changes with age and acute CA, equal amounts of cDNA were incubated with FAM-labelled TaqMan specific probes (ThermoFisher, Mississauga, ON, CAN) for genes of interest (Table 1). Samples were run in duplicate on a 96-well plate and assessed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was normalized to the average

of the loading controls β2-Microglobulin, β-actin and GAPDH. To assess mRNA from CCA obtained tissues, extraction and cDNA conversion was identical as described above. cDNA was incubated with PerfeCTa SYBR Green (Quantabio, Beverly, MA, USA) and forward and reverse primers specific to the gene of interest (Table 1). Primers were assessed for specificity and optimized for concentration prior to collection of CCA transcript data. Gene expression was normalized to S12 as previous and current examination of samples in our laboratory has determined that this gene does not to change in response to either aging or CCA in the F344BNxF1 rat. Transcript expression was calculated using the $2^{-\Delta\Delta Ct}$ method relative to Young, or Young Control, where applicable.

Western Blotting. Equal amounts of protein (20-40µg) were loaded and separated on 8-15% gels followed by electrotransfer to a nitrocellulose membrane (Bio-Rad, Mississauga, ON, CAN). Membranes were incubated overnight at 4ºC with primary antibodies (Table 2). The next day membranes were washed in Tris-buffered saline with Tween-20 and incubated with appropriate secondary antibodies (Cell Signaling, Danvers, MA, USA) conjugated to horse radish peroxidase for one hour. Membranes were washed again and then developed using enhanced chemiluminescence (Bio-Rad, Mississauga, ON, CAN) on film (Mandel Scientific, Guelph, ON, CAN).

Statistics. Analyses were made using GraphPad Prism 6.0 with a student's t-test or two-way ANOVA where applicable. CCA mRNA data was evaluated using a two-way repeated measures ANOVA as CON and CCA conditions were obtained from the same animals. Data are presented as mean±SEM.

RESULTS

Young and aged characteristics. Aged animals exhibited characteristics of sarcopenic muscle including a 44% reduction in the TA/body mass ratio (Table 3). Additionally, *in situ* tetanic force production corrected for muscle mass was significantly suppressed by 27%, indicating reduced muscle quality (Table 3). Mitochondrial content, assessed through COX activity, was significantly reduced by ~25% in aged animals in conjunction with a 36% reduction in State III respiration, measured through high resolution respirometry (Fig. 1A, B). Assessment of the respiratory control ratios (State III/State IV) revealed no difference in intrinsic respiration between young (4.52±0.43) and aged (4.06±0.43) groups. Analyses of mRNA revealed that PGC-1α and COX IV transcripts were supressed by ~30% while no change in Tfam was observed in aged muscle (Fig. 1E). Evaluation of key transcription factors that drive PGC-1α expression revealed significant decreases by ~30% in NFE2L2 (Nrf2) and USF1 in aging muscle (Fig. 1C, D). In contrast, ATF2 and YYI were enhanced ($p<0.05$) with aging by 1.5 and 1.7-fold, respectively. No changes were noted in either GATA4 or CREB (Fig. 1C, D). Since PGC-1α transcript levels were suppressed in aged muscle, assessment of PGC-1α mRNA stability was performed. Interestingly, PGC-1α stability was significantly decreased in the aged muscle milieu compared to young animals (Fig. 1F). Additionally, we noted a likely increase in the mRNA destabilization factor, CUG binding protein 1 (CUGBP1) while the stabilizing factor human antigen R (HuR) remained unchanged in aged muscle (Fig. 1E).

Muscle and nerve force production. In an effort to examine the potential for age-related denervation in the TA muscles, young and aged animals were subjected to twitch and tetanic contractions via the sciatic nerve or intramuscular stimulation. In young muscle, tetanic contractions elicited 3.6-fold greater force production than twitch contractions ($p<0.05$, Fig. 2A).

No differences were noted in the amount of force generated through nerve and muscle stimulation for either twitch or tetanic contractions in young animals (Fig. 2A). Aged muscle produced significantly less force than young muscle during both tetanic (60-74% decrease) and twitch (47-63% decrease) contractions ($p<0.05$, Fig. 2B). Tetanic force development was 2.6-fold greater than twitch contractions in aged muscle ($p<0.05$, Fig. 2A). No differences were observed in force development elicited through nerve or muscle stimulation in the aged group (Fig. 2A), suggesting the absence of any functional denervation or deficits in signal transmission within the TA muscle of these aged animals.

In situ signaling with aging. Young and aged animals were then exposed to 40 mins of progressive *in situ* contractile activity (CA) with or without a two hour recovery period. A typical force output with progressive fatigue of the TA muscle is illustrated in Fig. 2B. This protocol was adopted because it elicits intensity-dependent increase in muscle oxygen consumption (23), mimicking the increase in oxygen consumption observed in muscle subjected to an increasing, graded workload test. Two key signaling kinases, AMPK and p38, were assessed immediately after contractile activity and following the recovery period. A significant enhancement of P-AMPK by 2.5-fold ($p<0.05$, Fig. 3A) was noted in young muscle immediately after CA (Fig. 3A). This elevation returned to control levels following quiet recovery. Aged muscle exhibited lower AMPK phosphorylation across all conditions in comparison to young muscle. In response to CA, P-AMPK increased by 2-fold in aged muscle ($p<0.05$, Fig. 3A). To determine if the strength of the signaling response was similar in young and aged muscle, the delta was calculated (STIM-CON). An attenuation in AMPK signaling by 46% ($p<0.05$, Fig. 3A, inset) was observed in aged compared to young muscle. P-p38 was significantly lower across all conditions in aged animals compared to

young ($p<0.05$, Fig. 3B). A trend ($p=0.06$) for an increase in P-p38 was observed with acute CA in both age groups.

PGC-1α promoter activity and target transcripts. Activity of the upstream 1.5kb rat PGC-1α promoter was assessed through luciferase activity following CA and recovery. Young muscle exhibited a significant 1.8-fold increase in promoter activity with CA which returned to baseline levels subsequent to the recovery period (Fig. 4A). Aged muscle displayed reduced promoter activity basally by ~66% ($p<0.05$, Fig. 4A). Following CA in aged muscle, a 2.4-fold increase was observed. Similar to young muscle, promoter activity returned to baseline values after the 2 hour recovery. Calculation of the delta (STIM-CON) to compare the difference between the magnitude of young and aged promoter activation with acute CA revealed no difference ($p>0.05$; Fig. 4A, inset).

PGC-1α mRNA remained unchanged after the contractile activity and recovery period in young muscle. However, aging muscle exhibited lower transcript levels at rest and following CA compared to young CON muscle as assessed through post-hoc analysis ($p<0.05$, Fig. 4B). Interestingly, there was no significant difference in PGC-1α mRNA when aged REC was compared to young CON (Fig. 4B). COX IV expression is coactivated by PGC-1α, thus we also assessed transcript levels of this nuclear-encoded mitochondrially-destined gene. COX IV transcript was unchanged in young muscle. In aged muscle, a main effect for a decrease in COX IV mRNA was noted across all conditions (Fig. 4C).

DNA methylation factors. Regulation of gene expression can be influenced by epigenetic alterations, such as methylation of the DNA. Assessment of global DNA methylation on isolated genomic DNA revealed significantly greater levels in aged, compared to young muscle (Fig. 5A). With CA and recovery, no significant changes were noted (Fig. 5B). However, methylation

appeared to be greater with age across all rest, CA and recovery periods ($p=0.057$; Fig. 5B). Next we assessed the transcript content of two DNA methyltransferases responsible for adding methyl groups to DNA, Dnmt3a and 3b. Dnmt3a mRNA was significantly reduced by ~54% while Dnmt3b was unchanged with aging (Fig. 5C). Protein assessment of Dnmt3b showed an increase by ~1.9-fold ($p<0.05$) in aged muscle (Fig. 5D).

Effect of CCA on transcript expression. In a separate set of animals, we assessed whether accumulating bouts of CA could restore transcript levels of PGC-1α in aged muscle. As expected, aged muscle displayed significantly reduced expression of PGC-1α mRNA ($p<0.05$, Fig. 6A). CCA induced a significant upregulation ($p<0.05$, Fig. 6A) in PGC-1α expression in both young (1.3-fold) and aged (1.6-fold) muscle. Interestingly, the levels of PGC-1α mRNA in aged muscle subject to 7 days of CCA were not different from those observed in young resting muscle (Fig. 6A). We also evaluated COX IV following CCA. Similar to PGC-1α, COX IV was reduced in aged muscle ($p<0.05$, Fig, 6B). CCA evoked a trend ($p=0.07$) to increase COX IV mRNA levels. We also examined Nrf2 mRNA in response to aging and CCA. No difference in transcript expression was observed with age. CCA induced a similar elevation in Nrf2 ($p<0.05$, Fig. 6C) in both young and aged groups.

DISCUSSION

With advancing age, mitochondria are often reported to diminish in volume and function within skeletal muscle. The mechanisms responsible for age-related mitochondrial loss are not fully understood and continue to be elucidated. To produce fully functioning, high quality organelles, coordination between the nuclear and mitochondrial genomes is necessary to produce protein products in the correct stoichiometry. Central to the regulation of the expression of nuclear

genes encoding mitochondrial proteins (NuGEMPs) is the transcriptional coactivator PGC-1α. PGC-1α coactivates a wide array of nuclear receptors/transcription factors, including those found on numerous NuGEMPs, as well as its own promoter. Thus, maintaining the levels of PGC-1α is integral to promoting mitochondrial biogenesis. With aging, PGC-1α expression has been observed to decline (10, 13, 25, 31, 32, 38). However, the cellular mechanisms underlying this deficiency with age have not been documented. In this study, we sought to understand whether PGC-1α gene transcription was altered in aging muscle, and if PGC-1α transcription could be induced to increase, as has been documented previously (4, 45) with exercise in skeletal muscle of young subjects.

The F344BNxF1 rat is considered an appropriate aging model as these animals exhibit sarcopenia similar to humans, albeit in a shorter timeframe (5). Indeed, we observed that aged muscle was reduced in quantity and quality, as mass and force generation were significantly diminished compared to the younger group. This also appeared to take place in the absence of any functional denervation or defects in neuromuscular transmission within the aged muscle. Furthermore, reductions in skeletal muscle mitochondrial content and quality were also apparent with aging. These observations are in agreement with previous work using the same rodent model of aging (36, 38). Additionally, the transcripts for PGC-1α and COX IV exhibited blunted expression, also pointing to a decrement in mitochondrial biogenesis in aged muscle. Transcript levels are a product of the extent of transcription, and are modulated by the presence of stabilizing or destabilizing factors. In an effort to understand the effect of the aging milieu on transcript stability of PGC-1α, assessment of mRNA stability was made using cytosolic extracts from young and aged muscle. Interestingly, PGC-1α stability was significantly decreased in aged muscle. Thus,

the decrease in PGC-1α mRNA is likely a consequence of reduced transcription, and/or increased instability.

To evaluate PGC-1α gene transcription in muscle, we utilized a construct consisting of 1.5kb upstream of the rat PGC-1α canonical/proximal promoter attached to a luciferase reporter. This construct was electrotransfected bilaterally into the TA muscles of young and aged animals. The transcription of PGC-1α via the canonical promoter was reduced by ~66% with age in resting muscle. This undoubtedly contributes to the diminished levels of PGC-1α mRNA and protein often found in aged muscle (10, 13, 25, 31, 32, 38), in combination with enhanced decay of transcripts. Taken together, the observed decreases in transcription, stability, and mRNA levels suggest that the aged muscle environment is unfavourable to maintain PGC-1α levels, and this may ultimately contribute to a reduction in mitochondrial content.

This also suggests that factors which regulate the basal expression of PGC-1α are less effective with age, and restoration of canonical promoter activity would likely serve to increase PGC-1α transcript content. AMPK and p38 are two kinases documented to regulate PGC-1α promoter and protein activity (3, 26–28, 46). Lower levels of AMPK and p38 phosphorylation were observed basally in aged muscle in agreement with previous observations (36, 47). Additionally, epigenetic alterations of promoter methylation may affect gene transcription. Previous work has documented enhanced levels of methylation in skeletal muscle both globally, and on specific NuGEMP promoters with aging (49, 57). In agreement, our study noted enhanced global genomic methylation in aged muscle. This enhanced methylation profile is likely mediated, in part, via the action of Dnmt3b, a *de novo* methyltransferase which adds methyl groups to the 5' position of cytosine residues that precede guanines (CpGs). Additionally, Dnmt3b has been found to methylate the PGC-1α promoter on non-cytosine residues in diabetic patients which correlates

with decreased PGC-1α mRNA (6). Furthermore, knockdown of Dnmt3b prevents hypermethylation using an *in vitro* muscle model. In our study, Dnmt3b levels were increased in aging muscle, while the mRNA of the other *de novo* methyltransferase, Dnmt3a, was reduced. Unfortunately, we were unable to detect Dnmt3a at the protein level. While we were unable to directly assess promoter-specific methylation patterns of the PGC-1α promoter, our data nonetheless suggest that the increase in global methylation may be partly attributable to the rise in Dnmt3b observed in aged muscle. The increase in global methylation may also be partly explained by lack of demethylation. More recently, the events which regulate active demethylation have begun to be described, involving a series of oxidation reactions affecting 5-mC and the base excision repair pathway (19, 55). Future work examining the expression of the regulators of demethylation would add to our understanding of the role of epigenetics in aging skeletal muscle.

We next assessed the protein expression of transcription factors that have been documented to have putative binding sites on the PGC-1α promoter. Previous reports, along with TRANSFAC and JASPAR database searches, have identified that USF1(27), GATA4 (27), CREB (16), ATF2 (3, 4), Nrf2 and YY1(11) have binding sites on the PGC-1α promoter. Basally, we observed that the expression of these transcription factors were imbalanced within the aging milieu compared to young muscle. Interestingly, some of these factors including ATF2, USF1 and GATA4 have been identified to regulate PGC-1α gene expression in response to muscle contractile activity signaling. However, this has thus far only been documented in young muscle, in exercise mimetic signaling or in cell culture models. Taken together, the reductions in PGC-1α promoter activity in resting muscle of aged animals may be attributable to repressed kinase activity, imbalanced transcription factor expression and elevated DNA methylation patterns. Thus, rectification of these deficits may improve transcription of PGC-1α, and ultimately mitochondrial biogenesis.

With aging, it has been noted that physical activity levels often decline in tandem with advancing age. Whether ageing *per se* or changes in physical activity levels contribute to the declines in PGC-1α and/or mitochondrial biogenesis is not well understood. In an effort to understand whether a single bout of physical activity could initiate the signaling required to remedy the deficit in PGC-1α promoter activity, we exposed the animals to an acute bout of *in situ* contractile activity of 40 mins, with or without a two hour recovery period. We selected this paradigm as it is recruits all fibre types and it is progressive in intensity for both young and aged muscle. Furthermore, tetanic contractions were chosen as higher intensity exercise often leads to more robust kinase signaling pathways that impinge on PGC-1α (15). To confirm that the contractile activity paradigm was able to activate known exercise-responsive signaling pathways, we evaluated the phosphorylation of AMPK and p38, kinases that are also known to affect PGC-1α transcription with contractile activity (4, 27, 46, 54). AMPK phosphorylation was remarkably elevated in young muscle immediately following CA, and returned to basal levels subsequent to the recovery period. This is in agreement with previous observations that have documented enhanced AMPK signaling following acute exercise in young muscle (15, 37). In contrast, aged muscle had reduced AMPK activity overall, including the response to contractile activity and recovery. We observed a trend for an increase in P-p38 with contractile activity regardless of age. Thus, it appears that aged muscle retains the ability to activate kinase-signaling networks in response to contractile activity, however in comparison to young muscle, the response is diminished. Perhaps with successive, accumulating bouts of contractile activity, kinase signaling could be restored to similar levels observed in young muscle.

An acute bout of progressive contractile activity was employed to evaluate PGC-1α transcription in the setting of increased muscle workload intensity and oxygen consumption (23).

This acute paradigm produced an increase in PGC-1α promoter activity in both young and aged muscle suggesting that exercise is a positive stimulus towards the restoration of PGC-1α promoter activity in aged muscle. Despite the relatively pronounced change in promoter activity observed in young muscle, PGC-1α mRNA was not significantly changed immediately following this single, short bout of CA. However, following the 2 hour recovery period PGC-1α mRNA levels were no longer significantly different in aged muscle compared to those observed in young resting muscle. While others have observed increases in the mRNA of PGC-1α following acute exercise, the timing of the recovery may influence the observed result. The recovery time period chosen was based on previous literature documenting changes in transcript expression in young muscle (3, 45). However, this time point representing the optimal increase in PGC-1α and its targets are likely different in aged and young muscle, given the divergent signaling milieu and transcription kinetics in these aged groups.

Although our research has documented the transcriptional activity of the canonical PGC-1α promoter, an alternative promoter exists for PGC-1α, as well as splicing events that generate numerous truncated variants. Future work assessing the role and function of the alternative promoter and the variants in the context of aging would provide additional insight into the regulation of PGC-1α expression. However, since aged muscle displayed a decrement basally in the activity of the canonical promoter, it is reasonable to develop strategies that reinstate a higher level of promoter activity for PGC-1α. Thus, we evaluated whether repeated bouts of contractile activity would rescue the levels of PGC-1α mRNA and its downstream targets. Importantly, chronic contractile activity elicited rejuvenating effects on both PGC-1α and COX IV in aged muscle, and increased Nrf2 levels similarly in both young and old muscle. This suggests that repeated bouts of exercise can promote the enhanced expression of transcripts that play an integral

role in the expression of the PGC-1α gene. Indeed, these results are in agreement with previous literature which has identified that PGC-1α mRNA and other transcripts can accumulate in individuals regardless of age, after exposure to training paradigms (51). However, whether the adaptation is similar in both young and aged muscle is controversial (9, 24). Restoration of a basal defect in PGC-1α mRNA is regarded as beneficial, however we have yet to discern whether this change results in increased protein product, which is the functional unit that will elicit the mitochondrial adaptations through the coactivation of target genes. Indeed, recent evidence has pointed to the concept that the improvements in mitochondrial content with exercise training in aged individuals are due in part to enhanced gains in protein abundance, rather than solely mRNA (48). Continued work assessing the relationship between chronic exercise, transcriptional activity, mRNA and protein levels would shed more insight on this matter.

We also evaluated the genomic methylation landscape following acute contractile activity and recovery in young and aged muscle. It has been previously identified that global methylation as well as methylation of the PGC-1α promoter in young muscle is reduced in response to acute exercise (7). This demethylation of PGC-1α occurred immediately following acute exercise, preceding the rise in corresponding mRNA which peaked at 3 hours post-recovery, suggesting that reduced methylation is coupled to transcript expression. While we did not observe the same decrease in global methylation following acute contractile activity in either of our age groups, this may be explained by the timing of sample acquisition, exercise intensity, and/or species differences. Despite this, we did measure a significant upregulation of PGC-1α transcriptional activity in both age groups. Evaluation of our promoter construct revealed the presence of numerous CpG sites, suggesting that it could be subject to methylation/demethylation events. Future work evaluating the specific methylation pattern of the PGC-1α promoter in aged muscle

using bisulfite sequencing would be ideal to understand the global hypermethylation that occurs with aging, as well as the rise in transcription following acute contractile activity. However, it should also be noted that a direct correlation between methylation and transcript expression may not always be apparent, since the expression of mRNA following its production can be subject to mRNA stability factors which either stabilize or subject to the transcript to degradation. As such, the lack of "coupling" between the observed increase in PGC-1α transcription and mRNA abundance and may be partly explained by changes in mRNA stability.

In conclusion, we have demonstrated that PGC-1α promoter activity is reduced in resting aging muscle concomitant with decreased signaling pathways and regulatory transcription factors. This suggests that the signals towards biogenesis are impaired within the aged muscle milieu. However, aged muscle appears to retain the ability to respond to acute exercise signals, ultimately leading to a mitochondrial adaptation when these signals are repeated chronically. This suggests that the decrements in mitochondrial content and quality that are often found in aged individuals can be rectified, at least in part, by the adoption of a strategy that includes a program of regular physical activity. Broadly, this has impact for the health of sedentary aged individuals, as gains in mitochondrial content and quality can still be brought about through the instigation of exercise.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

D.A.H. and H.N.C conceived the study; H.N.C, M.P. and L.D.T. performed experiments; D.A.H. and H.N.C analyzed data; D.A.H. and H.N.C. interpreted results of experiments; H.N.C prepared figures; H.N.C drafted manuscript; D.A.H. edited and revised manuscript; D.A.H. approved final version of manuscript.

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FIGURE LEGENDS

Figure 1. *Alterations of Mitochondrial Content and Factors Regulating PGC-1α in Aged Muscle.* **A** Mitochondrial content was assessed through the marker COX activity in young and aged TA muscle. n=14. **B** Fibre bundles from the red gastrocnemius were used to measure oxygen flux in young and aged muscle during basal and active respiration. n=7-8. **C, D** Transcription factors identified to bind putative sites on the PGC-1α promoter were assessed through immunoblotting. A representative image of each protein is presented and proteins of interest were corrected for Aciculin. Data are presented as fold change relative to young. n=4-11. **E** Protein assessment of CUGBP1, a destabilizing factor, and HuR, a stabilizing protein were assessed in young and aged extracts. Aciculin was used as a loading control. n=2. **F** PGC-1a mRNA stability in young and aged muscle. Representative ethidium bromide stained gel of amplified PGC-1α mRNA following incubation with cytosolic fractions from young or aged muscle for 0, 15 or 30 mins (top). Semi-logarithmic quantification of the experiments (bottom; n=3). The reaction in the absence of cDNA is presented as the negative control. **G** Transcript expression of PGC-1α, cytochrome oxidase subunit IV (COX IV) and mitochondrial transcription factor A (Tfam) were assessed in basal young and aged TA muscle. Data are presented as fold change relative to young. n=11-15. *$p<0.05$ aged vs. young. ¶$p<0.05$ interaction effect. Contrast and brightness changes to immunoblots were applied equally to each image. Data are presented as mean±SEM. COX, cytochrome oxidase; TA, tibialis anterior; Nrf2, Nuclear Factor Erythroid 2–Related Factor 2; USF1, Upstream Transcription Factor 1; CREB, cAMP response element-binding protein; GATA4, GATA Binding Protein 4; ATF2, Activating Transcription Factor 2; YY1, yin yang 1; CUGBP1, CUG binding protein 1; HuR, human antigen R; Y, young; A, aged.

Figure 2. *In Situ Force Generation in Young and Aged Muscle.* **A** Young and animals were anesthetized and the TA muscle was exposed and attached to a force transducer. To assess force generation capabilities the TA was stimulated via the sciatic nerve and through intramuscular field stimulation. Twitch and tetanic contractions were performed in both scenarios of each age group. n=8-16. *p<0.05 aged vs young. †p<0.05 tetanic vs. twitch. Data are presented as mean±SEM. **B** A representative tracing of progressive fatigue through *in situ* stimulation. The protocol was 40 mins in duration with increasing intensity every ten 10 minutes in the following order; 0.25 TPS, 0.5 TPS, 1 TPS and 2 TPS. TPS, trains per second; TA, tibialis anterior.

Figure 3. *Signaling with in situ Contractile Activity.* Phosphorylation of AMPK **A** and p38 **B** were assessed immediately following *in situ* contractile activity or subsequent to the recovery period. Phosphorylated protein abundance was corrected for their corresponding total protein content. The inset graph in **A** depicts attenuated signaling of AMPK in aged muscle following acute stimulation. *p<0.05 aged vs. young. †p<0.05 STIM vs. CON. n=3-8 for AMPK and p38. CON/C, control; STIM/S, in situ acute stimulation; REC/R, 2 hour recovery; AMPK, AMP-activated protein kinase; p38, p38 mitogen-activated protein kinase; P, phospho; T, total; A.U., arbitrary units. Data are presented as mean±SEM.

Figure 4. *PGC-1α Promoter Activity and Transcript Expression.* **A** An ~1.5kb PGC-1α upstream promoter driving luciferase expression was electroporated bilaterally into the TA muscles of young and aged animals. Luciferase activity was corrected for renilla luciferase to control for transfection efficiency. Basal, acute contractile activity and recovery samples were assessed for luciferase n=6-19. Inset graph depicts the delta of STIM-CON. Transcript expression of PGC-1α (n=4-13) **B** and COX IV (n=6-14) **C** were measured using qPCR TaqMan chemistry. Transcript data is presented as fold relative to young control. Assays IDs are available in Table 1. *p<0.05 aged vs. young.

†p<0.05 STIM vs. CON. RLU, relative light units; CON, control; STIM, acute *in situ* stimulation; REC, 2 hour recovery; YC, young control. n.s. not significant. Data are presented as mean±SEM.

Figure 5. *DNA Methylation and Methyltransferase Expression in Aging Muscle.* Global methylation of young and aged genomic DNA basally **A** (n=7) and with acute *in situ* stimulation and recovery **B** (n=3-7). Dnmt expression at the transcript **C** (n=6-8) and protein level **D** (n=4). Immunoblots were adjusted for contrast and brightness equally. *p<0.05 aged vs. young. CON, control; STIM, acute *in situ* stimulation; REC, 2 hour recovery; Y, young; A, aged; Dnmt, DNA methyltransferase; A.U., arbitrary unit. Transcript expression is presented as ΔΔCt relative to young. Data are presented as mean±SEM.

Figure 6. *Effects of CCA on mRNA abundance.* Young and aged animals were subjected to seven consecutive days of CCA and transcripts for PGC-1α **A** , COX IV **B**, and Nrf2 **C** were assessed using qPCR SYBR Green chemistry (n=5-6). Transcript data is presented as fold relative to young control. Primer sequences can be found in Table 1. Control and CCA are paired within each age group. *p<0.05 aged vs. young. †p<0.05 CCA vs. CON. CON, control; CCA, chronic contractile activity; YC, young control. Data are presented as mean±SEM.

Table 1. List of mRNA Primers and Assays.

Target	Forward	Reverse
mRNA assays used basally and for in situ contractile activity		
PGC-1α	TaqMan Probe Assay ID	Rn00580241_m1
COX IV	TaqMan Probe Assay ID	Rn00665001_g1
Tfam	TaqMan Probe Assay ID	Rn00580051_m1
Dnmt3a	TaqMan Probe Assay ID	Rn01027162_g1
Dnmt3b	TaqMan Probe Assay ID	Rn01536418_g1
GAPDH	TaqMan Probe Assay ID	Rn01775763_g1
β-actin	TaqMan Probe Assay ID	Rn00667869_m1
β2-Microglobulin	TaqMan Probe Assay ID	Rn00560865_m1
Primers used for mRNA evaluation of CCA animals		
PGC-1α	5’-CAT CGC AAT TCT CCC TTG TAT-3’	5’-CAG ACT CCC GCT TCT CAT ACT-3’
COX IV	5’-GGC AAG AGA GCC ATT TCT ACT T-3’	5’-GTA GTC ACG CCG ATC AAC ATA-3’
Nrf2	5’-AGT CGC TTG CCC TGG ATA TT-3’	5’-GCT CCA TGT CCT GCT GTA T-3’
S12	5'-ATG GAC GTC AAC ACT GCT CT-3'	5'-ATG CAA GCA CGC AGA GAT-3'

Table 2. List of Antibodies.

Target	Manufacturer	Product ID	Lot #
Nrf2 (NFE2L2)	Santa Cruz	sc-722	C2013
USF1	Santa Cruz	sc-8983	B2713
CREB	Cell Signaling	9192	4
GATA4	Santa Cruz	sc-9053	I1404
ATF2	Cell Signaling	9226	2
YY1	Santa Cruz	sc-7341	L0208
CUGBP1	Santa Cruz	sc-20003	B2210
HuR	Santa Cruz	sc-5261	E0710
P-AMPK$\alpha_{1/2}$	Cell Signaling	2535	16
T-AMPK$\alpha_{1/2}$	Cell Signaling	2532	19
P-p38	Cell Signaling	9211	23
T-p38	Cell Signaling	9212	23
Dnmt3b	Novus Biologicals	NBP1-40651	YJ031903DS
Aciculin	Gift	(8)	

Table 3. Animal Characteristics.

	YOUNG	AGED	Fold Difference (Aged/Young)
Body Mass (g)	430.5±7.31 (24)	480.5±12.82* (24)	1.1
TA Mass (mg)	845.9±22.95 (23)	522.5±17.98* (23)	0.62
TA/Body Mass (mg/g)	1.97±0.025 (23)	1.12±0.045* (23)	0.57
Heart Mass/Body Mass (mg/g)	2.29±0.029 (23)	2.64±0.091* (22)	1.2
Tetanic/TA Mass (mN/mg)	3.86±0.24 (23)	2.82±0.30* (20)	0.73
Tibia Length (mm)	42.6±0.4 (15)	43.5±0.42 (14)	1.02
TA mass/Tibia Length (mg/cm)	0.18±0.004 (15)	0.13±0.005* (14)	0.72

*$p<0.05$ aged vs. young. Values are reported as means ± SEM. n = number in parentheses.

Fig. 1

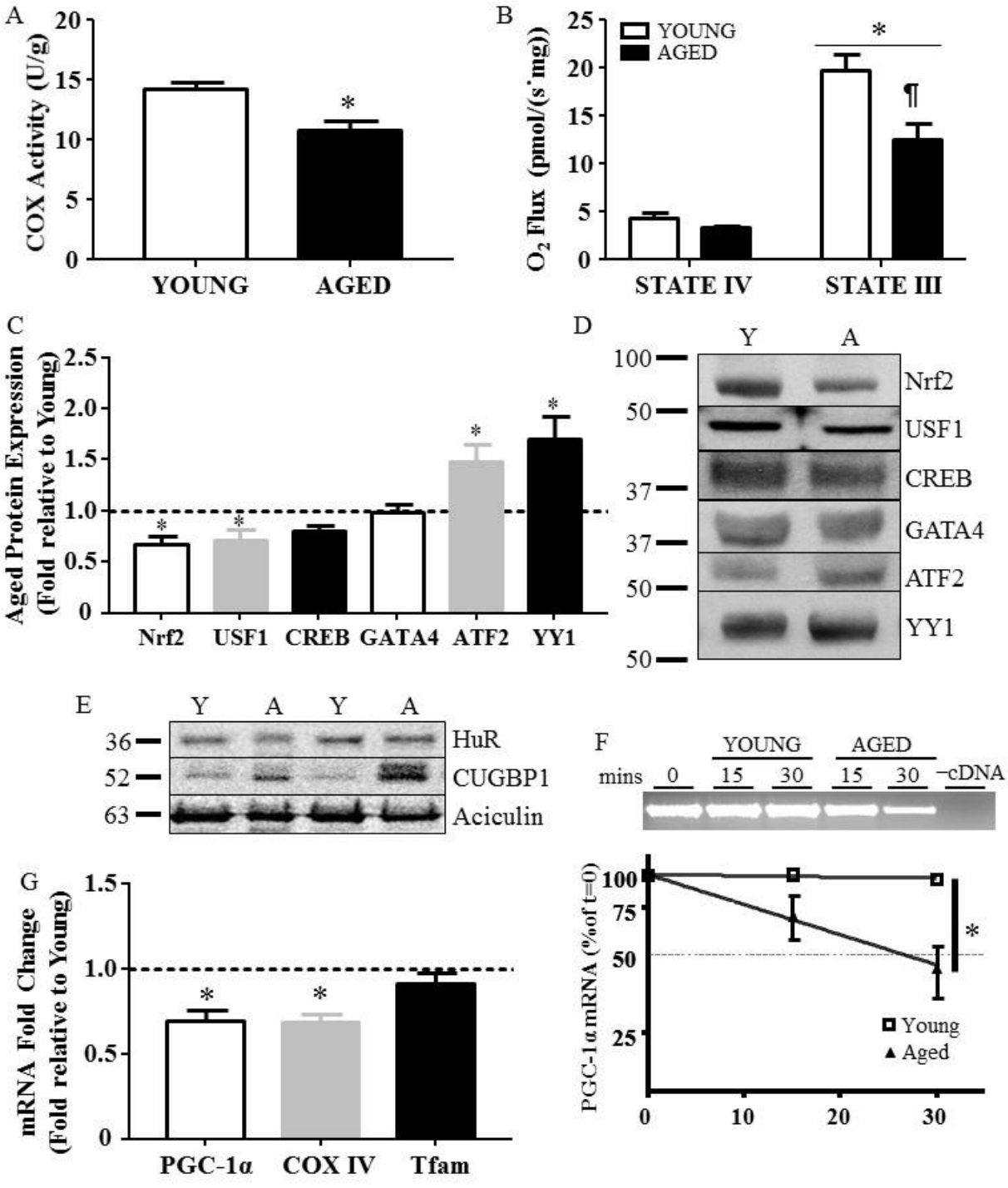
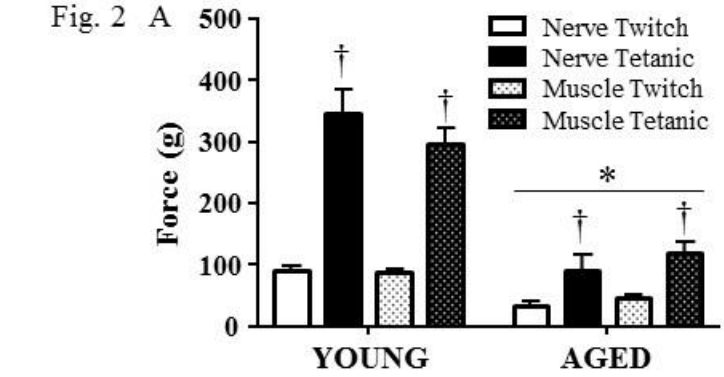

A
COX Activity (U/g)
YOUNG
AGED
B
YOUNG
AGED
O2 Flux (pmol/(s*mg))
STATE IV
STATE III
C
Aged Protein Expression (Fold relative to Young)
Nrf2
USF1
CREB
GATA4
ATF2
YY1
D
Y
A
Nrf2
USF1
CREB
GATA4
ATF2
YY1
E
Y
A
Y
A
HuR
CUGBP1
Aciculin
F
YOUNG
AGED
mins
0
15
30
15
30
−cDNA
G
mRNA Fold Change (Fold relative to Young)
PGC-1α
COX IV
Tfam
PGC-1α mRNA (%of t=0)
Young
Aged

Fig. 2 A


500
400
300
200
100
0
Force (g)
Nerve Twitch
Nerve Tetanic
Muscle Twitch
Muscle Tetanic
†
†
†
†
*
YOUNG
AGED

B

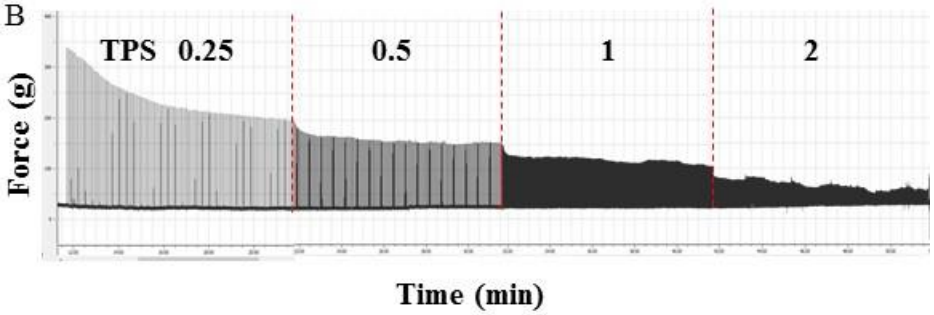
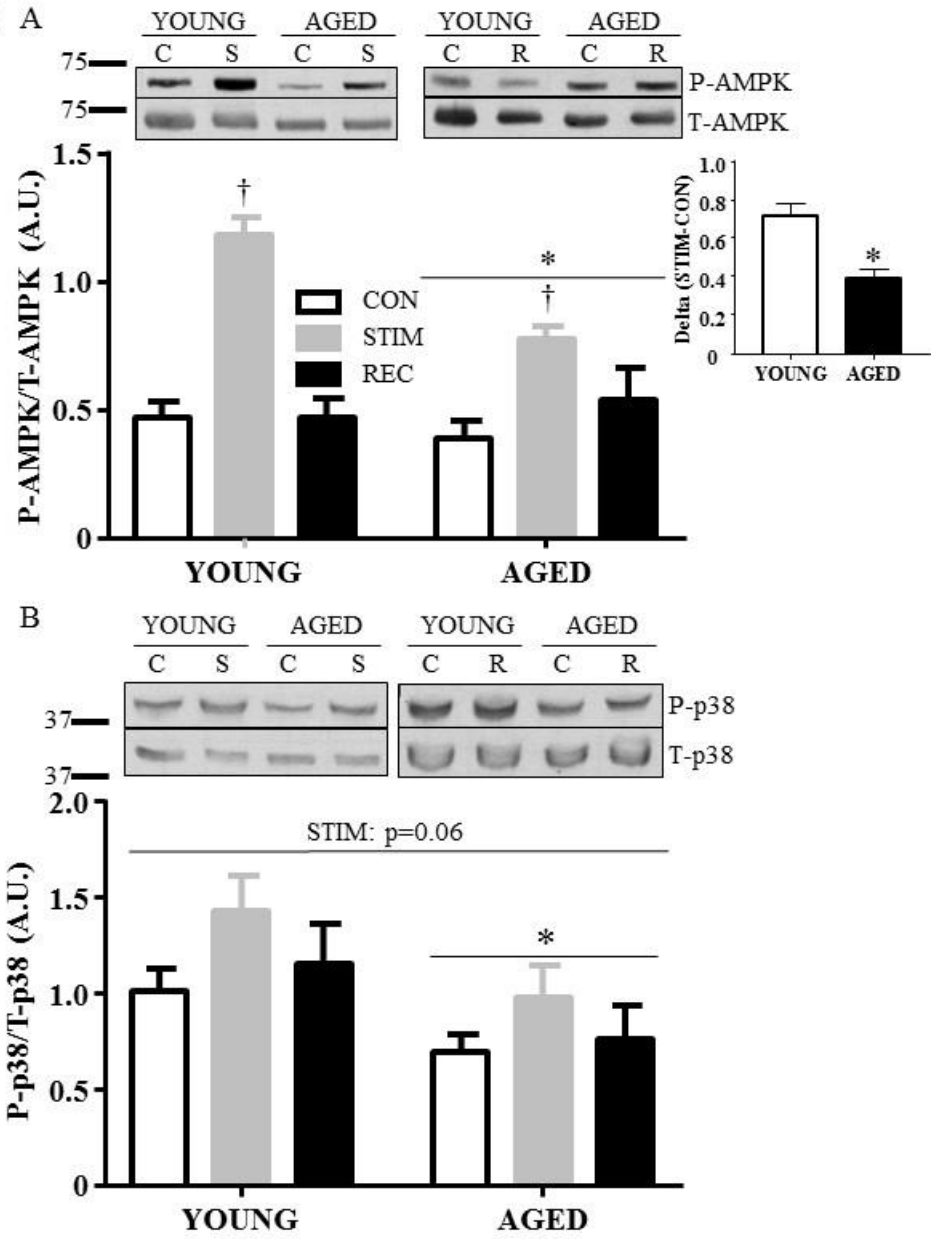

TPS 0.25
0.5
1
2
Force (g)
Time (min)

Fig. 3 A


YOUNG
AGED
YOUNG
AGED
C
S
C
S
C
R
C
R
75
75
P-AMPK
T-AMPK
P-AMPK/T-AMPK (A.U.)
1.5
1.0
0.5
0
†
*
†
CON
STIM
REC
YOUNG
AGED
Delta (STIM-CON)
1.0
0.8
0.6
0.4
0.2
0
*
YOUNG
AGED

B

YOUNG
AGED
YOUNG
AGED
C
S
C
S
C
R
C
R
37
37
P-p38
T-p38
STIM: p=0.06
P-p38/T-p38 (A.U.)
2.0
1.5
1.0
0.5
0
*
YOUNG
AGED

Fig. 4

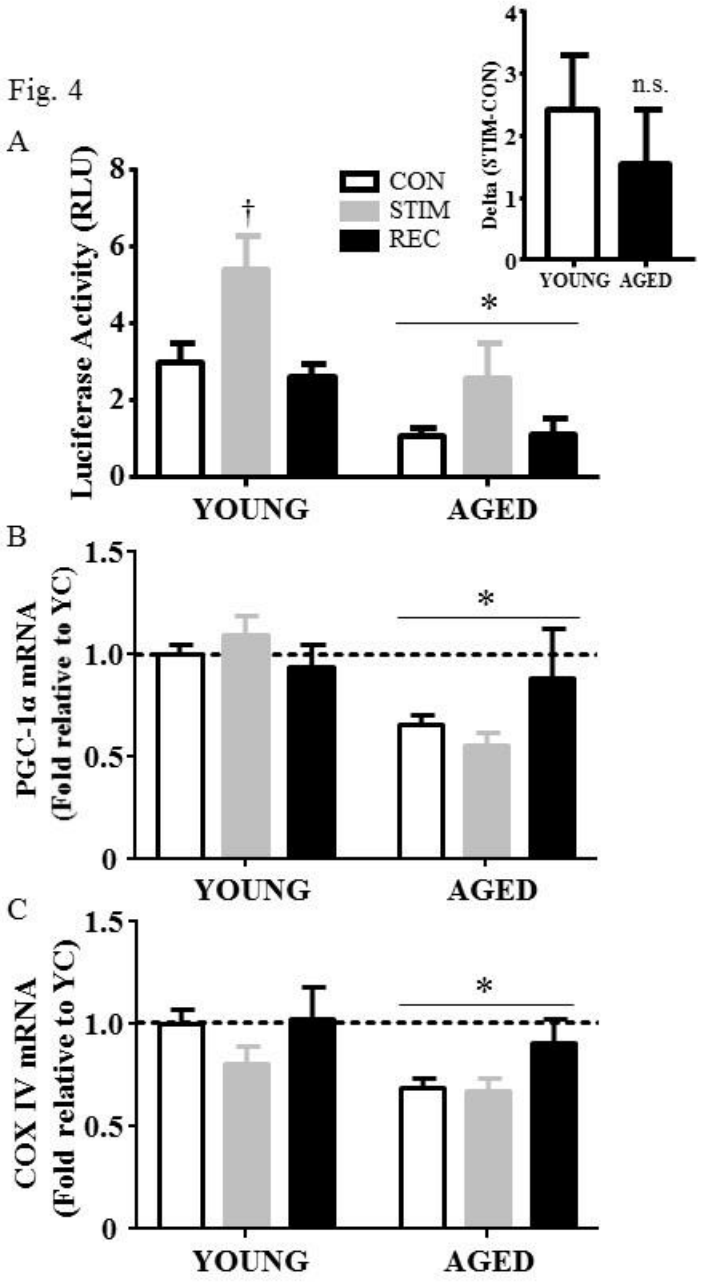

A
Luciferase Activity (RLU)
CON
STIM
REC
†
*
YOUNG
AGED
Delta (STIM-CON)
n.s.
YOUNG AGED
B
PGC-1α mRNA
(Fold relative to YC)
*
YOUNG
AGED
C
COX IV mRNA
(Fold relative to YC)
*
YOUNG
AGED

Fig. 5

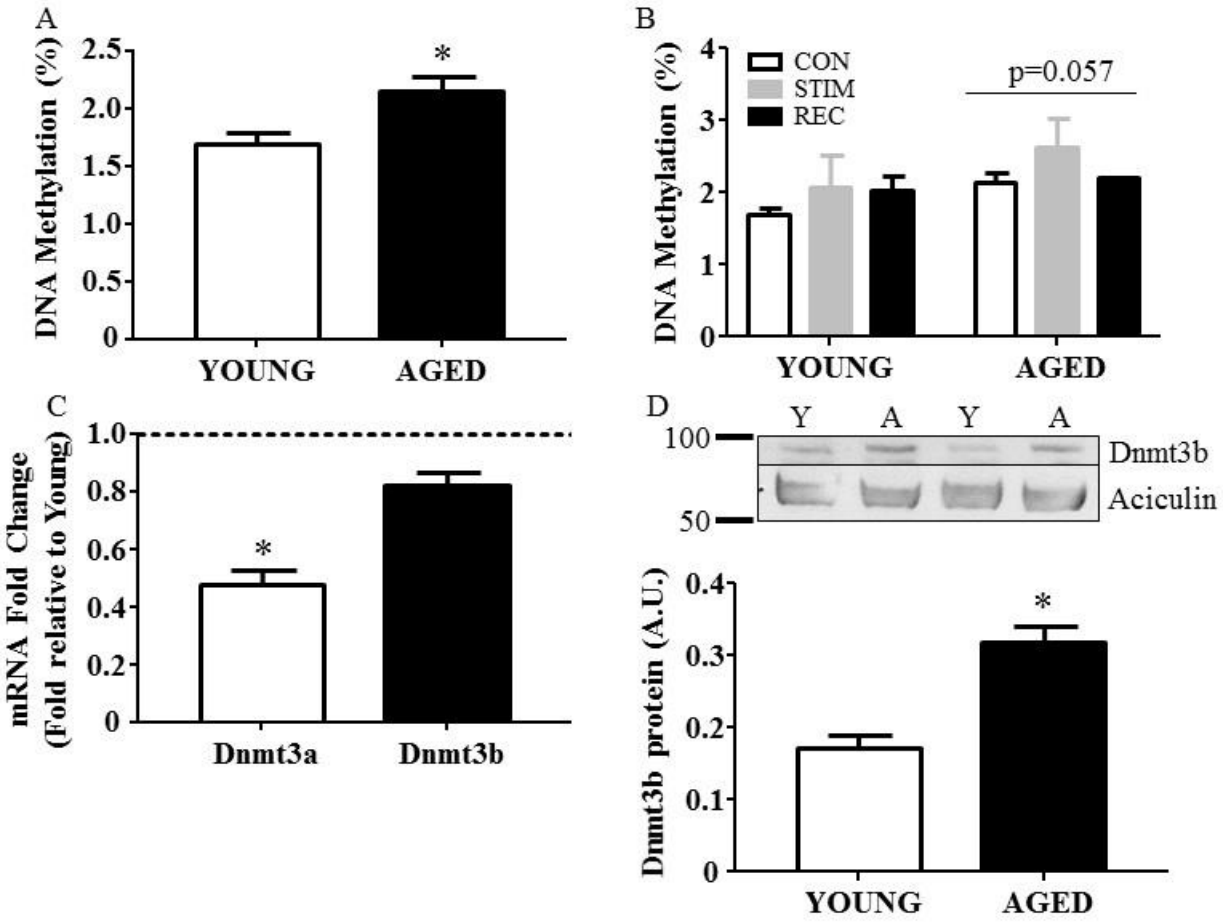
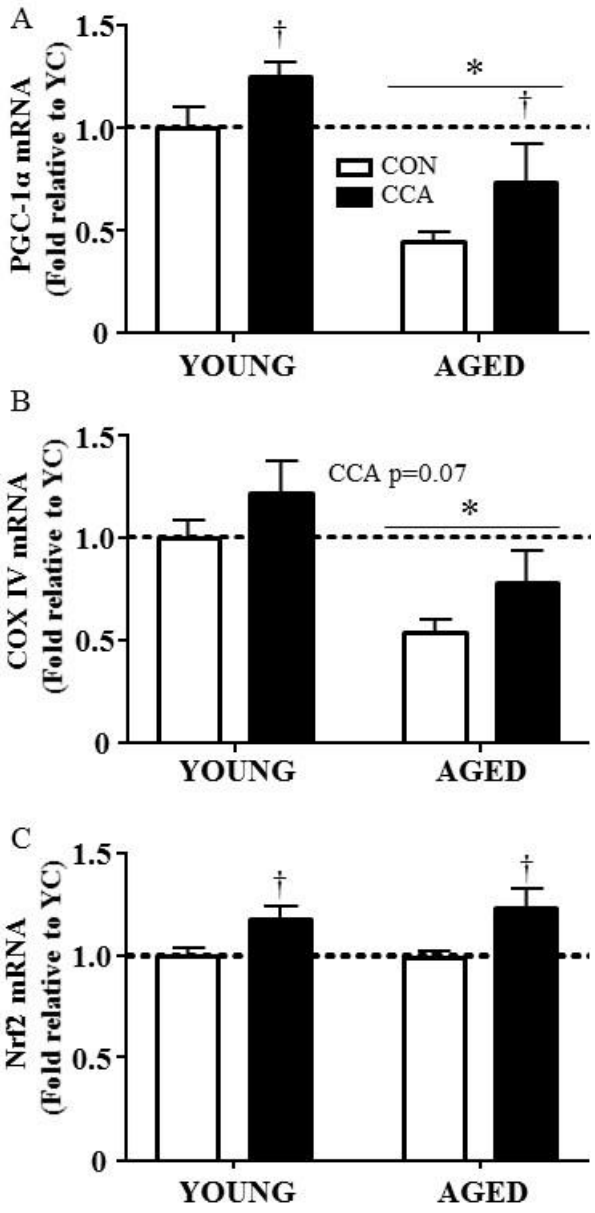

A
DNA Methylation (%)
2.5
2.0
1.5
1.0
0.5
0
*
YOUNG
AGED
B
DNA Methylation (%)
4
3
2
1
0
CON
STIM
REC
p=0.057
YOUNG
AGED
C
mRNA Fold Change
(Fold relative to Young)
1.0
0.8
0.6
0.4
0.2
0
*
Dnmt3a
Dnmt3b
D
Y
A
Y
A
100
50
Dnmt3b
Aciculin
Dnmt3b protein (A.U.)
0.4
0.3
0.2
0.1
0
*
YOUNG
AGED

Fig. 6


A
PGC-1α mRNA (Fold relative to YC)
1.5
1.0
0.5
0
†
*
†
CON
CCA
YOUNG
AGED
B
COX IV mRNA (Fold relative to YC)
1.5
1.0
0.5
0
CCA p=0.07
*
YOUNG
AGED
C
Nrf2 mRNA (Fold relative to YC)
1.5
1.0
0.5
0
†
†
YOUNG
AGED

CHAPTER FOUR

Autophagy and Mitophagy Flux in Young and Aged Skeletal Muscle Following Chronic Contractile Activity

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KEY POINTS SUMMARY

- A healthy mitochondrial pool is dependent on the removal of dysfunctional organelles via mitophagy, but little is known about how mitophagy is altered with aging and chronic exercise;
- Chronic contractile activity (CCA) is a standardized exercise model that can elicit mitochondrial adaptations in both young and aged muscle, albeit to a lesser degree in the aged group;
- Assessment of mitophagy flux revealed enhanced targeting of mitochondria for degradation in aged muscle, in contrast to previous theories;
- Mitophagy flux was significantly reduced as an adaptation to CCA suggesting that an improvement in organelle quality reduces the need for mitochondrial turnover;
- CCA enhances lysosomal capacity and may ameliorate lysosomal dysfunction in aged muscle.

ABSTRACT

Skeletal muscle exhibits deficits in mitochondrial quality with age. Central to the maintenance of a healthy mitochondrial pool is the removal of dysfunctional organelles via mitophagy. Little is known on how mitophagy is altered with aging and chronic exercise. We assessed mitophagy flux using colchicine treatment *in vivo* following chronic contractile activity (CCA) of muscle in young and aged rats. CCA evoked mitochondrial biogenesis in young muscle, with an attenuated response in aged muscle. Mitophagy flux was higher in aged muscle and was correlated with the enhanced expression of mitophagy receptors and upstream transcriptional regulators. CCA decreased mitophagy flux in both age groups, suggesting an improvement in organelle quality. CCA also reduced the exaggerated expression of TFEB evident in aged muscle, which may be promoting the age-induced increase in lysosomal markers. Thus, aged muscle possesses an elevated drive for autophagy and mitophagy which may contribute to the decline in organelle content observed with age, but which may serve to maintain mitochondrial quality. CCA improves organelle integrity and reduces mitophagy, illustrating that chronic exercise is a modality to improve muscle quality in aged populations.

Keywords: mitochondria, exercise, aging

INTRODUCTION

During the natural course of advancing age the loss of muscle mass and function, commonly referred to as sarcopenia, remains an incompletely described phenomenon (Leon, 2017; Sakuma *et al.*, 2017). Implicated in the maintenance of skeletal muscle with aging is the presence of a healthy population of mitochondria (Romanello & Sandri, 2016; Rygiel *et al.*, 2016; Alway *et al.*, 2017). These organelles are widely recognized to produce the bulk of cellular energy, regulate metabolism, act as mediators for apoptosis and participate in a plethora of cellular signaling cascades. With engagement in habitual aerobic exercise, expansion of the mitochondrial reticulum and improved integrity of the organelles in skeletal muscle has been well described to occur in young, healthy individuals (Holloszy, 1967; Hoppeler *et al.*, 1985; Menzies *et al.*, 2013).

Although controversial, aging muscle often presents with a population of compromised mitochondria. Observed impairments include reduced organelle content (Chabi *et al.*, 2008; Ljubicic *et al.*, 2009; O'Leary *et al.*, 2013; St-Jean-Pelletier *et al.*, 2017), impaired transcription of PGC-1α (Carter *et al.*, 2018), altered synthesis of mitochondrial proteins (Rooyackers *et al.*, 1996; Miller *et al.*, 2012), poor respiratory function (Conley *et al.*, 2000; Ljubicic *et al.*, 2009), higher reactive oxygen species (ROS) emission (Chabi *et al.*, 2008; Ljubicic *et al.*, 2009), loss of $\Delta\Psi_m$ (Chabi *et al.*, 2008), decreased calcium retention (Gouspillou *et al.*, 2014), increased mtDNA mutational load (Melov *et al.*, 1995) and greater release of pro-apoptotic proteins (Chabi *et al.*, 2008; Gouspillou *et al.*, 2014). These changes may potentially contribute to the observed decline in muscle performance with aging, however it remains unclear if these changes are due solely to aging or are a product of changes in physical activity (Kent-Braun & Ng, 2000; Picard *et al.*, 2011; Joseph *et al.*, 2012; Hepple, 2014; Leduc-Gaudet *et al.*, 2015; St-Jean-Pelletier *et al.*, 2017).

Furthermore, while beneficial, exercise training may not produce the same magnitude of mitochondrial adaptation in aged muscle when compared to younger cohorts (Carter *et al.*, 2015; Hood *et al.*, 2016), however controversy on this topic remains (Robinson *et al.*, 2017). Previous work has detailed that the signaling towards nuclear genes encoding mitochondrial proteins (NuGEMPs) is blunted, suggesting that mitochondrial biogenesis remains low in aged muscle (Ljubicic & Hood, 2009). While intact mitochondrial biogenesis is essential to produce/maintain healthy organelles, the removal of damaged/dysfunctional organelles also has a critical role in the health of mitochondria and skeletal muscle. This selective removal of mitochondria is termed mitophagy, and is part of the larger, evolutionarily conserved autophagy pathway. Our understanding of autophagy and mitophagy and the role they play in skeletal muscle health with exercise and aging, remains in its infancy.

Autophagy is an intracellular recycling mechanism, whereby damaged or redundant cellular components are engulfed in a double membrane structure called the autophagosome, which is ultimately delivered to the lysosome for digestion of its contents by pH-sensitive enzymes. Through the use of genetic ablation of critical autophagy genes, it has been described that intact autophagy is necessary to preserve muscle mass (Masiero *et al.*, 2009), and the degeneration of muscle that occurs in the absence of autophagy is reminiscent of the maladaptive changes that occur with aging (Carnio *et al.*, 2014). Indeed, the literature often discusses the concept that autophagy declines with aging, contributing to deleterious organ and tissue health through aggregation of cellular debris (Brunk & Terman, 2002; Terman *et al.*, 2006, 2010; Rubinsztein *et al.*, 2011; Kroemer *et al.*, 2015; Sakuma *et al.*, 2016; Sebastián *et al.*, 2016). However, many of these ideas are extended from observations noted in lower organisms or in non-muscle tissue, and whether the same conclusions can be drawn for skeletal muscle remains unknown. In aged skeletal

muscle, it has been documented that the expression of many autophagy proteins increases (O'Leary *et al.*, 2013; Sakuma *et al.*, 2016). Furthermore, there is an increased presence of the mitophagy receptor Parkin on isolated organelles (O'Leary *et al.*, 2013). However, we now appreciate that the documentation of changes in upstream autophagy proteins levels provides a less than complete picture of the process of autophagy within cells. A difficultly in assessing autophagy and mitophagy *in vivo* is that these are highly dynamic processes, and static measures are often subject to discrepancies in interpretation (Klionsky, 2016; Yoshii & Mizushima, 2017). Thus, measures of autophagosome flux are required, and an accepted technique for this obligates the use of an autophagy-inhibited condition for all experimental conditions, along with the corresponding vehicle controls, to allow for the calculation of flux using isolated organelles (Ju *et al.*, 2010). However, no studies to date have yet to capture the dynamic process of autophagy/mitophagy flux in aging skeletal muscle with exercise.

In addition, very little information has been garnered about the effects of chronic exercise on autophagy and mitophagy, particularly in aged muscle. Recent evidence has demonstrated that autophagy and mitophagy flux increase subsequent to a single bout of acute exercise in young, untrained animals (Grumati *et al.*, 2011; He *et al.*, 2012; Vainshtein *et al.*, 2015*b*; Laker *et al.*, 2017). This is likely a precipitating factor for the eventual remodelling of the mitochondrial network that would occur with repeated, successive bouts of exercise. However, how autophagy and mitophagy flux adapt to exercise following training has not been conclusively described. Two studies have begun to examine mitophagy with chronic exercise training in a young cohort (Lira *et al.*, 2013; Ju *et al.*, 2016) and both of these studies have concluded that autophagy flux was higher following exercise training. However, only one study used an autophagy inhibitor to capture the dynamic turnover of autophagosomes through autophagy, and neither study isolated

mitochondria to examine the rate at which mitochondria may be targeted for mitophagy following training. Thus, the purposes of our study were 1) to examine autophagy and mitophagy autophagosome flux in aged muscle, and 2) to understand how flux may adapt following chronic contractile activity (CCA) in skeletal muscle of young and aged animals.

MATERIALS AND METHODS

Animals: All animal procedures were conducted in strict accordance with the standards set by the Canadian Council on Animal Care and with the approval of York University Animal Care Committee (YUACC). Young (5-6 months) and aged (35-36 months) male Fisher 344 Brown Norway F1 Hybrid rats were obtained from the National Institute of Aging (NIA, Bethesda, MD, USA). Upon arrival, animals were acclimated to the facility in accordance with YUACC protocol guidelines. Food and water were provided *ad libitum* and food intake for each age group was monitored for two weeks subsequent to the acclimation period. Animal characteristics are described in Table 1.

Chronic contractile activity: Animals were sedated with isoflurane anesthesia and surgical implantation of a stimulator unit was performed as previously described (Adhihetty *et al.,* 2007; Ljubicic *et al.*, 2009). Oral temperature of the animals was continuously monitored during the procedure using a CODA monitor (Kent Scientific, Torrington, CT, USA) and a heating pad was used to keep body temperature stable at ~37ºC. The fur was shaved over the caudal rib cage as well as over the left hind limb. Antiseptic (Povidone-iodine 10%) was applied to the exposed skin and all surgical procedures were performed under aseptic conditions. The first incision (1-2cm) was made vertically between the last rib and pelvic girdle. A second incision was made horizontally over the cephalic aspect of the left hind limb. Blunt dissection liberated the skin from underlying tissues and a subcutaneous passage was made from the vertical incision to the

horizontal incision. The abdominal musculature was lifted with forceps and cut. The opening was large enough to accommodate insertion of the stimulator unit. The sterile stimulator unit [purchased from JC Jarvis, Liverpool, U.K.; (Jarvis & Salmons, 1991; Salmons & Jarvis, 1991; Mayne *et al.*, 1993)] was inserted into the abdominal cavity and placed caudally toward the ilium with the Dacron mesh facing towards the incision. The wires were passed through the subcutaneous tunnel to the left hind limb. The deep musculature of the abdominal wall was closed with the Dacron mesh incorporated into the suture line with sterile 5.0 silk suture. The superficial musculature was closed with separate sutures and the skin was stapled together. Each muscle and skin layer received antibiotic treatment. At the hind limb, the biceps femoris was lifted with forceps and cut. Gentle blunt dissection was used to locate the peroneal nerve. The loop ends of the electrodes were affixed to the deep musculature, with one wire flanking each side of the peroneal nerve. Using a digital stroboscope, the stimulation unit was turned on and the tibialis anterior (TA) and extensor digitalis muscles (EDL) were palpated to ensure muscle recruitment. The stimulation unit was turned off and the muscle incision at the left hind limb was carefully closed with sutures so as not to disrupt the electrodes. The skin was stapled closed and antibiotic was applied to both layers. Animals were given pain medication (Meloxicam, 2mg/kg) subcutaneously for three consecutive days with the dose decreasing by half each day. Antibiotic (amoxicillin) was also provided in the drinking water for 7 days (0.3mg/L). Animals were closely monitored for mobility and behaviour. After one week of recovery, the CCA protocol began. The left TA and EDL were stimulated for 3h per day (9am-12pm) for nine consecutive days with 21 hours of recovery between bouts, while the contralateral limb served as an internal control (CON). To inhibit autophagy, sterile solutions of colchicine (COL;0.4mg/kg/day; Sigma, Oakville, ON, CAN) or vehicle (VEH; 0.9% saline) were injected into the intraperitoneal cavity during the last three days of CCA.

Animals were randomly assigned to the treatment groups. No signs of distress as a result of the injections were observed. Moreover, this duration of colchicine treatment is sufficient to inhibit autophagy for the measurement of flux (Ju *et al.*, 2010; Mofarrahi *et al.*, 2013; Vainshtein *et al.*, 2015*a*, 2015*b*), yet will not induce myopathy, which is generally observed with treatments lasting 10 days or greater at the same dosage (Ching *et al.*, 2013). Animals were anesthetized with isoflurane between 8:30-9am, 21 hours after the last stimulation period, and relevant tissues were removed and weighed. Animals were killed by exsanguination after median sternotomy.

Mitochondrial isolations: IMF mitochondria were isolated from the distal 2/3 of the TA as previously described (Chabi *et al.*, 2008; Ljubicic *et al.*, 2009). Briefly, tissue was minced and subjected to mechanical homogenization, differential centrifugation and protease treatment (nagarse; 0.025ml/g; Sigma, Oakville, ON, CAN) to liberate the organelles from the dense myofibril network. Mitochondrial pellets were resuspended in ice-cold buffer (100 mM KCl, 10 mM MOPS, 0.2% BSA) supplemented with two phosphatase inhibitor cocktails (Sigma, Oakville, ON, CAN) as well as protease inhibitors (Roche, Mississauga, ON, CAN). Samples were stored at -80ºC until later use.

Protein extraction and western blotting: The proximal one-third of the TA was snap frozen in liquid nitrogen upon removal and stored at -80ºC. The tissue was pulverized to a fine powder at the temperature of liquid nitrogen. Protein extracts were made by combining a small amount of powder with extraction buffer (20mM HEPES, 2mM EGTA, 1% Triton-X 100, 50% glycerol and 50mM β-Glycerophosphate) supplemented with protease (Roche, Mississauga, ON, CAN) and phosphatase (Sigma, Oakville, ON, CAN) inhibitor cocktails, rotating end-over-end for 1 hour at 4ºC followed by sonication on ice. The solution was spun in a microfuge at 4ºC for 15 min and the supernate was collected. Protein concentration was determined by the Bradford method. Equal

amounts of protein (20-40µg) were loaded into SDS-PAGE gels for separation. Protein was wet transferred to nitrocellulose membrane (Bio-Rad, Mississauga, ON, CAN) and subsequently blocked for 1 hour in 5% skim milk powder (w/v) dissolved in tris-buffered saline with Tween-20 (TBST). Primary antibodies were incubated on the membrane overnight at 4ºC. The next day the membrane was washed 3X for 5 minutes each, with TBST and incubated at room temperature with the appropriate secondary antibody conjugated to horse-radish peroxidase (Santa Cruz, Mississauga, ON, CAN). The protein density was visualized using enhanced chemiluminescence (Bio-Rad, Mississauga, Ontario, CAN) on film or with an ImageStation 4000MM Pro (Carestream, Concord, ON, CAN). Long and short exposures were performed to isolate the linear range. Primary antibodies are detailed in Table 2.

Autophagy and mitophagy flux calculation: To determine the relative degree of autophagosome turnover or mitochondrial-targeted turnover (mitophagy), colchicine and vehicle treatment conditions were employed. Western blotting of LC3-II and p62 were performed in whole muscle extracts or IMF mitochondrial subfractions (isolated as described above) with all conditions represented on one SDS-PAGE gel. Protein abundance was quantified from blots using Image J and values were corrected for corresponding loading controls (aciculin for whole muscle extracts, VDAC for mitochondria). The mean CON values were subtracted from the COL values of corresponding conditions (e.g. young CON COLC – mean young CON VEH) to yield autophagosome flux values.

High resolution respirometry: Half of the EDL muscle was used for high resolution respirometry (Oroboros O2k, Austria). Briefly, the instrument chambers were calibrated prior to sample isolation. The muscle sample was excised and immediately placed in ice-cold BIOPS buffer (10 mM Ca-EGTA, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM

DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) followed by gentle mechanical separation of small muscle fibre bundles using fine forceps under a dissection microscope while on ice. Fibre bundles were incubated at 4ºC for 30 mins in saponin (50µg/mL) with gentle rocking. Fibre bundles were washed and weighed then added to the oxygen chamber in Miro5 buffer (110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, pH 7.1, and 0.1% BSA). The chambers were hyperoxygenated with 100% O_2 followed by stabilization for a background reading at a temperature of 37°C. Glutamate (10mM) and malate (2mM) were added followed by ADP (2.5mM) to assess basal and maximal Complex I-stimulated respiration. Following this, succinate (10mM) was added to assess Complex I and II maximal respiration. Oxygen flux values were corrected for background readings and fibre mass. Respiratory control ratios were assessed to ensure quality of the fibres and mitochondrial respiration.

COX activity: As previously described (Chabi *et al.*, 2008; Ljubicic *et al.*, 2009; O'Leary *et al.*, 2013), muscle lysates from young and aged TA were generated and incubated in the presence of fully reduced cytochrome c (Sigma, Oakville, ON, CAN). Equal amounts of sample were incubated with the reduced cytochrome c solution and the rate of oxidation over time was observed as the change in absorbance at 550nm using a plate reader (Synergy HT, Bio-tek, Thorold, ON, CAN).

Electron microscopy: Cubes (2 mm^2) from the control and CCA EDL muscles were placed in a 2% glutaraldehyde solution in 0.1 mM sodium cacodylate, pH 7.3. Samples were embedded, stained and cut at the Advanced Imaging Centre at The Hospital for Sick Children (Toronto, ON, CAN) using standard procedures. About 35-40 images for each condition (n=2) were captured on a FEI Tecnai 20 transmission electron microscope.

Histochemistry: The remainder of the EDL was placed in Optimal Cutting Temperature compound (OCT) and frozen in isopentane cooled on liquid nitrogen. When completely frozen, the samples were stored in the -80ºC until future use. Samples were thawed to -20ºC and mounted on a chuck in a cryostat (Cryotome SME; ThermoFisher, Mississauga, ON, CAN) with OCT. Samples were oriented for cross-sectional cuts, cut in quadruple 10µm thick sections, and transferred to clean microscope slides. All experimental conditions were represented on each microscope slide. Slices were then stained for cytochrome oxidase (COX) or succinate dehydrogenase (SDH) and dried as previously described (Menzies *et al.*, 2013; Vainshtein *et al.*, 2015*a*). Samples were covered with a thin glass coverslip and mounting media. Cross sections were imaged at 10x magnification using a Nikon Eclipse 90i upright microscope.

Statistics: Statistics were assessed using GraphPad Prism 6 software. A student's t-test or two-way ANOVA with Tukey post-hoc test were used where applicable. Due to unequal group sizes repeated measures assessment or a three-way ANOVA, was unable to be performed. For Figures 4 and 6, separate two-way ANOVAs were performed for 1) vehicle-treated young and aged with CCA and 2) colchicine-treated young and aged with CCA.

RESULTS

Effect of aging and CCA on mitochondrial content: Aged animals were significantly larger than the young group and contained larger fat mass ($p<0.05$; Table 1), although no changes in food intake were noted. Additionally, aged animals had larger hearts, indicative of modest hypertrophy ($p<0.05$; Table 1). Examination of muscle mass (TA and EDL) revealed that aged muscle was significantly smaller compared to muscle from the young animals ($p<0.05$, Table 1). No change in muscle mass was noted following CCA or with colchicine injections in either age group. To confirm the induction of mitochondrial biogenesis in the vehicle-treated age groups, COX enzyme

activity was assessed as a representative measure of mitochondrial content. In young animals, CCA induced a 1.7-fold increase ($p<0.05$) in COX activity versus the contralateral muscle (Fig. 1A). Furthermore, post-hoc analysis revealed COX activity in young animals following CCA was significantly higher than in aged CON and CCA muscle by 2-fold and 1.5-fold, respectively ($p<0.05$, Fig. 1A). PGC-1α protein expression was enhanced in young VEH-treated muscle following CCA by 1.6-fold ($p<0.05$, Fig. 1B). In contrast, CCA did not produce a significant enhancement of this key transcriptional coactivator in aged VEH-treated muscle (Fig. 1B).

In support of these biochemical findings, qualitative histochemical staining for SDH in VEH-treated muscle revealed similar observations as reflected by darker staining of fibres in young CCA muscle. A darker shade of SDH staining was also visible in aged muscle subject to CCA compared to the respective control. It is interesting to note that the aged muscle contained many fibres with much more variable cross-sectional area (Fig. 1C). Additionally, qualitative electron micrographs of VEH-treated muscle demonstrated enhanced thickness of the SS layer (yellow arrow) and IMF density (green arrow) following CCA in young animals (Fig. 1D). With aging, the SS layer became notably thinner and less dense, however CCA produced a modest enrichment of this SS layer (yellow arrow) in aged muscle consistent with SS mitochondrial biogenesis. These results are similar to our previous findings (Iqbal *et al.*, 2013).

Using permeabilized muscle fibres from VEH-treated animals, basal State IV respiration was decreased with age (main effect, $p<0.05$, Fig. 1E). No change was observed with CCA for basal respiration in either age cohort (Fig. 1E). Active, Complex I-stimulated State III respiration was also diminished with age (main effect, $p<0.05$, Fig. 1F). The same effect was observed for Complex I + II-stimulated State III respiration (Fig. 1G). CCA also induced elevations ($p<0.05$)

in State III Complex I-stimulated respiration in both age groups (Fig. 1F). When these respiration data were normalized to COX activity, no significant differences among the conditions were noted (data not shown), indicating that the effects of age and CCA are reflective of changes in mitochondrial content per gram of muscle, rather than a result of alterations in mitochondrial composition.

Alterations in upstream autophagy regulators: Aging resulted in a significant increase in forkhead box O3 (FoxO3) level in skeletal muscle. However, FoxO3 remained unaffected by CCA, regardless of age (Fig. 2A, B). A main effect of CCA was observed on Beclin 1 protein expression, which was particularly evident by the 2.1-fold increase observed in young muscle ($p<0.05$; Fig. 2A, C). Aging also produced a large induction of Beclin 1 ($p<0.05$) compared to young muscle in both CON and CCA conditions. p53 was undetectable in whole muscle extracts of young animals (data not shown), but was significantly enriched in aged muscle. CCA led to a marked 1.7-fold increase ($p<0.05$) in aged muscle (Fig. 2D).

Autophagosome turnover in young and aged muscle: No change in microtubule-associated proteins 1A/1B light chain 3-II (LC3-II) protein expression was observed following CCA in young muscle (Fig. 3A, B). Aged muscle displayed greater LC3-II levels (main effect, $p<0.05$) than young muscle (Fig. 3A, B). Following CCA, no change in LC3-II levels were noted in either young or aged muscle. As expected, colchicine treatment significantly enhanced LC3-II accumulation in both young and aged muscle, ranging from 1.9 to 2.5-fold (main effect, $p<0.05$; Fig. 3A, B) compared to respective vehicle-treated conditions. With age, there was a greater accumulation of LC3-II with autophagy inhibition compared to young muscle with colchicine treatment ($p<0.05$). Interestingly, calculation of LC3-II autophagosome flux (COL-VEH), revealed no alterations in

young muscle after CCA (Fig. 3C). However, aging muscle presented with increased autophagic flux ($p<0.05$), which also remained unchanged following CCA (Fig. 3C).

In agreement with the autophagy flux calculations, no difference was detected in the LC3-II/LC3-I ratio after CCA compared to CON in young muscle (Fig. 3D). A 1.6-fold elevation in the LC3-II/LC3-I ratio was detected in aged muscle basally compared to young resting muscle ($p<0.05$). This ratio remained unchanged in aged muscle in response to CCA. In agreement with LC3-II protein expression, we observed a predictable increase in the LC3-II/LC3-I ratio (main effect, $p<0.05$) under colchicine-treated conditions in both young and aged muscle, regardless of CCA. Neither CCA nor colchicine had an effect on LC3-I levels in either age group. However, there was a main effect of aging to increase the expression of LC3-I ($p<0.05$; Fig. 3E).

Mitophagy markers with aging and CCA: In young muscle, CCA increased BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) expression by 1.9-fold ($p<0.05$, Fig. 4A, B). Aging produced a marked elevation in BNIP3 protein expression by 4.8-fold ($p<0.05$) and this remained unchanged following CCA. BNIP3-like (BNIP3L/NIX) protein levels were not altered after CCA in young muscle (Fig. 4A, C). Similar to BNIP3, a significant 4.0-fold accumulation of NIX was noted in aged, resting muscle, and these high levels persisted following CCA (Fig. 4A, C). The expression of Parkin in whole muscle extracts exhibited a trend for an increase in aging muscle ($p=0.06$) and remained unaffected by CCA in both age groups (Fig. 4D). Post-hoc analysis of Optineurin revealed a dramatic enhancement by 3.9-fold ($p<0.05$) with CCA in young muscle compared to its respective CON (Fig. 4A, E). Optineurin levels were elevated basally in aged CON muscle by 2.7-fold ($p<0.05$), compared to young CON muscle. Optineurin levels were unresponsive to CCA in the muscle of aging animals, in contrast to the large CCA-induced

enhancement found in young muscle (Fig. 4A, E). Both age and CCA significantly increased the levels of Optineurin above that found in young CON muscle ($p<0.05$).

Mitophagy in young and aged muscle with CCA: Localization of LC3-II to IMF mitochondria was not changed following CCA in young muscle (Fig. 5A, B). Similarly, no change in LC3-II positive IMF mitochondria was noted with aging, irrespective of CCA when compared to young (Fig. 5A, B). Colchicine-mediated autophagy inhibition resulted in significant accumulations of LC3-II on both young and aged mitochondria compared to vehicle, ranging from 1.4- to 3.6-fold (main effect, $p<0.05$; Fig. 5B). IMF mitochondria from aged muscle accumulated more LC3-II following colchicine treatment (main effect, $p<0.05$) when compared to the young colchicine-treated group. Notably, CCA caused a significant decrease in colchicine-induced LC3-II accumulation in both young and aged IMF mitochondria by ~20% ($p<0.05$; Fig. 5B). Calculation of LC3-II flux revealed a 1.6-fold enhancement ($p<0.05$) of basal mitophagy in aged, as compared to young muscle (Fig. 5C). Interestingly, CCA produced decreases ($p<0.05$) in LC3-II IMF flux compared to CON muscle by 40% and 24% in young and aged muscle, respectively (Fig. 5C).

p62 localization to IMF mitochondria was very low in muscle of young animals, and accumulated to significantly higher levels on mitochondria from aged muscle ($p<0.05$, Fig. 5D, E). CCA elicited 40-90% reductions in p62 localized to IMF mitochondria in young and aged muscle in the absence of colchicine. Colchicine treatment resulted in marked increases of p62 between 6.6- to ~200-fold in both age groups ($p<0.05$; Fig. 5E). CCA had no significant effect on p62 mitophagy flux in young muscle (Fig. 5F). With aging, a 1.6-fold elevation ($p<0.05$) in IMF p62 flux was observed, and this remained unchanged following CCA. The localization of Parkin and Optineurin to IMF mitochondria was unaffected by either CCA, or with age (Fig. 5G).

Lysosomal alterations with aging and CCA: Divergent responses were observed for lysosomal-associated membrane proteins -1 and 2 (LAMP-1/2), two abundant lysosomal membrane proteins. LAMP-1 protein was induced in response to CCA in both young and aged muscle (main effect, $p<0.05$; Fig. 6A, B). No significant difference in LAMP-1 was observed in aged, compared to young muscle (Fig. 6B). In contrast, LAMP-2 was not significantly altered by CCA in either age group, but exhibited an enhanced accumulation in aged muscle by 4.7-fold ($p<0.05$; Fig. 6A, C). In this study, CCA produced no significant change in TFEB content in young muscle, however aging alone led to an exaggerated 2.8-fold ($p<0.05$) increase in TFEB protein in comparison to young CON (Fig. 6A, D). Interestingly, with the addition of CCA in the aged group, this increase was no longer present (Fig. 6D). Cathepsin D expression was not altered by CCA in either young or aged muscle, however aging muscle displayed a marked ~3-fold increase ($p<0.05$), compared to young muscle (Fig. 6A, E).

DISCUSSION

With advancing age, there is a loss of muscle mass and performance. Identification of the potential cellular and molecular regulators that underlie this maladaptive phenomenon is critical as the number of aged individuals in the population continues to increase. Understanding the mechanisms that contribute to sarcopenia would be insightful to tailor beneficial strategies, such as exercise, to help preserve well-being into the advanced years.

Mitochondria are essential organelles that have been documented to exhibit dysfunctional properties in aged muscle (Carter *et al.*, 2015). While debated, these organelles have been associated with the maladaptive features of sarcopenia and remain under intense investigation in the literature. It has been documented that the generation and maintenance of mitochondria

(biogenesis) in muscle with advancing age is hampered (Ljubicic & Hood, 2009; Ljubicic *et al.*, 2009). Additionally, subsequent to paradigms of exercise training, aged muscle appears to harbour a lower capacity for the generation of new, healthy organelles than younger counterparts (Hood *et al.*, 2016). Although this remains controversial, recent work has indicated that aged muscle can increase the transcription of PGC-1α in response to contractile activity (Carter *et al.*, 2018), as well as enhance its translational capacity in response to a training paradigm (Robinson *et al.*, 2017). Nonetheless, little research has focussed on the selective removal of organelles through mitophagy, a process also critical to maintain organelle and muscle integrity. Furthermore, since mitophagy is highly dynamic, capturing the turnover or "flux" of this process is paramount, rather than relying on snapshot protein measurements of pathway components, or of the LC3-II/LC3-I ratio (Mizushima & Yoshimori, 2007; Klionsky, 2016; Castets *et al.*, 2016; Yoshii & Mizushima, 2017). Thus, this study had two central aims: 1) to directly determine how autophagosome/mitophagy flux is changed in aged muscle, and 2) to determine how autophagosome/mitophagy flux adapt in response to a model of chronic exercise in both young and aged animals.

To gain insight into these aims, we utilized an established rodent model of aging (Lushaj *et al.*, 2008; Ballak *et al.*, 2014), the Fisher 344 Brown Norway Hybrid rat and compared young (6 months) to aged (35-36 months) animals. The aged cohort of this strain displayed hallmark features of aged muscle including reduced muscle mass, lower mitochondrial content, decreased respiration and the presence of lipofuscin. Additionally, previous studies utilizing this model have characterized selected dysfunctional properties of mitochondria, including increased ROS production and greater apoptotic susceptibility (Chabi *et al.*, 2008; Ljubicic *et al.*, 2009).

Autophagy occurs at low levels basally, and is known to be upregulated with a variety of stimuli, including acute exercise (He *et al.*, 2012; Vainshtein *et al.*, 2015*b*). In order to capture the degree of autophagosome turnover through autophagy and mitophagy in young and aged muscle, we employed the autophagy inhibitor, colchicine. Colchicine disrupts microtubules and impairs the ability of autophagosomes to travel to the lysosome for fusion and degradation of their sequestered contents (Amenta *et al.*, 1977; Ju *et al.*, 2010). Our preliminary data using electron microscopy indicated that autophagy inhibition through colchicine treatment produces an increased level of vacuolar inclusions within both young and aged muscle, as expected, but no disruption in sarcomere structure which might limit muscle contractility (Carter et al. unpublished observations). This drug has previously been used successfully for the measurement of autophagy and mitophagy flux in skeletal muscle (Ju *et al.*, 2010, 2016; Mofarrahi *et al.*, 2013; Vainshtein *et al.*, 2015*a*, 2015*b*; Baehr *et al.*, 2016). Thus, we compared the difference in the levels of LC3-II in colchicine-treated to vehicle-treated conditions to provide us with an approximation of the amount of autophagosomes with cargo, such as mitochondria, destined for lysosomal degradation. In the case of mitophagy, the isolation of mitochondria and subsequent western blotting for LC3-II reveals an approximation of the degree of organelles targeted for selective sequestration and recycling. Pilot work in Sprague-Dawley rats with 3 days of colchicine injections, followed by the assessment of LC3-II accumulation revealed the success of the method. We observed significant increases in the amount of LC3-II in both whole muscle and isolated mitochondria, compared to vehicle-treated animals. Overall, these observations confirm that colchicine was effective in blocking the autophagy pathway in rat skeletal muscle.

We employed chronic contractile activity (CCA) as a model of exercise training to assess the response of autophagy/mitophagy. We have previously used this model with success to induce

mitochondrial biogenesis in young and aged muscle (Ljubicic *et al.*, 2009). Indeed, in our current study we observed a robust induction of mitochondria in young muscle following nine consecutive days of CCA, as demonstrated by elevated COX activity, enhanced respiration, darker SDH and COX histochemical staining and expansion of the SS and IMF populations observed through qualitative electron microscopy. Additionally, a significant induction of PGC-1α was present in young muscle following CCA. Together, these observations confirm the high degree of malleability in young skeletal muscle, and indicate that the CCA paradigm was effective to induce mitochondrial biogenesis.

In agreement with our previous observations (Ljubicic *et al.*, 2009), CCA in aged animals also induced mitochondrial biogenesis, however the degree of adaptation was reduced compared to that observed in young muscle. This was evident from attenuated increases in the key mitochondrial transcriptional coactivator, PGC-1α, along with reduced increases in COX activity in aged, compared to young muscle. Additionally, qualitative histochemical staining and electron micrographs revealed increases in mitochondria following CCA in aged muscle, but to a lesser degree than their younger counterparts. This is particularly interesting since the same absolute workload is applied to both age cohorts, suggesting that the responsiveness of aged muscle has diminished kinetics for mitochondrial biogenesis (Carter *et al.*, 2015; Hood *et al.*, 2016). While these observations appear to indicate that the capacity for exercise-induced biogenesis is reduced, this has remained debateable. For instance, a recent study in aged human muscle found that chronic exercise training is capable of inducing an enhanced abundance of mitochondrial proteins through increased protein synthesis (Robinson *et al.*, 2017), suggesting that the capacity for the translation of mitochondrial proteins is not impaired with age. Further research on the simultaneous investigation of multiple steps within the gene expression pathway (eg. transcription, translation,

post-translational trafficking) will be useful in determining whether exercise can serve to accelerate the pathways that may limit the mitochondrial adaptations in aged muscle.

To assess autophagy, we used LC3-II as a key identifier of autophagosomes. We assessed the levels of LC3-II under all conditions in whole muscle extracts with the intent of procuring an estimate of the amount of autophagosome flux occurring in young and aged muscle, with or without CCA. Treatment with colchicine greatly elevated LC3-II levels in both young and aged cohorts, confirming that autophagy was successfully blocked. The benefit of performing flux calculations is to avoid the misinterpretation that could occur simply by assessing LC3-II levels in untreated tissues. Greater LC3-II levels could occur via two possible mechanisms, either by greater processing of LC3-I to LC3-II, or by the impaired degradation of autophagosomes which harbour LC3-II, leading to enhanced levels of this marker (Klionsky, 2016). Thus, having a condition which inhibited autophagy allow us to gain an appreciation for the amount of autophagosomes destined for degradation.

Contrary to our hypothesis, LC3-II autophagy flux in aged muscle was greater than in young muscle. There were no changes in LC3-II autophagy flux brought about by CCA. These data were also reflected in the evaluation of p62 flux which show the same pattern (data not shown). If the LC3-II/I ratio had only been evaluated, then the increase in this ratio in aged muscle could have been potentially interpreted in two ways: 1) greater autophagy flux (i.e. more LC3-II, more autophagosome formation, more drive for autophagy), or 2) an impairment in degradation, and the subsequent accumulation of autophagosomes. Thus, by having a flux measurement this study we gained a clearer picture of the autophagy events occurring in aged muscle. Previous reports in young animal models with exercise training have concluded that autophagy flux is increased

following training (Lira *et al.*, 2013; Ju *et al.*, 2016), yet our data, using direct flux measurements, or the LC3-II/I ratio, reveal no change under these experimental conditions. The discrepancies in these results may have to do with the divergent training paradigms, animal models and muscles examined, and are likely influenced by the choice of pathway marker employed as a surrogate for autophagy flux measures.

The literature has also documented that specific muscle fiber types (e.g. fast vs slow) exhibit differences in autophagy/mitophagy turnover. Some studies have concluded that oxidative fibres have higher rates of flux (Lira *et al.*, 2013), while others have noted greater rates in predominantly glycolytic muscle (Mofarrahi *et al.*, 2013; Paré *et al.*, 2017). Fibre type changes in response to our short protocol of CCA (3 hrs/day, 9 days) are unlikely to confound the results of this study, since a greater volume and time of stimulation is required to produce a shift in myosin heavy chain expression (Putman *et al.*, 2001), and our previous work has documented enhanced fatigue resistance, but no change in half relaxation time or time to peak tension in young or aged skeletal muscle following this CCA protocol (Ljubicic *et al.*, 2009).

Beyond bulk autophagy flux, we have a profound interest in the selective autophagy pathway directed toward dysfunctional mitochondria, termed mitophagy. Initially, we assessed the levels of receptors known to be involved in the targeted removal of these organelles in young and aged muscle. BNIP3, and its related family member NIX, are located in the outer mitochondrial membrane and each harbour a LC3-interacting region (LIR). In aged muscle, these receptors were significantly upregulated. Parkin, an E3 ubiquitin ligase, is recruited to depolarized mitochondria by the kinase PTEN-induced putative kinase 1 (PINK1) (Matsuda *et al.*, 2010; Koyano *et al.*, 2014; Matsuda, 2016). Upon recruitment, Parkin will ubiquitinate outer mitochondrial membrane

proteins, such as VDAC (Sun *et al.*, 2012) and Mfn2 (Gegg *et al.*, 2010; Chen & Dorn, 2013), to signal the organelle for degradation. Similar to previous results, Parkin exhibited a trend for upregulation with aging while Optineurin, a receptor which is recruited to ubiquitin-tagged mitochondria subsequent to phosphorylation (Wong & Holzbaur, 2015; Richter *et al.*, 2016), remained unchanged with age. Despite this, the ratio of enhanced levels of these receptors with aging suggests that the mitochondrial selection for mitophagy is high in aged muscle.

To evaluate mitophagy flux directly, mitochondria were isolated from the TA muscle and extracts were used to assess the organelle-specific localization of mitophagy markers. In agreement with our whole muscle measures, LC3-II localization was enriched in colchicine-treated conditions of both young and aged muscle. Flux analysis revealed that, in contrast to expectations, aged muscle exhibited greater LC3-II mitophagy flux than young muscle. These findings were supported by our measurements of enhanced p62 mitophagy flux and localization on mitochondria, as well as the elevated expression of mitophagy receptors in mitochondria of aged muscle, as discussed above. Therefore, the enhanced rate of mitochondrial removal via this mitophagy pathway could be a contributing factor to the reduction in organelle content which can be observed in aging muscle. On the other hand, the discrepancies evident in the literature on mitochondrial function (e.g. respiration) with age may depend, in large measure, on whether mitophagy is sufficiently up-regulated to selectively remove the dysfunctional organelles, resulting in the maintenance of only a healthy pool of organelles, under the conditions of study.

Of great interest within our results was the observation that the adaptive response to CCA resulted in a significant reduction in mitophagy flux. It is known that under a variety of conditions in which respiratory dysfunction exists, exercise training or chronic contractile activity have led to an

improvement in mitochondrial function, leading to reduced ROS signaling and AMPK activation (Taivassalo *et al.*, 2001; Adhihetty *et al.*, 2009; Ljubicic *et al.*, 2009; Carter & Hood, 2012; Menzies *et al.*, 2013; Conley *et al.*, 2013). These are well-known triggers for the activation of mitophagy. Thus, the adaptation to a reduced mitophagy flux is likely due to an improvement in the quality of the organelles brought about by CCA, and the attenuated signaling that accompanies this adaptation. Despite this decrease in mitophagy flux, CCA did elicit an increase in the expression of BNIP3 and Optineurin receptors, exclusively in young muscle. In aged muscle, the same response to CCA was not observed, and in the case of Optineurin, aged muscle was remarkably unresponsive. Previous work examining BNIP3 with exercise training has also observed increases in BNIP3 in whole muscle extracts (Lira *et al.*, 2013; Ju *et al.*, 2016). This increase in BNIP3 has been interpreted to mean that mitophagy must be increased as well. Our observations contradict previous conclusions which suggest that mitochondrial removal is enhanced following exercise training (Lira *et al.*, 2013; Ju *et al.*, 2016). However, in these prior cases only indirect measures of mitophagy were used to derive this conclusion.

Transcriptional regulation of autophagy components is necessary for changes in autophagy flux (Füllgrabe *et al.*, 2016). Thus, we assessed the expression of the well-known transcriptional regulators, FoxO3 (Mammucari *et al.*, 2007) and p53 (Maiuri *et al.*, 2010; Wang *et al.*, 2013). In young muscle no CCA-induced alterations were detectable in either protein. However, each of these proteins was significantly upregulated with age, in agreement with other studies (Tamilselvan *et al.*, 2007; Ziaaldini *et al.*, 2015; Wagatsuma *et al.*, 2016). Interestingly, p53 was further enhanced with CCA in old muscle. Coupled with our observations of enhanced flux in aged muscle, the increase in these proteins may suggest enhanced transcription for autophagy genes in the aging milieu. p53 has also been demonstrated to exert a role in mitochondrial biogenesis with

exercise (Saleem *et al.*, 2009; Saleem & Hood, 2013), thus the enhanced expression with aging and CCA may also contribute to the production of new organelles with exercise. However, FoxO3 and p53 are subject to post-translational modifications as well as changes in cellular distribution (e.g. nuclear/cytosolic shuttling) and further work investigating the cellular location and phosphorylation status of these transcription factors is required to fully understand their role in autophagy/mitophagy with exercise and aging.

Further along the upstream autophagy pathway, numerous inputs converge upon Beclin 1, an essential regulator of the core autophagy machinery. In aged muscle, greater Beclin 1 was observed, similar to previous observations (Wohlgemuth *et al.*, 2010; O'Leary *et al.*, 2013; Sakuma *et al.*, 2016). Depending on which protein is partnered with Beclin 1, autophagy can either be activated or inhibited. For instance, Bcl-2 is negative regulator of autophagy when associated with Beclin 1 (Salminen *et al.*, 2013). Other reports have noted that Bcl-2 declines in aging muscle (Ziaaldini *et al.*, 2015), further supporting the conclusion autophagy is enhanced in aged muscle. We also observed an increase in Beclin 1 protein expression in young muscle exposed to CCA. Previous reports have identified an increase in Beclin 1 following both acute and chronic exercise (He *et al.*, 2012; Ju *et al.*, 2016), however some opposing results have also been documented (McMillan *et al.*, 2015; Mejías-Peña *et al.*, 2016; Kim & Hood, 2017). He and colleagues (2012) observed a decreased association of Beclin 1 with Bcl-2 within 30 min of acute exercise, despite a rise in total protein levels of Beclin 1. Thus, further examination of Beclin 1 interactions following training would provide insight on the significance of enhanced Beclin 1 expression following CCA.

The terminal step of the autophagy/mitophagy pathway is degradation of cargo with the low pH environment of the lysosome. We examined markers of the lysosome in young and aged conditions, and also with CCA. In muscle of young animals, we have previously observed that CCA leads to an early adaptive increase in lysosomal markers, indicative of organelle biogenesis prior to mitochondrial adaptations (Kim & Hood, 2017). In the current study, we observed a CCA-induced increase in LAMP-1, supporting this increase in the capacity of muscle with respect to the terminal step of autophagy. In contrast, we have previously reported that aged muscle exhibits morphological evidence of lysosomal impairment, as evident from observations of lipofuscin in electron micrographs (O'Leary *et al.*, 2013). These lipofuscin granules were also evident in muscle from aged, but not young, animals in the current study (Carter et al., unpublished observations). This was coincident with enhanced expression of lysosomal protein markers. These two observations may suggest that aged skeletal muscle contains an accumulation of defective lysosomes, congruent with the lysosomal theory of aging (Wiederanders & Oelke, 1984; Brunk & Terman, 2002). The increase in autophagy and mitophagy flux with aging, measured up to the point of lysosomal cargo delivery, suggests that the lysosomes are overburdened and may become unable to process the incoming cargo, resulting in the formation of lipofuscin granules. Interestingly, CCA significantly lowered the expression of the master regulator of lysosomal biogenesis, TFEB, in aged muscle, suggesting that CCA promotes a corrective phenotype to both mitochondria and lysosomes in aged muscle. Future examination of TFEB cellular localization (Erlich *et al.*, 2018), as well as its phosphorylation status, along with direct assessments of lysosomal function and degradation capacity, will be required to complete the assessment of autophagy and mitophagy flux with exercise in aging muscle.

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Author Contributions

D.A.H. and H.N.C. conceived the experiment design, analyzed the data and wrote the manuscript. H.N.C., Y.T., A.T.E. and D.Z. performed the experiments.

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TABLES

Table 1. Young and Aged Animal Characteristics.

	YOUNG		AGED	
	CON	CCA	CON	CCA
Body Mass (g)	396.3±9.1		461±13.8*	
Food Intake (g/day)	16.13±0.54		15.14±0.5	
Heart Mass/BM (mg/g)	2.45±0.03		2.84±0.08*	
Epi. Fat/BM (mg/g)	5.33±0.46		10.72±0.87*	
TA Mass/BM (mg/g)	1.82±0.03	1.87±0.07	0.91±0.07*	0.95±0.06*
EDL Mass/BM (mg/g)	0.45±0.004	0.45±0.01	0.26±0.02*	0.28±0.01*

*$p<0.05$ aged vs young (n = 11-16 per group). No effect of colchicine was noted on these measures.

Table 2. List of Antibodies.

Target	Manufacturer	Product	Lot#	Primary	Exposure
PGC-1α	Millipore	AB3242	2691399	1:1000	3
LC3-I/II	Cell Signaling	4108	3	1:500	2
p62	Sigma	P0067	015M4877V	1:3000	3
BNIP3	Dr. LA	Gift		1:1000	2
NIX	Santa Cruz	sc-166332	D0114	1:200	2
TFEB	Bethyl	A303-	n/a	1:4000	5
LAMP-1	Abcam	ab24170	GR3183900-	1:1000	2
LAMP-2	Abcam	ab13524	GR770-14	1:1000	3
Cathepsin	Santa Cruz	sc-6486	J1111	1:1000	3
Parkin	Cell Signaling	4211	4	1:1000	2
FoxO3	Cell Signaling	2497	6	1:1000	2
p53	Dr. S Benchimol			1:50	5
Beclin 1	Cell Signaling	3738	3	1:1000	2
Aciculin	(Belkin *et al.*,	Gift		1:2000	1
Optineurin	Santa Cruz	sc-166576	L2915	1:1000	3
VDAC	Abcam	ab14734	GR243577-6	1:2000	1

FIGURE LEGENDS

Figure 1. Mitochondrial Content with CCA. A COX activity in vehicle-treated young and aged muscle following CCA. n=6-8 **B** PGC-1α protein expression expressed as fold change of CCA over CON. n=5-6 **C** SDH staining was performed on 10μm sections. A representative image based on the assessment of two animals per condition is shown at 10X magnification. **D** Representative electron micrographs of mitochondria based on the assessment of two animals per condition (young and aged VEH-treated muscle) are shown. Yellow arrows denote SS mitochondria and green arrows indicate IMF mitochondria. **E-G** Respiration was measured on intact saponin-permeabilized muscle fibre bundles for basal, **E** and maximal, **F, G** respiration states. n=5-8. Data are presented as mean±SEM. ¶ $p<0.05$ CCA vs CON; *$p<0.05$ main effect of age; § $p<0.05$ main effect of CCA. CON, control; CCA, chronic contractile activity, COX; cytochrome oxidase; SDH, succinate dehydrogenase.

Figure 2. Upstream Autophagy Markers. A Whole muscle protein extracts were assessed in vehicle-treated muscle for FoxO3, n=7-8 **A, B**, Beclin 1, n=6 **A, C,** and p53, n=5 **D**. Representative blots are provided. Protein expression was normalized to Aciculin. Data are presented as mean±SEM. *$p<0.05$ main effect of age; †$p<0.05$ main effect of CCA. ¶ $p<0.05$ vs young CON. §$p<0.05$ vs age CON. CON, control; CCA, chronic contractile activity.

Figure 3. Autophagy Flux in Young and Aged Muscle with CCA. LC3-II protein expression was measured in whole muscle samples to determine autophagic flux, n=7 **A,B,C**. Flux was calculated by subtracting VEH LC3-II ratios from COL LC3-II ratios (COL – VEH; ratios were normalized to aciculin) n=7 **C**. The LC3-II/LC3-I ratio was also assessed to compare and contrast against previous publications, n=6-7 **D**. Expression of the precursor LC3-I was assessed under all

experimental conditions, n=6-7 **E**. Representative blots are shown. Data are presented as mean±SEM. *p<0.05 main effect of age; #p<0.05 main effect of COL. § p<0.05 aged COL vs young COL, main effect. CON, control; CCA, chronic contractile activity; COL, colchicine; VEH, vehicle.

Figure 4. Mitophagy receptor expression with CCA and aging. Protein expression of four known mitochondrial receptors for mitophagy were assessed through western blotting in whole muscle protein extracts of vehicle-treated animals, n=4-6 **A-E**. Representative blots are provided. Data was normalized to Aciculin. Data are presented as mean±SEM. *p<0.05 main effect of age; †p<0.05 main effect of CCA; ¶ p<0.05 vs young CON. CON, control; CCA, chronic contractile activity.

Figure 5. IMF Mitophagy flux. Mitochondria were isolated from the TA for all conditions. Localization of LC3-II, n=6-7 **A, B** and p62, n=4-5 **A, C** were assessed. Flux was calculated for both LC3-II, n=6-7 **C**, and p62, n=4-5 **F,** and calculated as described in Figure 4. Protein density was normalized to the mitochondrial marker VDAC. Translocation of receptors to the mitochondria were also examined, n=5 **G**. Data are presented as mean±SEM. *p<0.05 main effect of age; †p<0.05 main effect of CCA; #p<0.05, main effect of COL; § p<0.05 aged COL vs young COL, main effect. CON, control; CCA, chronic contractile activity; COL, colchicine; VEH, vehicle; IMF, intermyofibrillar; VDAC, voltage-dependent anion channel.

Figure 6. Lysosomal Markers with Aging and CCA. Lysosomes are the terminal step in autophagy/mitophagy. Assessment of protein indicators to lysosomal function were assessed including LAMP-1, n=5-6 **A, B**, LAMP-2, n=5-6 **A, C**, TFEB, n=6 **A, D** and Cathepsin D, n=6 **A, E** in vehicle-treated whole muscle extracts. Protein expression was normalized to Aciculin. Data

are presented as mean±SEM. *$p<0.05$ main effect of age; †$p<0.05$ main effect of CCA; ¶ $p<0.05$ age CON vs young CON. CON, control; CCA, chronic contractile activity; TFEB, transcription factor EB, LAMP, lysosomal-associated membrane protein.

Fig. 1

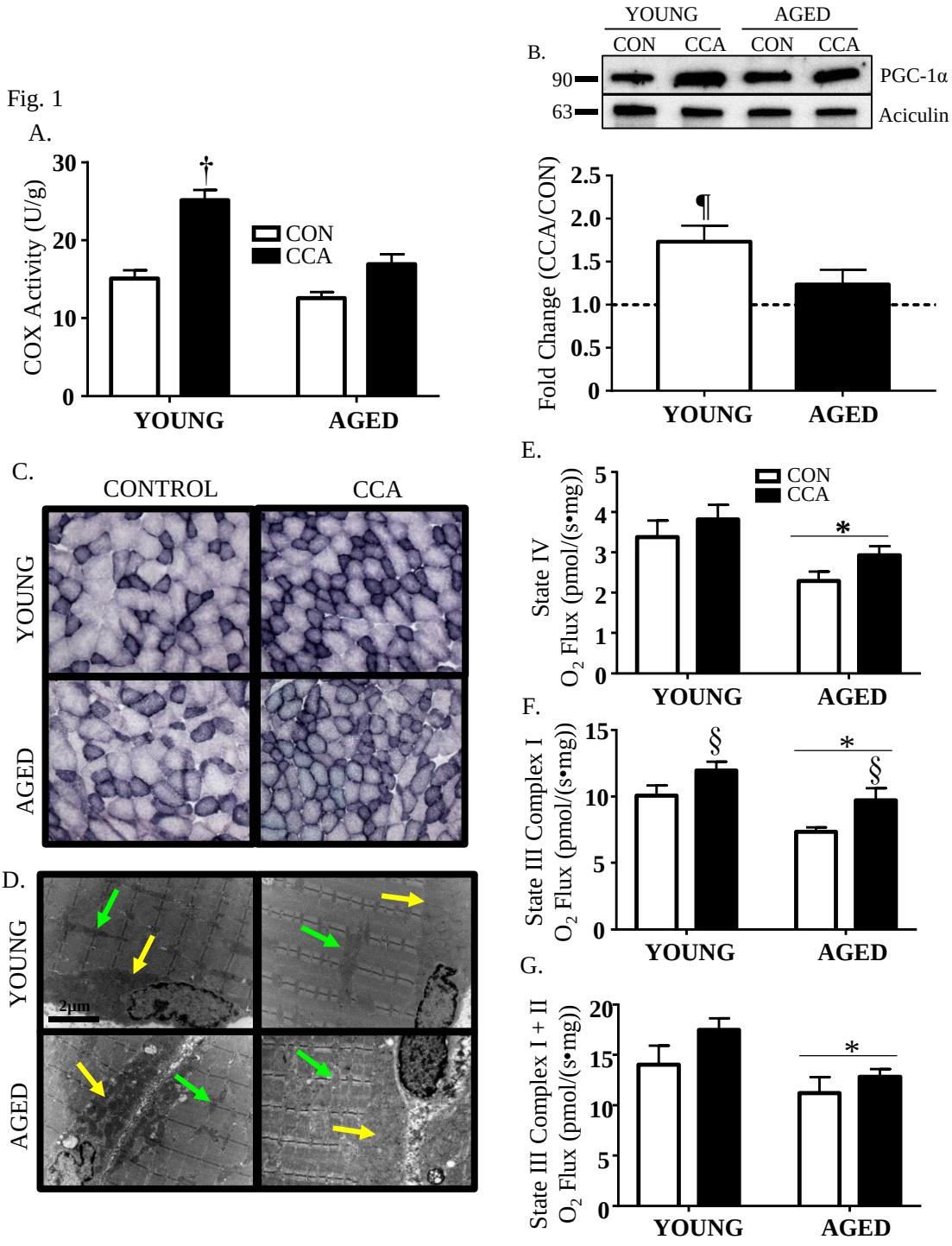

A.
COX Activity (U/g)
30
20
10
0
†
CON
CCA
YOUNG
AGED
B.
YOUNG
AGED
CON
CCA
CON
CCA
90
63
PGC-1α
Aciculin
Fold Change (CCA/CON)
2.5
2.0
1.5
1.0
0.5
0
¶
YOUNG
AGED
C.
CONTROL
CCA
YOUNG
AGED
D.
YOUNG
AGED
2μm
E.
State IV
O2 Flux (pmol/(s•mg))
5
4
3
2
1
0
CON
CCA
*
YOUNG
AGED
F.
State III Complex I
O2 Flux (pmol/(s•mg))
15
10
5
0
§
*
§
YOUNG
AGED
G.
State III Complex I + II
O2 Flux (pmol/(s•mg))
20
15
10
5
0
*
YOUNG
AGED

Fig.2

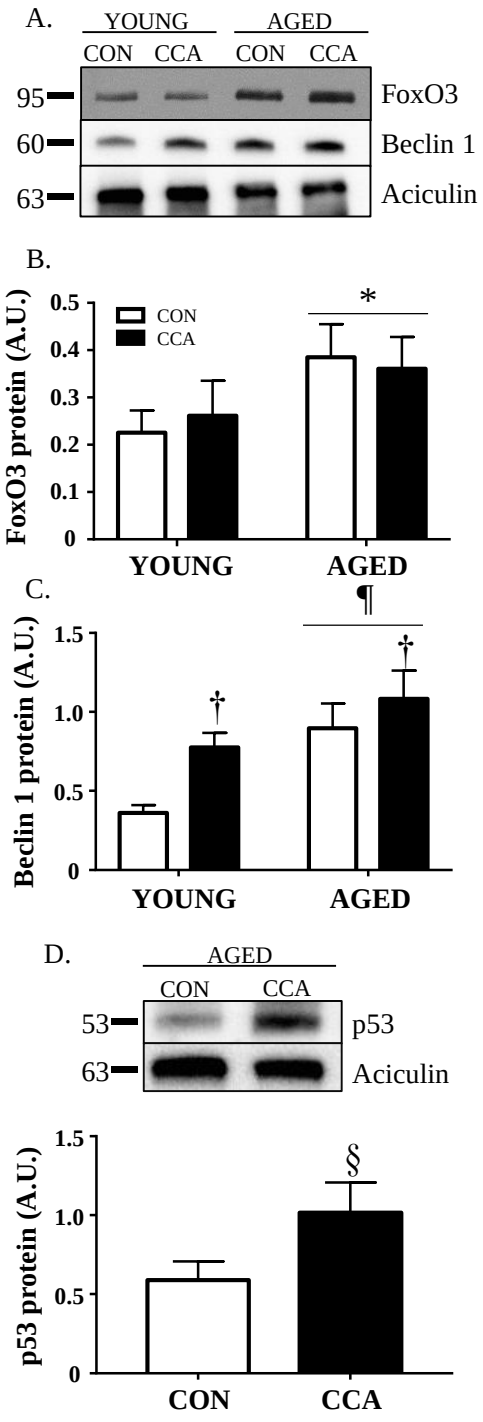

A.
YOUNG
AGED
CON
CCA
CON
CCA
95
60
63
FoxO3
Beclin 1
Aciculin
B.
FoxO3 protein (A.U.)
CON
CCA
*
0.5
0.4
0.3
0.2
0.1
0
YOUNG
AGED
C.
Beclin 1 protein (A.U.)
¶
†
†
1.5
1.0
0.5
0
YOUNG
AGED
D.
AGED
CON
CCA
53
63
p53
Aciculin
p53 protein (A.U.)
§
1.5
1.0
0.5
0
CON
CCA

Fig. 3

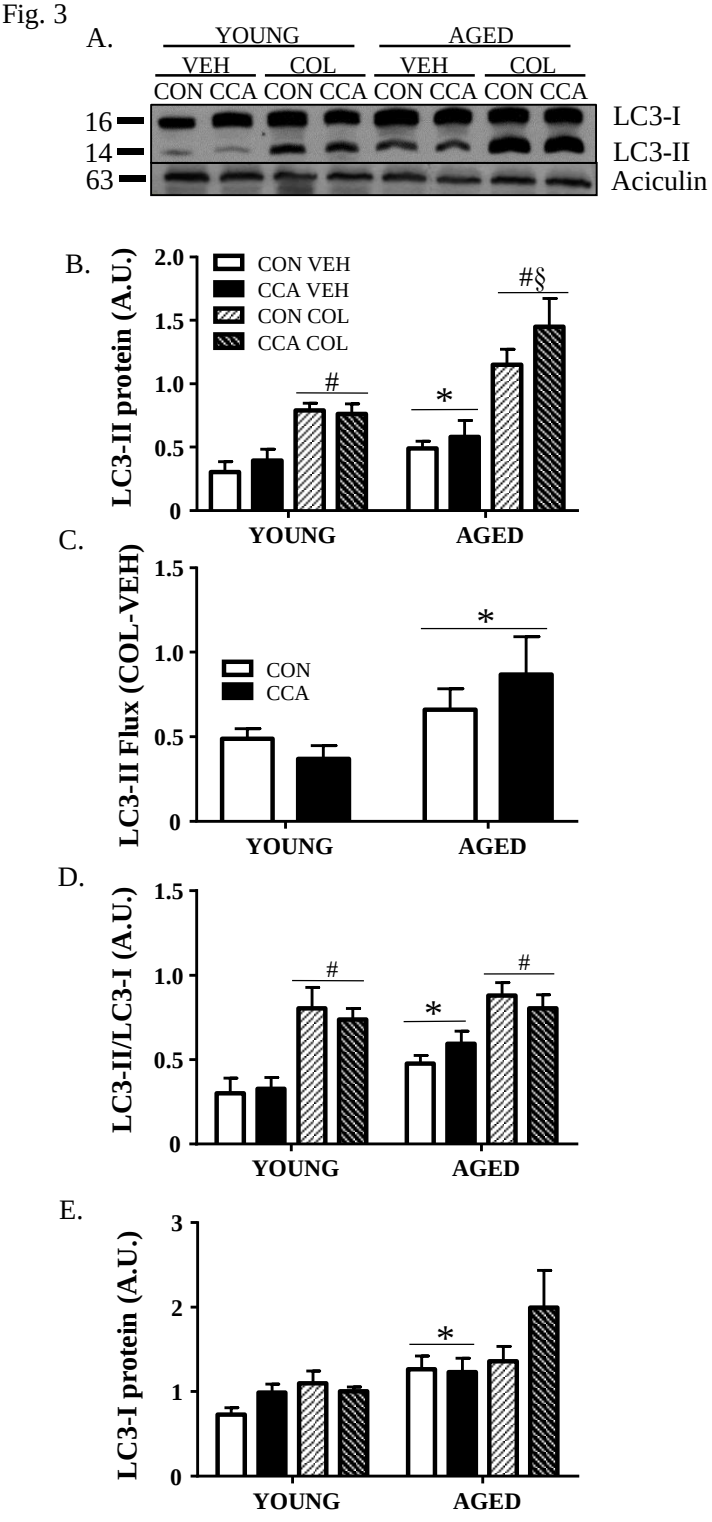

A.
YOUNG
AGED
VEH
COL
VEH
COL
CON CCA CON CCA CON CCA CON CCA
16
14
63
LC3-I
LC3-II
Aciculin
B.
LC3-II protein (A.U.)
CON VEH
CCA VEH
CON COL
CCA COL
#
*
#§
YOUNG
AGED
C.
LC3-II Flux (COL-VEH)
CON
CCA
*
YOUNG
AGED
D.
LC3-II/LC3-I (A.U.)
#
*
#
YOUNG
AGED
E.
LC3-I protein (A.U.)
*
YOUNG
AGED

Fig. 4

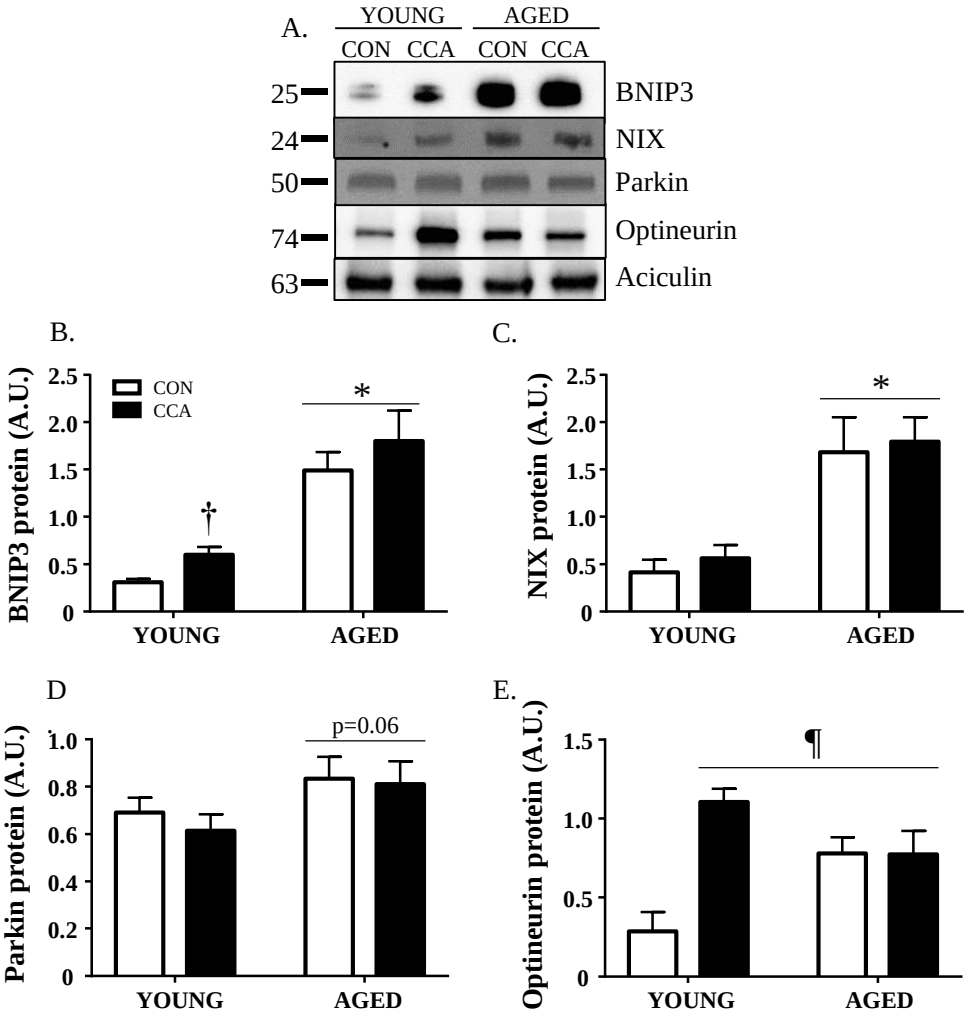

A.
YOUNG
AGED
CON CCA CON CCA
25
24
50
74
63
BNIP3
NIX
Parkin
Optineurin
Aciculin
B.
BNIP3 protein (A.U.)
CON
CCA
*
†
YOUNG
AGED
C.
NIX protein (A.U.)
*
YOUNG
AGED
D
Parkin protein (A.U.)
p=0.06
YOUNG
AGED
E.
Optineurin protein (A.U.)
¶
YOUNG
AGED

Fig. 5

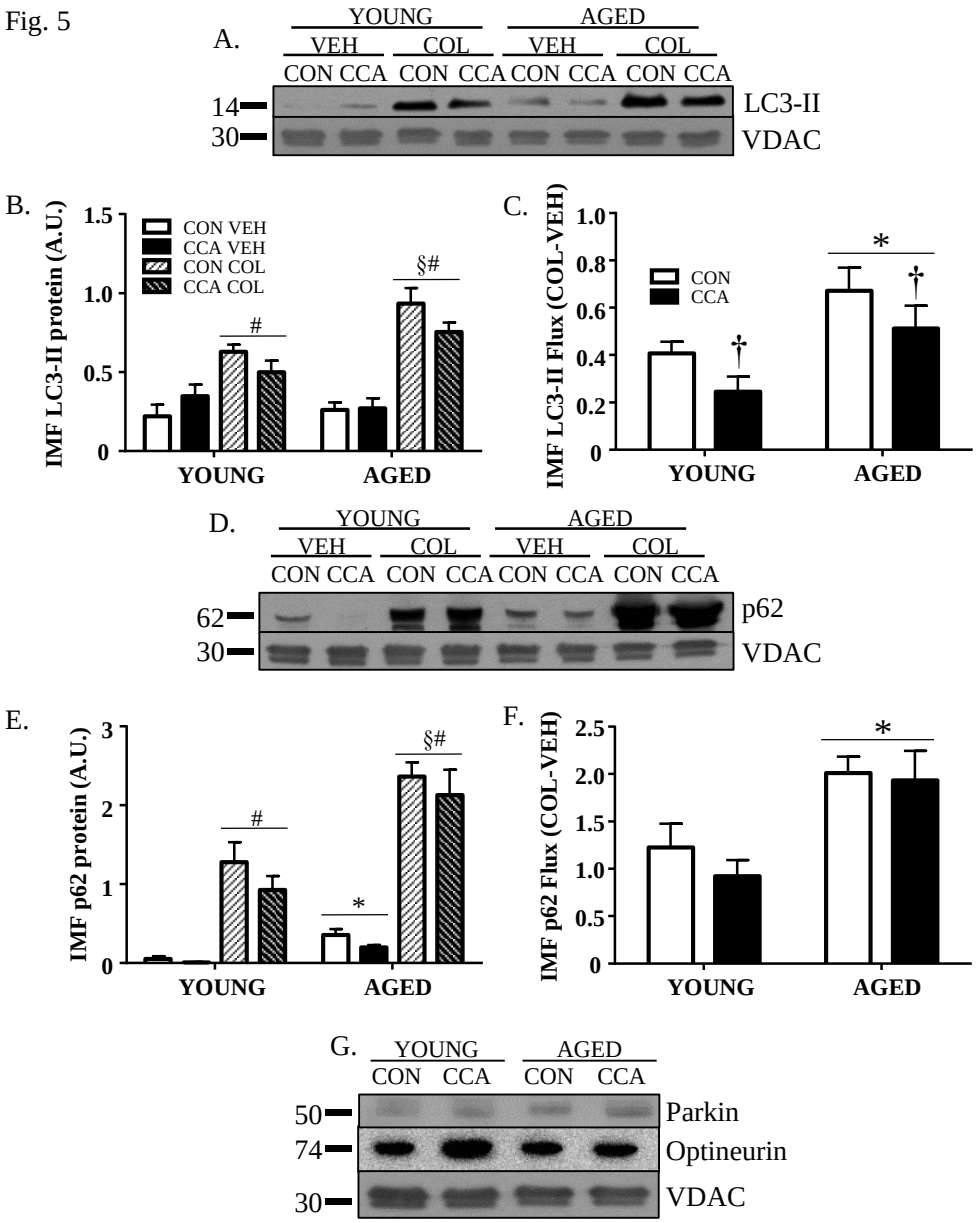
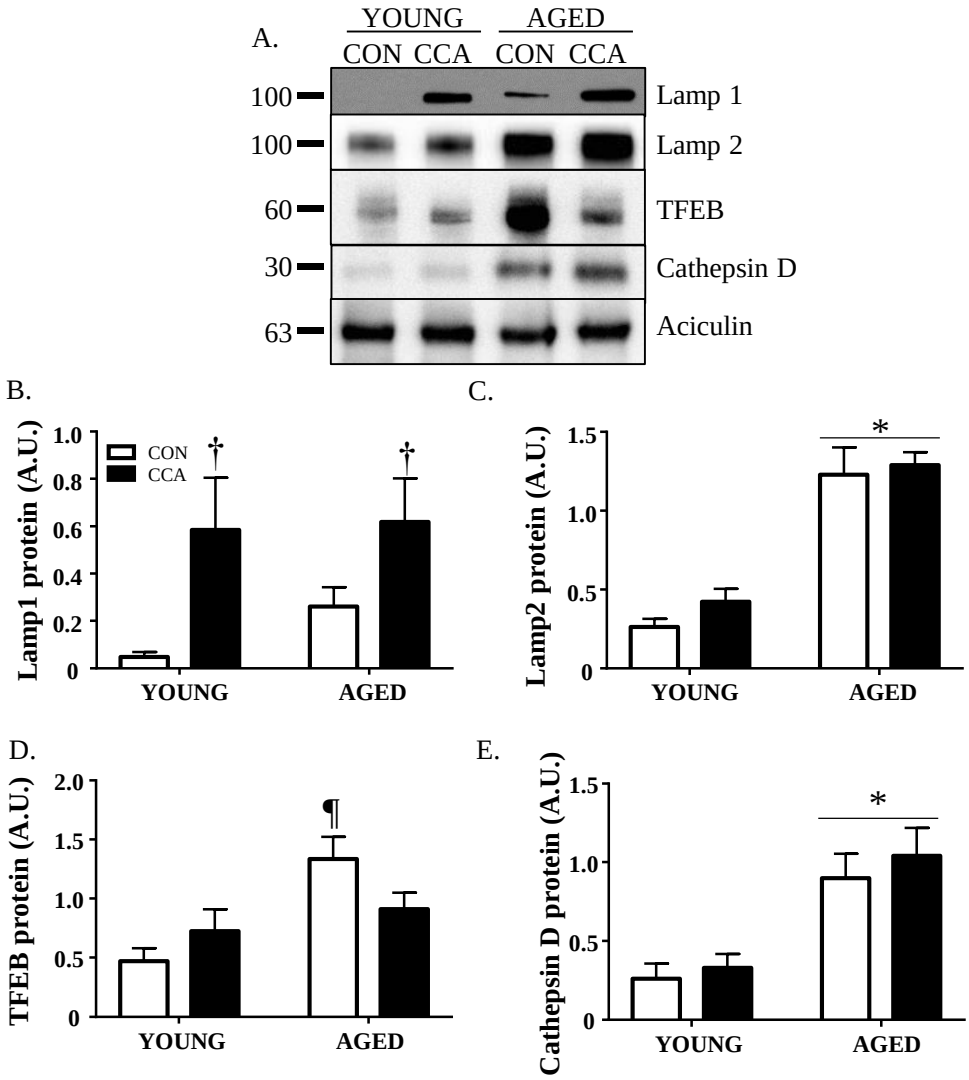

A.
YOUNG
AGED
VEH
COL
VEH
COL
CON CCA CON CCA CON CCA CON CCA
14
30
LC3-II
VDAC
B.
IMF LC3-II protein (A.U.)
CON VEH
CCA VEH
CON COL
CCA COL
#
§#
YOUNG
AGED
C.
IMF LC3-II Flux (COL-VEH)
CON
CCA
*
†
YOUNG
AGED
D.
YOUNG
AGED
VEH
COL
VEH
COL
CON CCA CON CCA CON CCA CON CCA
62
30
p62
VDAC
E.
IMF p62 protein (A.U.)
#
§#
*
YOUNG
AGED
F.
IMF p62 Flux (COL-VEH)
*
YOUNG
AGED
G.
YOUNG
AGED
CON CCA CON CCA
50
74
30
Parkin
Optineurin
VDAC

Fig. 6


A.
YOUNG
AGED
CON CCA CON CCA
100
100
60
30
63
Lamp 1
Lamp 2
TFEB
Cathepsin D
Aciculin
B.
Lamp1 protein (A.U.)
CON
CCA
†
†
YOUNG
AGED
C.
Lamp2 protein (A.U.)
*
YOUNG
AGED
D.
TFEB protein (A.U.)
¶
YOUNG
AGED
E.
Cathepsin D protein (A.U.)
*
YOUNG
AGED

CHAPTER FIVE: SUMMARY AND CONCLUSIONS

The process of aging leads to a decline in muscle mass, termed sarcopenia, which coincides with the presence of mitochondrial deficiencies. This may include increased mtDNA deletions/mutations, reductions in enzyme activities, reduced mitochondrial protein synthesis, greater ROS production, less ATP production through uncoupled respiration and greater apoptotic protein release (5). The precise mechanisms which mediate sarcopenia are still contested, however rectification of the organelles in aged muscle is an attractive target to mitigate potential damage and improve the quality of this tissue. Exercise is a potent stimulus for mitochondrial renovation in skeletal muscle (14) and relies on the transcriptional regulator PGC-1α (24), among others. Indeed, in young healthy individuals, adoption of regular exercise increases mitochondrial mass and quality leading to greater endurance capacity and reduced fatigability that presents with beneficial augmentations in PGC-1α expression and target genes (19). Mitochondrial enrichments occur through enhanced signaling towards mitochondrial biogenesis and the incorporation of newly synthesized gene products into the existing organelle structure, along with fusion of adjacent mitochondria. Furthermore, the process of mitophagy in response to acute exercise assists with the renovation of the network, pruning and degrading substandard organelles to leave only high quality mitochondria in the myocellular milieu. Together, the process of biogenesis and mitophagy contribute to mitochondrial turnover and maintenance of the organelle pool within skeletal muscle.

PGC-1α has received considerable attention over the years for its influence on mitochondria in skeletal muscle. Multiple studies have noted that PGC-1α mRNA and protein expression are decreased in aged muscle (8, 9, 15, 17, 26), but how this arises is currently unknown. Transcript and protein products are influenced by the rate of transcription of a gene. In young muscle after acute exercise, a notable enhancement of PGC-1α gene transcription has been

documented (1, 2, 23). However, whether PGC-1α transcription is altered in aged muscle, which may contribute to the reductions in its expression and ultimately mitochondria health, remained unexplored. Furthermore, how the gene may respond to acute exercise in the aged environment is relatively unknown. We sought to examine this concept in young and aged rats that approximately represented young adults and octogenarians, respectively. Basally, PGC-1α transcription was reduced in aged muscle compared to the younger counterparts, concomitant with reduced phosphorylation of signaling factors and imbalanced expression of a variety of transcription factors which regulate expression of PGC-1α (7). Following acute exercise, aged muscle retained the capacity to upregulate the transcription of PGC-1α, despite harboring lower signaling by AMPK and p38. Examination of PGC-1α mRNA following a paradigm of chronic exercise revealed that PGC-1α transcript levels in aged muscle with chronic contractile activity were no different than that found in young muscle at rest. This suggests that chronic exercise may be influential to restore signaling and transcription of the PGC-1α gene to evoke enhancements in mitochondrial mass and quality in aged muscle, hopefully reverting aspects of the organelle to a healthier state, reminiscent of young muscle. However, further research to examine this concept remains to be performed.

Promotion of mitochondrial synthesis is important for organelle quality but needs to be counterbalanced by the sequestration and removal of old or damaged segments of the mitochondrial reticulum through autophagy and mitophagy events. Acute exercise has been reproducibly documented to elicit enhanced turnover of mitochondria (12, 13, 29), however little examination of this phenomenon has occurred in aged skeletal muscle (3). Furthermore, investigations into how autophagy and mitophagy flux may be altered after exposure to chronic training are limited (16). Chronic exercise induces a healthier state of mitochondria through enhanced biogenesis, and during the first few bouts of the exercise there is an induction of

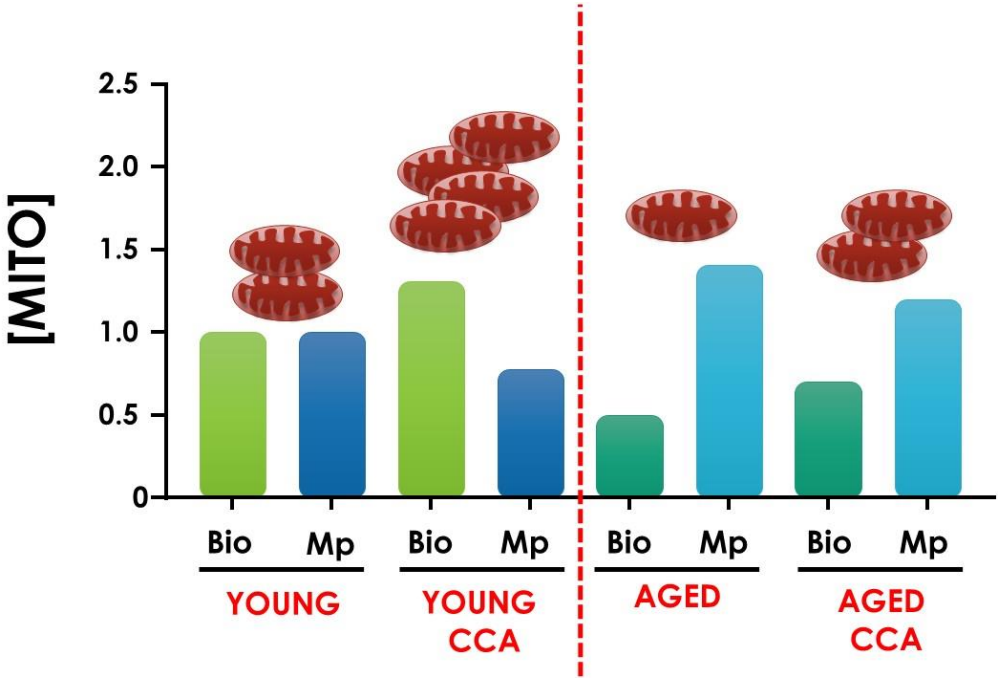


Fig. 1. Summary of Changes in Mitochondrial Content Elicited through Aging and Contractile Activity. To summarize our findings, this graph brings together an overview of the changes in biogenesis (green bars) and mitophagy (blue bars) in young and aged muscle with or without contractile activity. In young resting muscle, the degree of biogenesis and mitophagy are matched producing a steady state of mitochondrial content that supports the needs of the muscle. With acute and chronic contractile activity, numerous markers for mitochondrial biogenesis increase in young muscle, while mitophagy decreases. This difference between biogenesis and mitophagy rates skews the balance to favour an accumulation of organelles in young muscle. With aging, measures of mitochondrial biogenesis, including PGC-1α transcription, decline. This is coupled with an enhancement in mitophagy. The large difference between these opposing processes likely influences the decrease in mitochondrial content noted in aged skeletal muscle. Interestingly, following acute contractile activity, aged muscle remains responsive for the instigation of biogenesis through PGC-1α transcription. With accumulating bouts of contractile activity, an accretion of organelles is noted through a variety of biochemical measures with aging. To complement the rise in biogenesis, the rate of mitophagy is decreased in aged muscle following contractile activity. Thus, successive to contractile activity, the difference between mitophagy and biogenesis is reduced, allowing for an increase in organelle number in aged muscle. We conclude that contractile activity is a positive stimulus for the rectification of organelle number in aged muscle by enhancing biogenesis and mitigating mitophagy.

mitophagic remodeling (16). However, what occurs to the rates of autophagy/mitophagy flux subsequent to the adaptation, when mitochondrial content and quality are improved, is relatively unexplored. Furthermore, there is a paucity of data surrounding autophagy and mitophagy flux measurements in aged muscle with, or without exercise adaptations. Therefore, we sought to examine the changes that may transpire in autophagy and mitophagy in young and aged muscle following a model of chronic training, through chronic contractile activity (CCA).

Contrary to our hypothesis, we found that autophagy and mitophagy flux, measured to the point of the lysosome, as evaluated through colchicine treatment, were elevated in aged skeletal muscle compared to the young group (6). This coincided with enhanced upstream markers for autophagy and expression of receptors for organelle removal. Extension of these observations may impart the concept that enhanced mitophagy is a contributing factor to the reductions in mitochondrial content observed with aging. Interestingly, following CCA, mitophagy was reduced within both young and aged muscle. CCA induced a notable increase in mitochondrial content as assessed through biochemical and respiration assays. This suggests that with organelle improvement, there is a reduced requirement for the turnover of organelles. Most importantly, this benefit is apparent in aged muscle, suggesting that aged muscle retains the capacity to instigate alterations in both mitochondrial biogenesis and mitophagy following chronic exercise.

In the rodent aging model, declines in muscle mass and mitochondrial content are evident, and thus these animals serve as an acceptable model for aging human muscle. We have identified that there is transcriptional insufficiency of PGC-1α in the aged milieu, likely due to the reduction in signaling towards this NuGEMP coactivator. This may be partly responsible for the observed declines in mitochondrial content and quality in aged muscle. Also contributing to the reduction in mitochondrial content may be the enhancement in mitophagy observed in aged muscle.

Together, these two processes, biogenesis through PGC-1α, and the mitophagic program, appear to be imbalanced in aged muscle, skewing the balance towards favoring accelerated degradation compared to synthesis. Over time, this would ultimately lead to net loss of mitochondria, which corroborates the many findings of reduced content of these organelles in aging muscle.

Encouraging results surrounding the capabilities of aged muscle to respond to acute and chronic exercise can now be inferred. This is in agreement with previous works identifying that aged muscle responds in a favourable manner to the impetus of exercise with improvements in gene signatures (20) and mitochondrial content and function (4, 10, 30, 31, 11, 18, 19, 21, 22, 25, 27, 28). With a sufficient duration, intensity and frequency, mitochondrial adaptations are often noted to be similar to that which occurs in young muscle.

From our work, we have found that acute contractile activity in aged muscle is sufficient to elicit enhanced transcription of the PGC-1α gene, a dual regulator of biogenesis and mitophagy. Furthermore, with adaptation subsequent to a training paradigm, restoration of PGC-1α transcript levels and reductions in mitophagy are evident and acceptable consequences. Together, these observations permit an interpretation suggesting a restoration of the balance between biogenesis and mitophagy. This is favorable for aged muscle, as noted by the enhanced content of mitochondria and likely improvement in organelle quality. Thus, we overall conclude that engagement of aerobic style exercise has beneficial effects for mitochondria from aged muscle, through rejuvenation of the equilibrium between biogenesis and mitophagy.

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CHAPTER SIX: FUTURE DIRECTIONS

Based on our observations, continued work to explore and define the roles for PGC-1α, mitophagy and exercise with aging is warranted. We have developed additional questions that may be undertaken to broaden our understanding of the ability for exercise to improve mitochondria in aged skeletal muscle.

1) What is the time course for mitochondrial adaptations to exercise in aged muscle?

   Our findings suggest that mitochondrial biogenesis that occurs through PGC-1α in aged muscle can be augmented with exercise. Additionally, a decrease in mitophagy following CCA adaptation is also evident. However, what we currently have yet to elucidate is the time course of events. For example, do the changes involved in mitochondrial biogenesis occur before or concurrently with the changes in mitophagy? Furthermore, does the renovation of the mitochondrial milieu in aged muscle transpire in a different time frame than that of young muscle? Elucidation of these questions would be insightful to our understanding of the interwoven relationship between the biogenesis and mitophagic pathways. It also may assist in defining exercise parameters that would be tailored to aged muscle to evoke adaptations.

2) What are the expression patterns and influence of other mitophagy receptors/adaptors with aging and exercise?

   Recent research has advanced our understanding on the mechanisms which tether mitochondria to the autophagosomes through either receptors or adaptor proteins. Classically, p62 and LC3-II have been used to assess autophagy or mitophagy flux. However, we now know that numerous other adaptor proteins, such as Optineurin or NDP52, can serve as the scaffolds between the organelles and vesicle. We documented enhanced levels of the

mitophagy receptors NIX and BNIP3. However, we do not know how dominant these factors may be on mitophagy or whether we could use them as markers for flux calculations.

3) Are autophagy genes regulated by methylation events with aging or in response to exercise?

We have demonstrated that aged muscle has increased levels of global DNA methylation which may be prohibitive towards PGC-1α expression. However, we noted numerous factors involved in the autophagy and mitophagy pathways that were upregulated in aged muscle. This observation may partly suggest that these factors are not subject to the dampening effects brought about by enhanced methylation in aged muscle. It would be interesting to explore whether exercise may impart a shift in the methylome of mitochondrial and mitophagy genes in aged tissue, that may precede the noted adaptations in these respective pathways.

APPENDICES

APPENDIX A: ADDITIONAL DATA

Fig. 1. Transcript expression in aged vs young skeletal muscle.

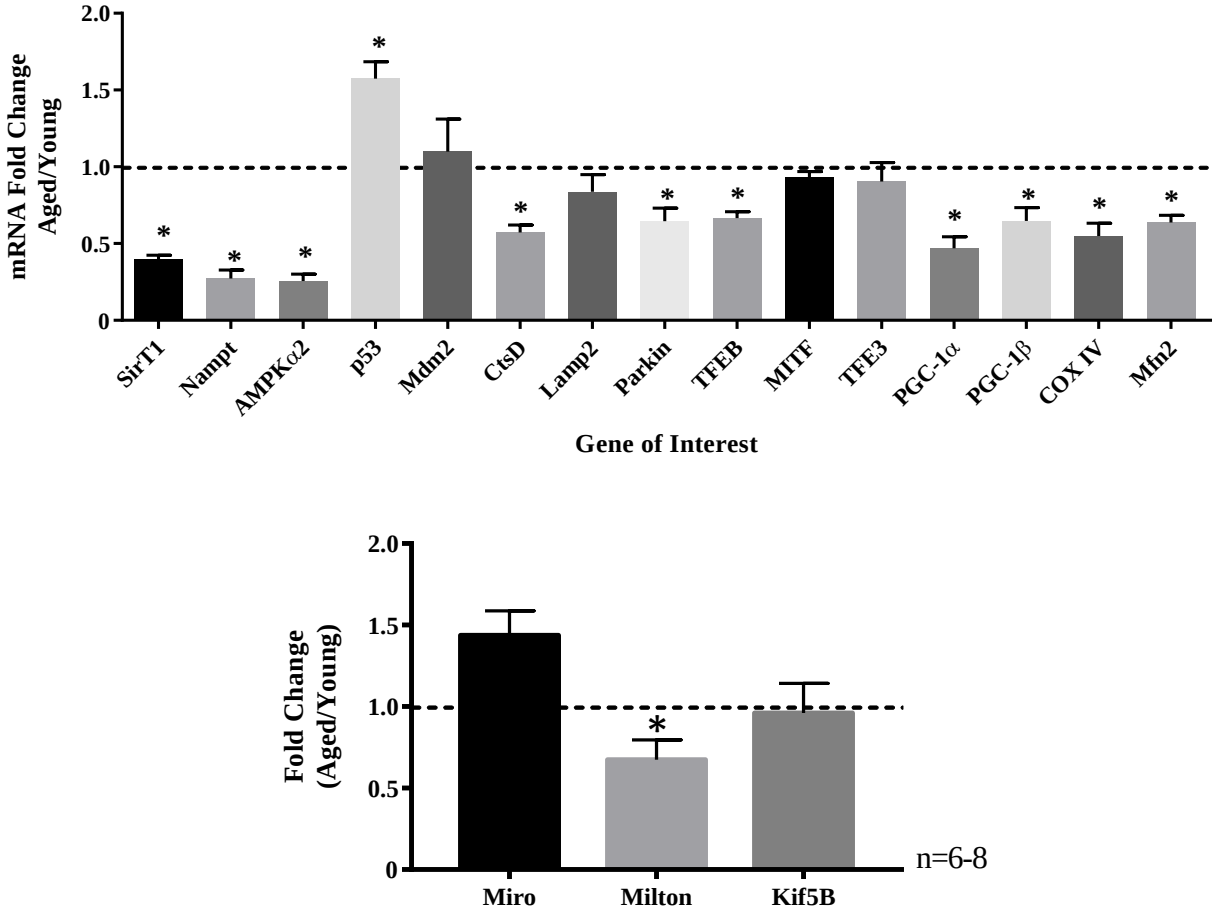

Fig. 1. mRNA expression in aged compared to young skeletal muscle. *p<0.05 aged vs young. N=6-8.

Fig. 2. Transcript expression in young and aged muscle following CCA.

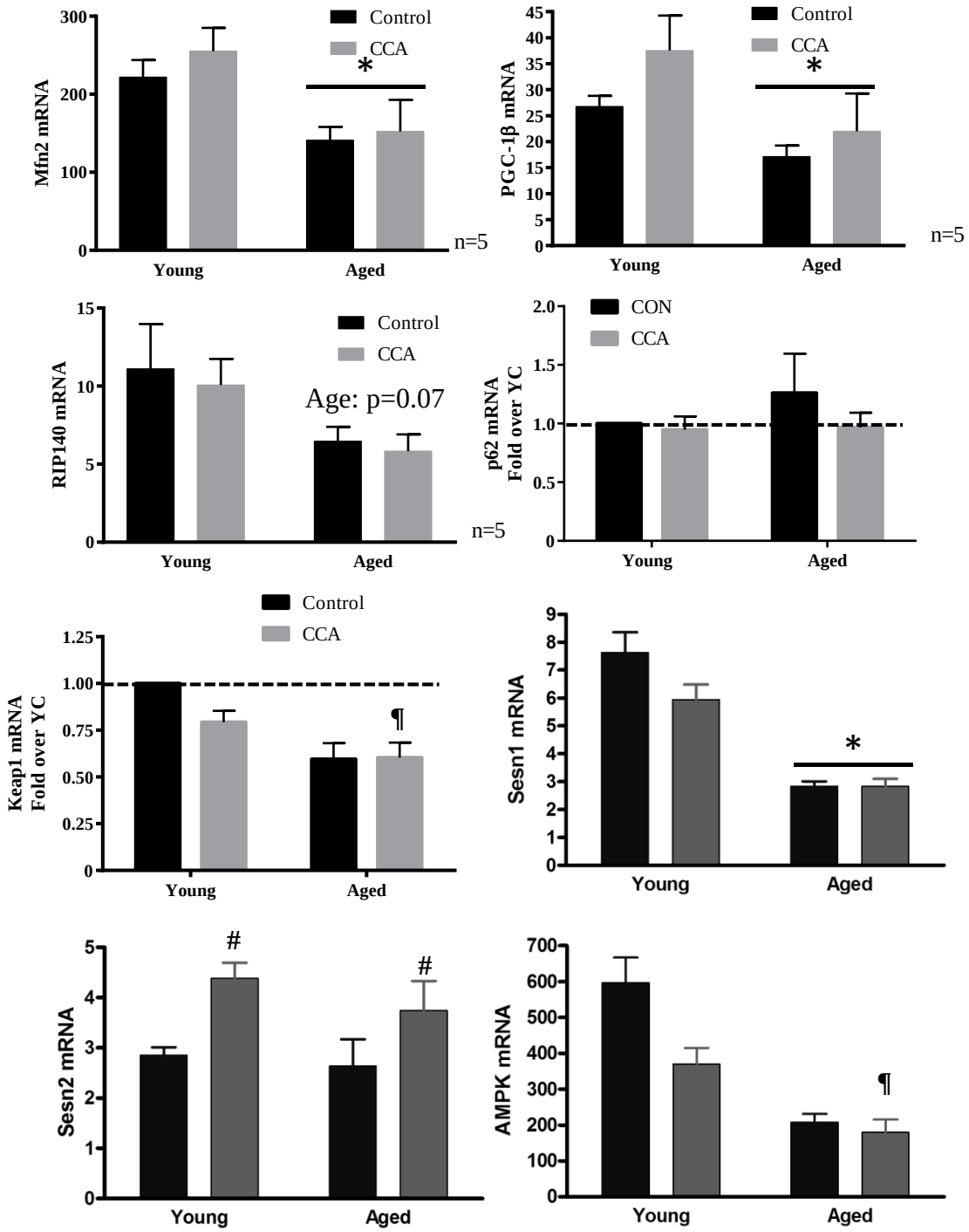

Fig. 2. mRNA expression following 7 days of CCA in young and aged muscle. *p<0.05 aged vs young, main effect. #p<0.05 CCA vs CON, main effect; ¶p<0.05, interaction effect. N=5.

Fig. 3. Expression of Sestrin mRNA and protein.

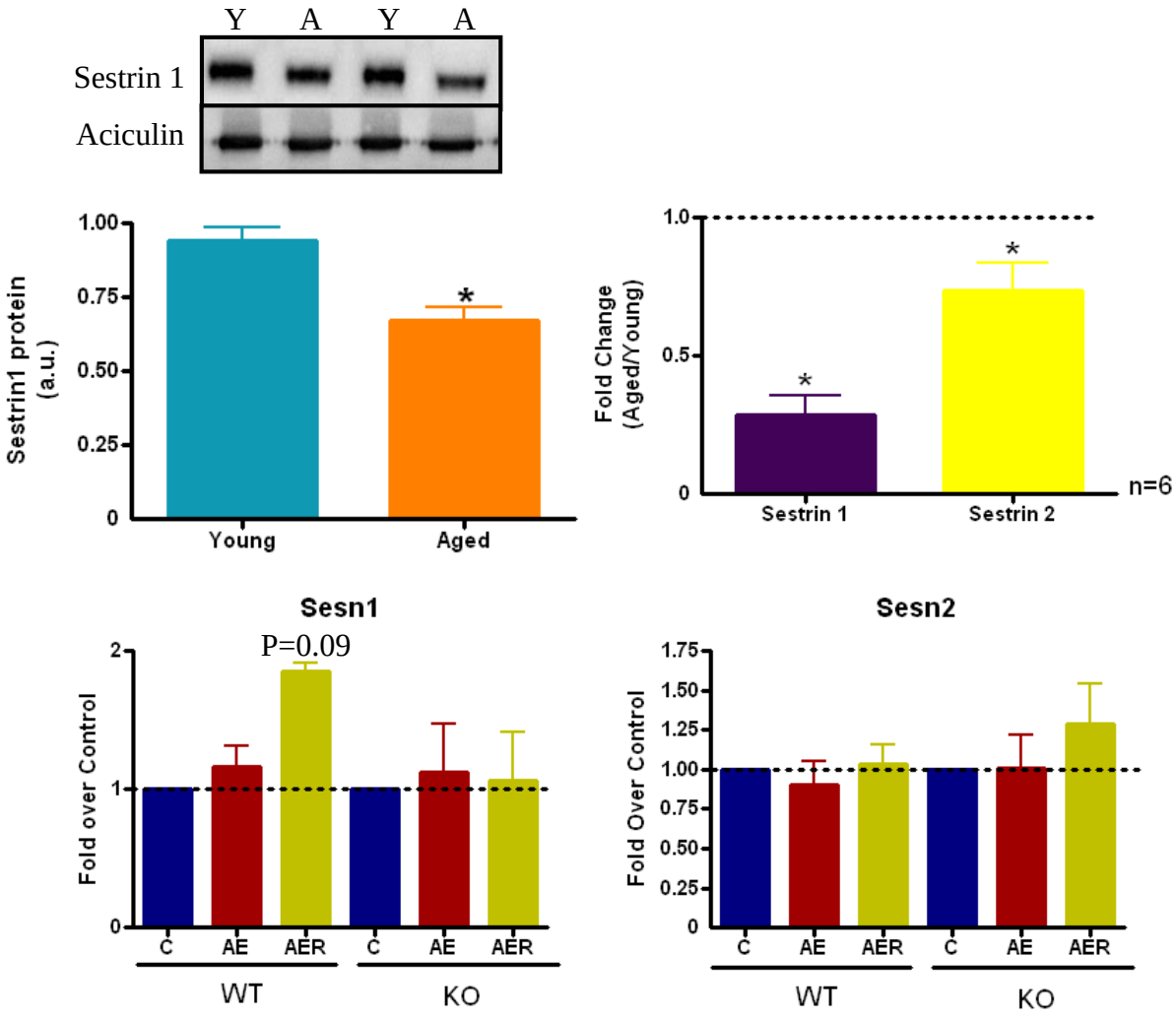

Fig. 3. Expression of sestrin mRNA and protein in aged tissues and p53 knockout mice with acute exercise and recovery. *p<0.05 aged vs young. N=5-6.

Fig. 4. Markers of mitochondrial content in young and aged muscles.

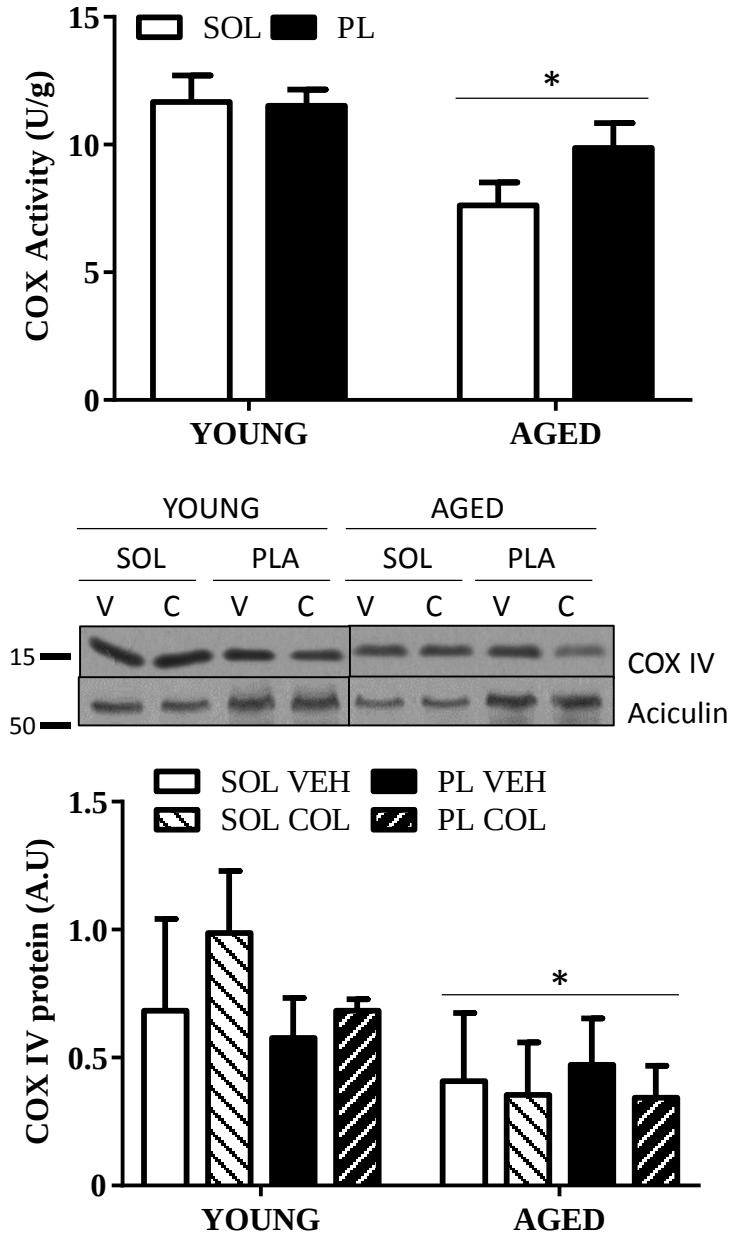

Fig. 4. Mitochondrial markers from young and aged slow- and fast-twitch skeletal muscle. *p<0.05 aged vs young.

Fig. 5. Autophagy flux in young and aged muscles.

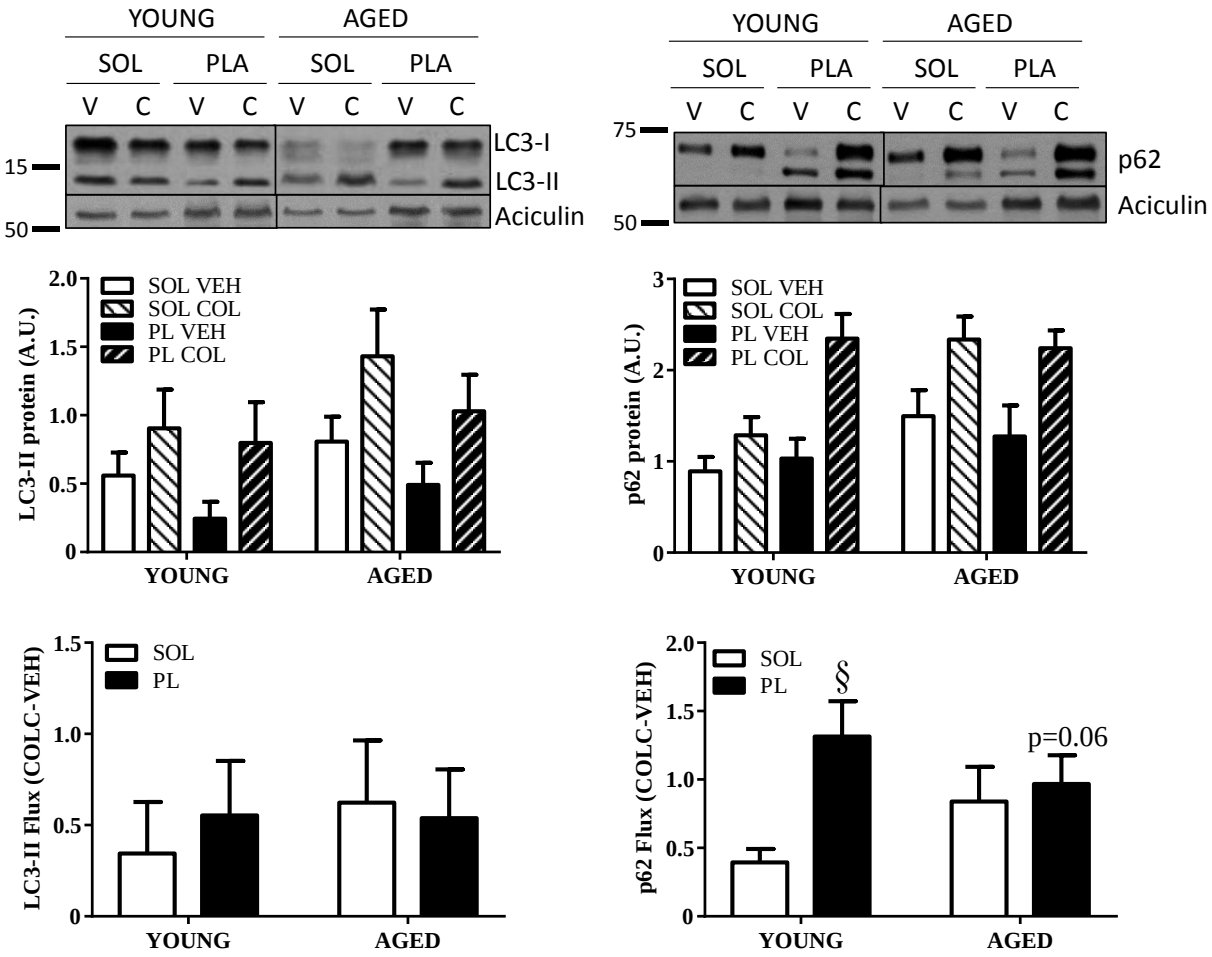

Fig. 5. Assessment of autophagy flux from slow- and fast-twitch muscle of young and aged rats. §p<0.05 young PL vs young SOL.

Fig. 6. Expression of NIX in young and aged muscles.

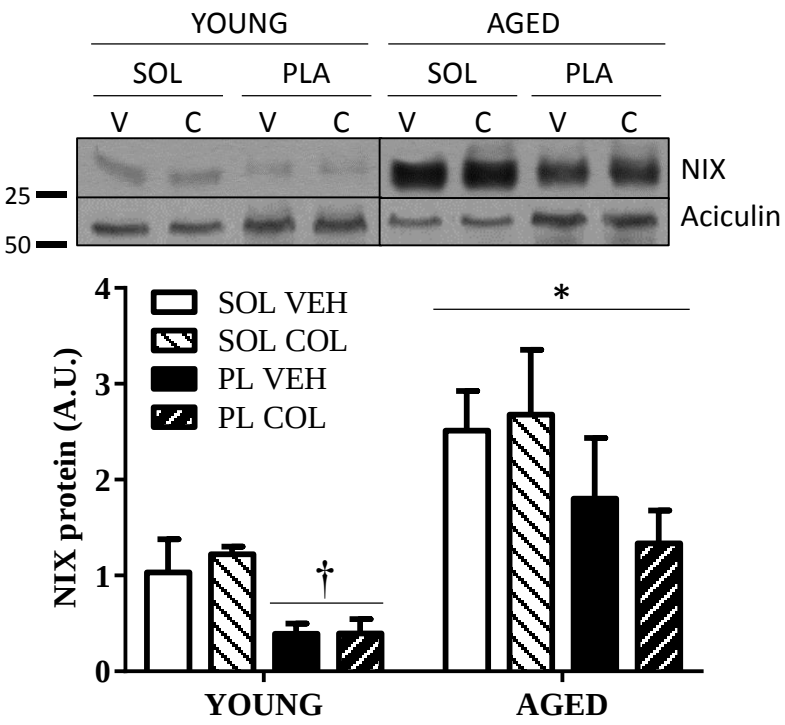

Fig. 6. Expression of the mitophagy receptor NIX in slow- and fast-twitch muscle from young and aged rats. †$p<0.05$ young PLA vs young SOL; $p<0.05$ aged vs young.

Fig. 7. COX activity in different tissues of young and aged rats.

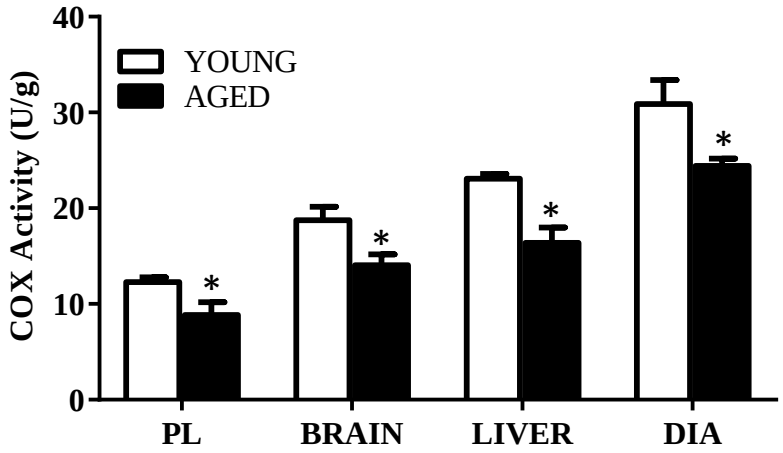

Fig. 7. COX activity in young and aged tissues. *p<0.05 aged vs young.

Fig. 8. Mitochondrial markers in young and aged skeletal muscle and brain.

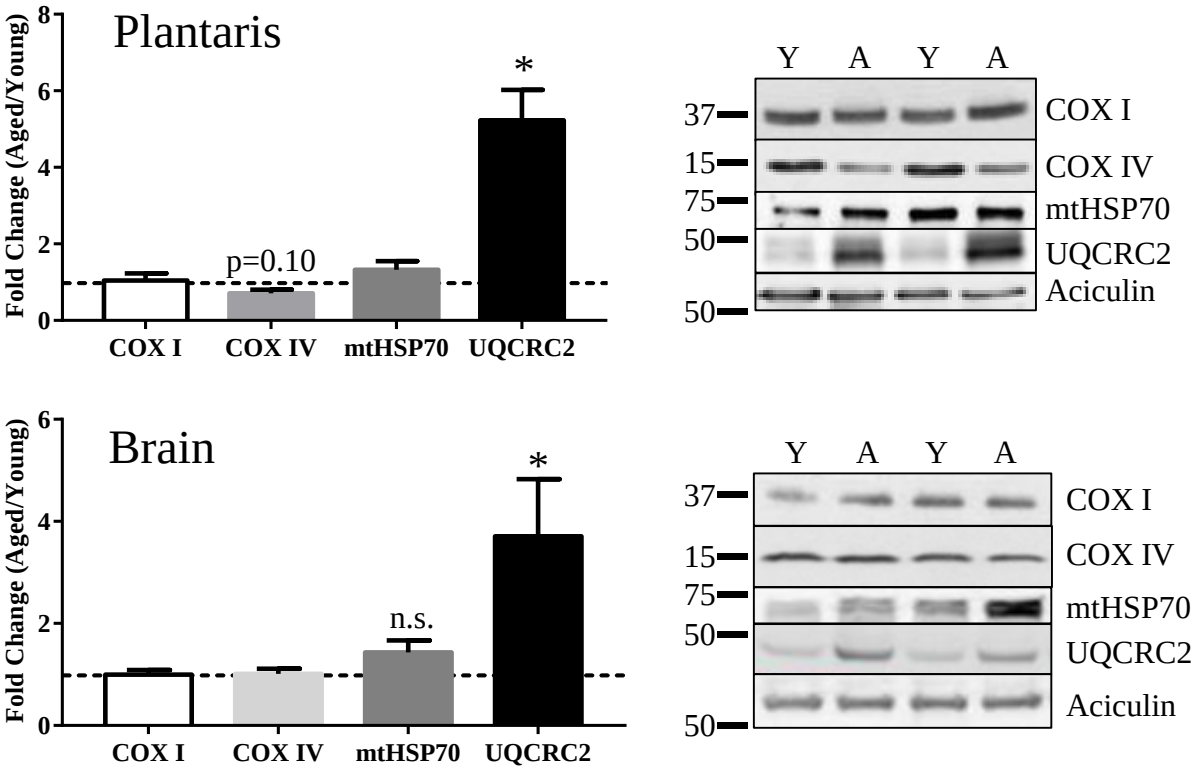

Fig. 8. Protein expression of mitochondria protein in young and aged plantaris muscle and whole brain tissue. *p<0.05 aged vs young.

Fig. 9. Mitochondrial markers in young and aged liver and diaphragm.

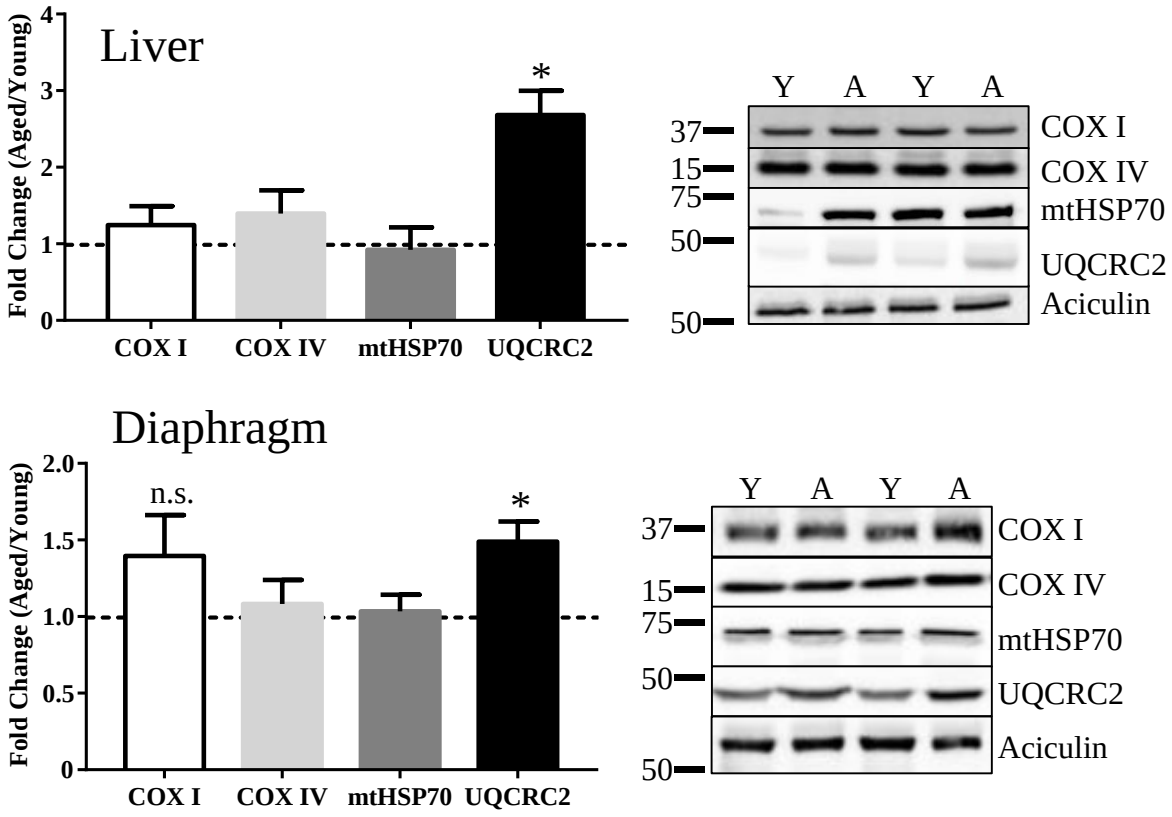

Fig. 9. Protein expression of mitochondrial markers in young compared to aged liver and diaphragm. *p<0.05 aged vs young.

APPENDIX B: DATA NOT SHOWN: CHAPTER FOUR

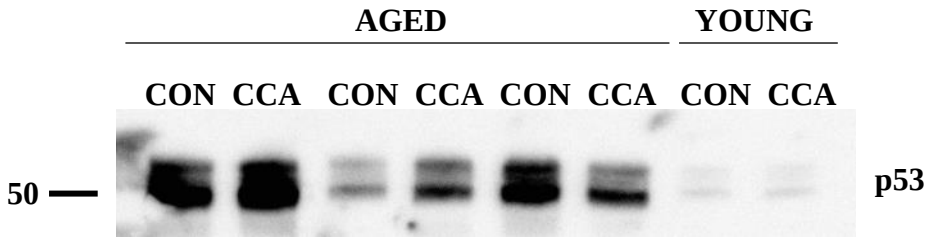

Fig. 1. Representative blot of p53 protein expression in young and aged whole muscle extracts with or without chronic contractile activity (CCA). p53 expression in young muscle is barely detectable compared to aged muscle. Both bands are considered to be p53.

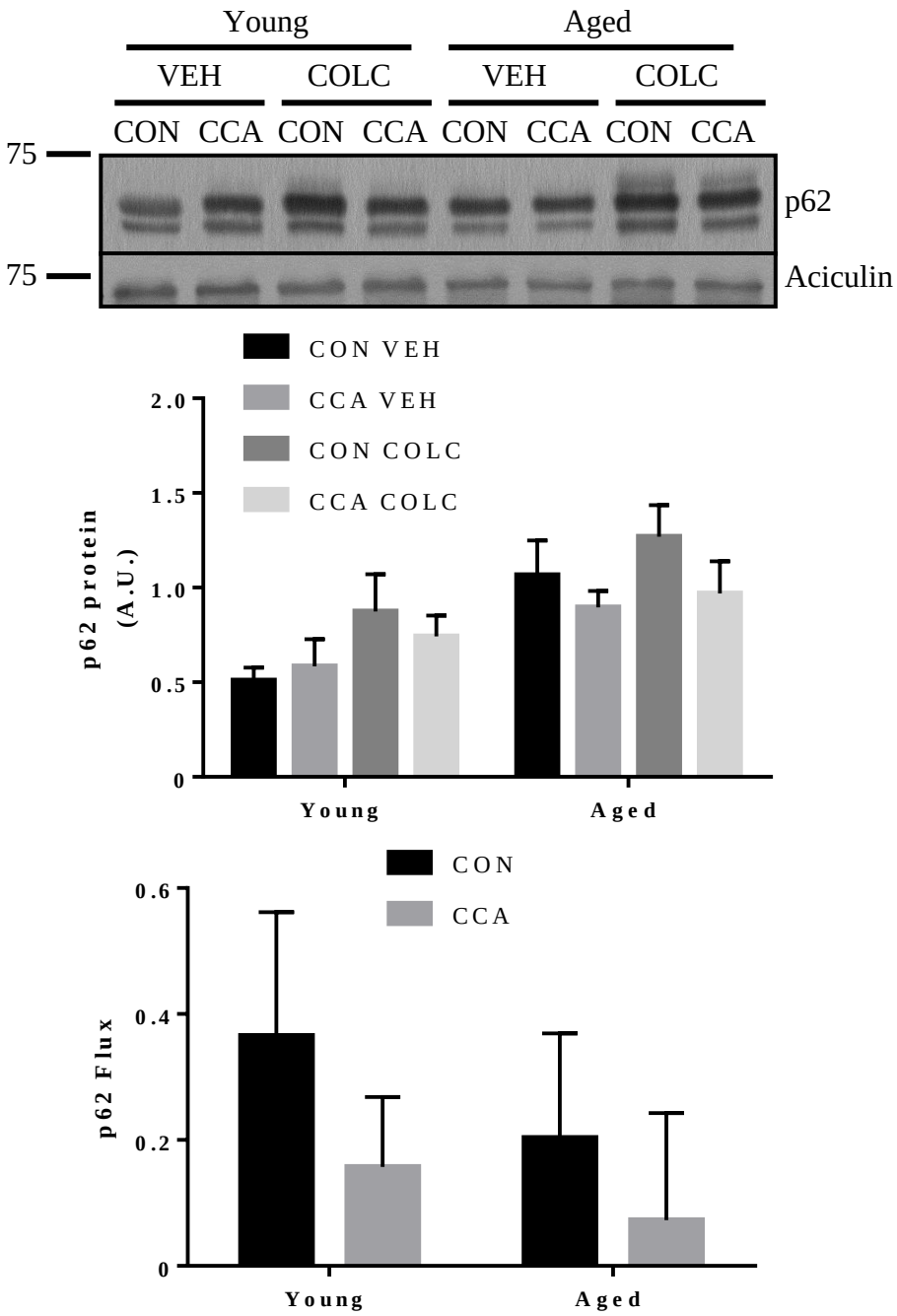

Fig. 2. p62 protein flux from young and aged whole muscle tissue extracts in control and chronic contractile activity (CCA) conditions. n=3.

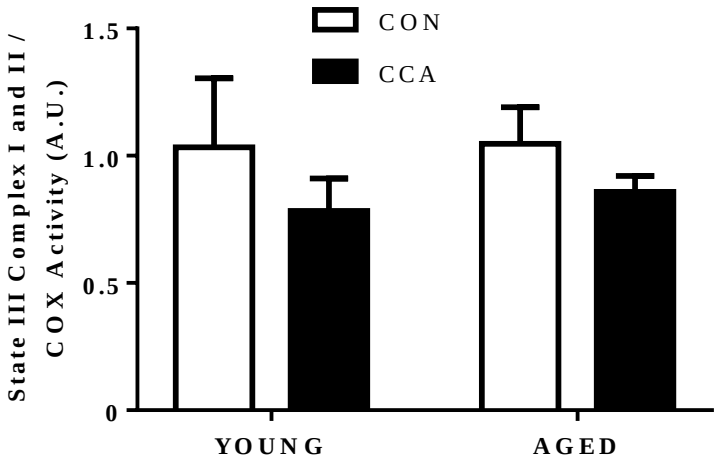

Fig. 3. Complex I and II, state III (maximal) respiration was corrected for corresponding COX activity values (i.e. oxygen consumption corrected for mitochondrial volume). No significant difference was noted between any of the conditions. Similar observations were made for State IV (basal) respiration as well as State III (maximal) Complex I-mediated oxygen consumption. n=5-8.

APPENDIX C: SCIENTIFIC CONTRIBUTIONS

Peer-reviewed articles

9. **Carter HN**, Kim Y, Erlich AT, Zarrin-khat, D and Hood DA. Autophagy and Mitophagy Flux in Young and Aged Skeletal Muscle Following Chronic Contractile Activity. *In revision, J Physiol*. Submission ID: JP-RP-2018-275998R2.

8. **Carter HN**, Pauly M, Tryon LD, Hood DA. (2018) Effect of contractile activity on PGC-1α transcription in young and aged skeletal muscle. *J Appl Physiol (1985)*. doi: 10.1152/japplphysiol.01110.2017. [Epub ahead of print]

7. Hood DA, Tryon LD, **Carter HN**, Kim Y, Chen CC. Unravelling the mechanisms regulating muscle mitochondrial biogenesis. *Biochem J.* 2016 Aug 1;473(15):2295-314.

6. **Carter HN,** Chen CW and Hood DA. (2015) Mitochondria, Muscle Health, and Exercise with Advancing Age. *Physiology (Bethesda).* 30(3):208-223

5. Hood DA, Tryon LD, Vainshtein A, Memme J, Chen C, Pauly M, Crilly MJ, **Carter H**. (2015) Exercise and the Regulation of Mitochondrial Turnover. *Prog Mol Biol Transl Sci.*135:99-127.

4. Saleem A, **Carter HN** and Hood DA. (2014) p53 is necessary for the adaptive changes in cellular milieu subsequent to an acute bout of endurance exercise. *Am J Physiol Cell Physiol.* 306(3):C241-9

3. **Carter HN** and Hood DA. (2012) Contractile activity-induced mitochondrial biogenesis and mTORC1. *Am J Physiol Cell Physiol.* 303(5):C540-7

2. O'Leary MF, Vainshtein A, **Carter HN**, Zhang Y, Hood DA. (2012) Denervation-induced mitochondrial dysfunction and autophagy in skeletal muscle of apoptosis-deficient animals. *Am J Physiol Cell Physiol.* 303(4):C447-54

1. Saleem A, **Carter HN**, Iqbal S, Hood DA. (2011) Role of p53 within the regulatory network controlling muscle mitochondrial biogenesis. *Exerc Sport Sci Rev*. 39(4):199-205.

Book Chapters

2. **Carter HN** and Hood DA. The relationship between muscle mitochondrial turnover and sarcopenia. CRC Press New York. Ed. Meynial-Denis D. *Submitted* March 30, 2017.

1. Hood DA, Saleem A, **Carter HN**, Vainshtein A, Ostojic O and Iqbal S. Molecular basis of exercise training adaptations in skeletal muscle. **Exercise Physiology in Canada.** MacIntosh BM, ed. (*Submitted)*

Commentaries

1. Hornberger TA, **Carter HN**, Hood DA, Figueiredo VC, Dupont-Versteegden EE, Peterson CA, McCarthy JJ, Camera DM, Hawley JA, Chaillou T, Cheng AJ, Nader GA, Wüst RC, Houtkooper RH. Commentaries on Viewpoint: The rigorous study of exercise adaptations: Why mRNA might not be enough. *J Appl Physiol* (1985). 2016 Aug 1;121(2):597-600.