THE INTERPLAY BETWEEN PGC-1α AND AUTOPHAGY DURING METABOLIC ALTERATIONS IN SKELETAL MUSCLE

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ABSTRACT

PGC-1α is a transcriptional co-activator well established as a potent regulator of oxidative metabolism in skeletal muscle. This co-activator mediates metabolic adaptation to exercise by orchestrating mitochondrial biogenesis. PGC-1α is also integral for the maintenance of muscle mass and function during atrophic conditions.

Autophagy is a highly conserved proteolytic pathway responsible for the degradation of dysfunctional organelles and protein aggregates through the lysosomal machinery. Autophagy is upregulated during metabolic distress and is essential for cell survival. While PGC-1α has been extensively studied in the context of skeletal muscle adaptations, its role in autophagy has not been dissected. To this end, the purpose of this dissertation was to examine the interplay between PGC-1α and autophagy in skeletal muscle in the spectrum of muscle contractile states.

We first assessed the role of PGC-1α in the regulation of autophagy and mitophagy during chronic muscle disuse in the form of denervation. To do this we denervated wildtype (WT), PGC-1α knockout (KO) as well as PGC-1α overexpressing (Tg) animals. We found that autophagy and mitophagy flux were compromised in KO animals both basally, and in response to denervation, resulting in a myopathic phenotype. In the Tg animals we uncovered enhanced levels of certain autophagy and lysosomal markers, but surprisingly noticed reduced targeting of mitochondria for degradation. Overall we concluded that PGC-1α is important for the fine-tuning of autophagy in skeletal muscle based on cellular metabolic state, but that it is not required for this process.

Next we evaluated the role of PGC-1α in mitochondrial turnover following an acute bout of exhaustive exercise. We found that in the absence of PGC-1α, exercise-induced mitochondrial turnover was compromised, as evident by reduced expression of mitochondrial genes and diminished autophagy/mitophagy induction, as well as flux.

Collectively, our data demonstrate that PGC-1α is instrumental in maintaining mitochondrial health in skeletal muscle through enhancing organelle turnover. Moreover, PGC-1α can fine-tune autophagy/mitophagy in a manner that is specific to cellular metabolic state.

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"No man is an island entire of itself; every man is a piece of the continent, a part of the main…" John Donne

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List of Abbreviations

ActRIIB	activin receptor type IIb
ADP	adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AIDS	acquired immune deficiency syndrome
Akt/PKB	protein kinase b
ALP	autophagy-lysosome pathway
AMBRA1	activating molecule in beclin-1-regulated autophagy protein 1
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATF	activating transcription factor
Atg	autophagy-related gene
ATP	adenosine triphosphate
Atrogin1/MAFbx	muscle atrophy F-box
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma-2
BH	Bcl-2 homology domain
BH3	bcl-2 homology domain 3
BMP	bone morphogenic protein
BNIP3	bcl2/adenovirus E1B 19 kDa protein-interacting protein 3
BW	body weight
Ca^{2+}	calcium
CaMK	calcium/ calmodulin dependent protein kinase
Caspase	cysteinyl aspartate protease
CLEAR	coordinated lysosomal expression and regulation
CMA	chaperone-mediated autophagy
Col	colchicine
Con	Control animals
COX	cytochrome c oxidase
COXIV	cytochrome c oxidase subunit IV
CREB	cAMP response element-binding protein
cyto C	cytochrome c
Den	denervated
DNA	deoxyribonucleic acid
DRP1	dynamin-related protein 1
E (1-3)	ubiquitin ligase
EDL	extensor digitorum longus
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase

ERR	estrogen receptor
ETC	electron transport chain
Ex	Exercise
Ex + R	Exercise and Recovery
FGF21	fibroblast growth factor 21
FIP200	FAK-family interacting protein of 200 kDa
Fis1	mitochondrial fission 1
FKHR	forkhead transcription factor
Fn-14	fibroblast growth factor-inducible 14
FoxO	Forkhead box O
FUNDC1	FUN14 domain containing 1
FYCO1	FYVE and coiled-coil domain containing 1
GABARAP	gamma-aminobutyric acid receptor-associated protein
GAPDH	glyceraldehyde-3 phosphate dehydrogenase
GATE-16	golgi-associated ATPase enhancer of 16 kDa
GCN5	general control of amino acid synthesis protein 5
GFP	green flourescent protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphate hydrolase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HFD	high fat diet
HIF-1α	hypoxia-inducible factor 1-alpha
Hsc	heat shock cognate
HSP	heat shock protein
IGF-1	insulin growth factor-1
JNK	C-Jun terminal kinases
kDa	kilodalton
KHC	kinesin heavy chain
KO	knock out
LAMP	lysosmal-associated membrane protein
LC3/Maplc3	microtubule-associated proteins 1 light chain 3
LIR	lc3 interacting region
LSD	lysosomal storage disease
MAPK	mitogen activated protein kinase
MDM2	Mouse double minute 2 homolog
MDV	mitochondria-derived vesicle
MEF-2	myocyte enhancer factor-2
MFN	mitofusin
MHC	myosin heavy chain
miR	microRNA

Miro	Mitochondrial Rho GTPase
MITF	microphthalmia-associated transcription factor
MPR	mannose 6-phosphate receptor
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
mTOR	mammalian/mechanistic target of rapamycin
mtPTP	mitochondrial permeability transition pore
mtUPR	mitochondrial unfolded protein response
Mul1	mitochondrial E3 ubiquitin protein ligase1
MuRF1/atrogin1	muscle ring finger1
MUSA1/Fbxo30	ubiquitin ligase of the SCF complex in atrophy-1
MVB	multivesicular body
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NBR1	neighbor of BRCA1
NCOR	Nuclear receptor co-repressor
NDP52	nuclear dot protein 52
NFAT	Nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIX/Bnip3L	Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3-like
NRF	nuclear respiratory factor
NUGEMPS	nuclear genes that encode for mitochondrial proteins
Opa	optic atrophy 1
p38	mitogen-activated kinase
p62	Sequestosome 1
PARIS/ZNF746	Parkin-Interacting Substrate /Zinc Finger Protein 746
PARL	Presenilins-associated rhomboid-like protein
PE	phosphatidylethanolamine
PGC-1α	PPAR γ coactivator-1α
PI3K	Phosphoinositide 3-kinase
PINK1	PTEN-induced putative kinase 1
PKA	protein kinase A
PKD	protein kinase D
POLG	polymerase γ
PPAR	peroxisome proliferator-activated receptor
PRMT1	protein arginine N-methyltransferase 1
Rab7	Ras-related protein
Rheb	Ras homolog enriched in brain
RIP140	receptor-interacting protein 140

RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SIRT1	NAD-dependent deacetylase sirtuin-1
SMURF1	SMAD Ubiquitination Regulatory Factor 12
SNARE	SNAP (Soluble NSF Attachment Protein) REceptor
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
SRC-1	steroid receptor coactivator-1
TA	tibialis anterior
Tfam	mitochondrial transcription factor A
TFE3	transcription factor E3
TFEB	transcription factor EB
Tg	transgenic
TGF-β	transforming growth factor beta
TGN	trans-golgi network
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TNFα	tumor necrosis factor α
TOM	translocase of outer membrane
TRAF6	TNF receptor associated factor 6
Trim32	tripartite motif-containing protein 32
tRNA	transfer RNA
TWEAK	TNF-like weak inducer of apoptosis
Ub	ubiquitin
UBL	ubiquitin like
ULK1/Atg1	unc-51 like autophagy activating kinase 1
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
UVRAG	UV radiation resistance-associated gene protein
VDAC	voltage dependant anion channel
Veh	vehicle
YY1	yin yang 1
ZKSCAN3	zinc finger with KRAB and SCAN domains

CHAPTER 1:

Introduction

Skeletal muscle is the largest of all human organs, and aside from its undisputed role in locomotion, it is also central to whole body metabolism and glucose homeostasis. Muscle possesses a unique ability to adapt to alterations in contractile activity and metabolic cues. This plasticity is responsible for many of the beneficial adaptations induced by exercise, and the metabolic derailments which accompany sedentary behaviour. Mitochondria, the cellular power plants, are at the fulcrum of cellular bioenergetics. These organelles are also major signaling hubs making day to day decisions that can influence cellular fate. Mitochondrial loss during muscle disuse contributes to atrophy, while enhanced organelle density and vigor with exercise are beneficial to cellular metabolism, muscle mass and overall health.

PGC-1α is a transcriptional regulator most renowned for its ability to drive the expression of oxidative genes in a variety of tissues and cells. The level of this co-activator rises and falls with mitochondrial content, and enhancing the expression of this protein is sufficient to block organelle loss as well as the decline in muscle mass under various atrophic conditions, such as diabetes, chronic heart failure, disuse, denervation and aging. PGC-1α is also responsible for mediating exercise-induced adaptations and has insulin sensitizing benefits.

Autophagy is a cellular quality control mechanism responsible for the recycling of toxic organelles and protein aggregates. Autopagy induction is cytoprotective during conditions of metabolic stress, but either deficient or hyperactive autophagy is detrimental to cellular health. Lack of autophagy exacerbates loss of muscle mass under atrophic conditions, resulting in the accumulation of aberrant organelles, and prohibits the glucose sensitizing benefits of exercise.

Overactive autophagy is equally detrimental, as it results in a net catabolic effect. Thus, tight control over autophagy regulation is imperative for the maintenance of muscle mass.

Although both PGC-1α and autophagy appear vital for mitochondrial homeostasis and muscle health, the interplay between them in the spectrum of muscle contractility has not been investigated as of yet. Elucidating the role of PGC-1α in autophagy regulation, and establishing how these two entities may communicate to influence mitochondrial turnover and skeletal muscle vitality is critical for expanding our understanding of muscle plasticity. With today's aging demographics and growing obesity epidemic, it is more important than ever to uncover the mechanisms responsible for the preservation of muscle health. The ability to target and manipulate the energy resources of skeletal muscle can have widespread implication for well-being and disease prevention, which is of great importance to public health.

CHAPTER 2:

Review of Literature

2.1 Mitochondria

2.1.1 Introduction

Once two organisms apart, now mitochondria are an integral part of the eukaryotic cell and represent the most efficient and cost effective energy producing mechanism, albeit with some toxic by products. Mitochondria are self-contained double-membrane organelles and are often regarded as the cellular "power plants". These organelles are believed to have provided the evolving eurokayotic cell with a significant advantage, giving rise to the complex variety of biological organisms that exist today. Mitochondria have entrenched themselves so far into cellular biology that they are now considered to contribute much more than just the energy needed for survival. Indeed, mitochondria have been recognized to play an integral role in cellular signaling, and are involved in cellular maintenance, growth, calcium signaling and storage, metabolite synthesis as well as life and death decisions (65). Therefore, a tight control over mitochondrial density, quality, and distribution is critical for cellular homeostasis. The consequences of aberrant mitochondria are dire, and have been implicated in a plethora of malignant conditions including cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, as well as aging (301). Alterations in mitochondrial content in skeletal muscle in response to changes in muscle milieu are believed to, at least in part, be responsible for the unique metabolic plasticity of muscle (82). This makes the processes of mitochondrial regulation an important field of study with great pleiotropic therapeutic potential in a large number of conditions.

2.1.2 Mitochondrial Biogenesis

Hints of the independent origin of mitochondria are evident from their peculiar nature. For instance, in contrast to other organelles, mitochondria are not formed "de novo" but rather originate only through the division of existing organelles, a process known as fission. Mitochondria also contain their own DNA, hosted in a circular plasmid within the mitochondrial matrix, very much like bacterial DNA. As further proof of their prior autonomous state these organelles possess their very own transcriptional and translational machinery. In the 1970s, all of these "coincidental" findings found their way into the now well accepted, theory of endosymbiosis postulated by Dr. Lynn Margulis (73). However, mitochondria do not contain anywhere near the amount of DNA needed to encode all ~1500 mitochondria-specific proteins. Only 13 proteins, 22tRNAs, and two rRNAs are actually transcribed within mitochondria (34). It is conceivable that throughout evolution the transcription of a growing number of mitochondrial proteins migrated into the nucleus. This is possibly due to the delicate nature of fairly unprotected mitochondrial DNA (mtDNA) and its close proximity to the electron transport chain (ETC), a major site of production of damaging reactive oxygen species (ROS) (305). Mitochondrial DNA is rendered fairly defenceless against the damaging effects of ROS due to its lack of protective histones, and fairly scarce DNA repair and proof-reading mechanisms. The vast majority of proteins required for mitochondrial function are, therefore, blueprinted by nuclear genes encoding for mitochondrial proteins (NUGEMPs) (252). Once transcribed in the nucleus these proteins are then translated in the cytosol and must be imported into mitochondria, and processed before being incorporated into their respective roles. Many of these NUGEMPs are destined for the ETC with the vital task of oxidative phosphorylation, while others may assist

with mitochondrial DNA replication and maintenance (i.e mitochondrial transcription factor A; Tfam) (70). Thus, mitochondrial biogenesis is a complex process that requires intricate cooperation of two separate genomes. Nuclear control of mitochondrial protein expression is achieved by the activation of several transcription factors, in response to diverse stimuli. Alterations in nutrient availability, hormones, growth factors and even temperature fluctuations can activate this process. Among the transcription factors involved in contributing to mitochondrial mass are the nuclear respiratory factors (NRF1 and NRF2) (315), the peroxisome proliferator activator receptor family (PPARα, PPARβ, PPARγ) (296) and estrogen-related receptors (ERR-α, ERR-β, ERR-γ) (53). But perhaps the most infamous factor involved in mitochondrial network growth and expansion is the transcriptional co-activator PPARγ coactivator-1α (PGC-1α). This transcriptional regulator was first described as a coactivator of the adipocyte-enriched nuclear receptor, PPARγ, in brown adipose tissue and skeletal muscle (211). PGC-1α was documented to drive the adaptive thermogenic response to cold exposure, by boosting mitochondrial machinery (211). It has since been demonstrated to robustly upregulate many aspects of mitochondrial content under various metabolic stresses by facilitating the activity of numerous transcription factors (98, 296, 315). It has also been recognized to mediate many of the benefits attributed to regular exercise (82).

2.1.2.1 PGC-1α

Since its discovery, PGC-lα has been studied extensively. Time and again it was demonstrated to orchestrate mitochondrial biogenesis under a variety of metabolic changes. As a transcriptional co-activator PGC-1α does not in itself bind DNA, nor does it possess any enzymatic activity. PGC-1α functions by binding to transcription factors on the promoters of mitochondrial genes within the nucleus and recruiting various histone acetyl transferases (HATs)

such as CREB binding protein (CBP)/p300 and SRC-1, thus allowing for chromatin remodelling. This greatly increases the access of the transcriptional machinery to DNA (174, 210). Furthermore, the coactivator can eliminate transcriptional repressors such as HDACs, and associate with RNA polymerase II, all with the ultimate goal of facilitating gene transcription (174). PGC-1α interacts with transcription factors NRF-1 and NRF-2 to upregulate the expression of genes involved in the electron transport chain (ETC), mitochondrial import machinery and transcription factors of mitochondrial DNA (mtDNA) (70). PGC-1α also enhances the activity of the hormonally-regulated ERRα, which drives the transcription of genes involved in many aspects of mitochondrial biology including fuel utilization, oxidative phosphorylation, and mitochondrial shaping machinery (53, 255). Finally, PGC-1α has been implicated in mammalian target of rapamycin (mTOR) kinase-mediated regulation of mitochondrial biogenesis. In this scenario mTOR forms a complex with transcriptional repressor YingYang1 (YY1) and PGC-1α on the promoters of several mitochondrial genes (50). Thus, PGC-lα coordinately regulates various transcription factors to facilitate the many facets involved in mitochondrial biogenesis.

Given its central role in cellular energy management, it is not surprising that PGC-lα can be regulated both transcriptionally and post-translationally to allow for both short- and long-term potentiation of metabolic machinery. Both aspects of modulation are highly responsive to energetic status, temperature, nutrient availability, hormonal stimuli and more (82, 155). Changes in cellular milieu result in alterations in ROS, NAD^+, AMP and Ca^{2+} levels, which launch signaling cascades resulting in the activation of metabolically-sensitive factors that converge on the PGC-1α promoter. Various transcription factors are implicated in this process, including cAMP responsive element binding protein (CREB) (88, 314), calcium-regulated

transcription factor 2 (ATF2) (6), p38 mitogen-activated protein kinase (MAPK), (6, 252), and Forkhead transcription factor (FKHR) (51), to name just a few. The activation of the energy sensor AMP-activated protein kinase (AMPK) genetically or pharmacologically though AICAR treatment results in the induction of both PGC-lα transcription, and activity (101, 102, 276) (Fig. 1). The coactivator also regulates its own levels in an autoregulatory loop (8, 81). Post-translational modifications of PGC-lα protein constitute an additional major regulatory axis as they affect protein stability, activity and subcellular localization. In skeletal muscle, PGC-1α activity can be enhanced by p38 MAPK-mediated phosphorylation, while phosphorylation by Akt results in an unstable protein that is less active (6, 51, 147, 272). Additional post-translational modifications of PGC-lα include acetylation, methylation and SUMOylation (the addition small ubiquitin-like modifier) (32). The AMPK-activated NAD^+-dependent histone deacetylase, silent information regulator 1 (SIRT1), can deacetylate the coactivator on its many lysine residues (8, 35, 185) which enhances the activity of the protein. In contrast, the acetylation of PGC-lα by general control of amino acid synthesis 5 (GCN5) acetyltransferase renders that protein inactive (143). PGC-lα is also a target of the transcriptional coactivator PRMT1. This protein enhances the function of PGC-lα by methylating its arginine residues (285). PGC-lα activity can also be antagonized by transcriptional corepressors which co-exist within the nucleus. Nuclear receptor interacting protein 140 (RIP140) and the nuclear receptor corepressor 1 (NCoR1) bind to PGC-1α, preventing its transcriptional activities (79, 205). Therefore, changes in tissue metabolic status regulate PGC-1α activity which, in turn, strongly modulates cellular metabolism, resulting in a metabolic feed-forward loop.

The overexpression of PGC-lα results not only in the upregulation of NUGEMPs and mitochondrial content, but also has more profound physiological consequences. Actually,

overexpression of PGC-1α has been documented to enhance endurance capacity, delay fatigue and improve whole body oxygen uptake (33, 150). A natural increase in the activator post-exercise supports its role in exercise-induced metabolic adaptations (10). Consistent with this, gain-of-function experiments documented improved metabolic profile through increased muscle glucose uptake and reduced glycolysis (2, 149, 150). In addition, forced expression of PGC-1α specifically in muscle was protective against various atrophic insults including the sarcopenia of aging (243, 307). Indeed, muscle mass was spared in PGC-1α overexpressing mice following denervation. This effect was mediated by PGC-1α suppression of FoxO3-induced atrophic program (243). Moreover, pharmacological activation of PGC-1α with bezafibrate was protective against metabolic disturbances caused by ETC deficiencies (306). Conversely, PGC-lα-deficient animals confirm the essential role of PGC-lα in mitochondrial biogenesis (142, 151, 323) as deficits in the coactivator result in poor mitochondrial content and dysfunctional organelles (4, 53). Furthermore, lack of PGC-lα results in increased oxidative stress and predisposes mitochondria to apoptotic cell death (4). Collectively, the manipulation of PGC-lα expression and activity revealed a dynamic role for the coactivator that appears to be centered around, but not limited to, the regulation of mitochondrial biogenesis.

2.1.3 Mitochondrial quality control and homeostasis

As a by-product of oxidative phosphorylation, mitochondria produce reactive oxygen species. These are composed of free radicals and unstable molecules derived from oxygen that can be toxic to cellular components. The rate of ROS production is estimated to be 1-5% of all oxygen consumed, but may increase beyond that under conditions of stress (24). In small amounts, ROS can act as signal transducers to activate mitochondrial biogenesis and enhance antioxidant defence mechanisms. However, high levels of ROS that surpass cellular antioxidant

capabilities can result in oxidative stress. This may lead to dangerous modifications to membranes, proteins, and lipids within the mitochondrion itself as well as spillover into neighbouring organelles. In order to minimize ROS production and to ensure optimal energy generation, mitochondria require robust and fool proof quality control mechanisms. Several mechanisms have been proposed to assist with this purpose. In concert, these processes ensure proper mitochondrial function basally, as well as increased mitochondrial efficiency during times of enhanced metabolic demands. These quality control mechanisms include: mitochondrial AAA Lon proteases (136, 163), mitochondria-derived vesicles (MDVs) (165, 271), mitochondrial unfolded protein response (mtUPR) (204), and mitophagy (288, 318). If these fail to restore mitochondrial function, apoptotic cell death might ensue, in order to avoid the spread of toxic waste to neighbouring cells.

2.1.3.1 Degradation of specific mitochondrial proteins and cargo

In accordance with their prior independence, mitochondria possess their own proteolytic system responsible for the safe removal of damaged or dysfunctional proteins. This process functions within the mitochondrial matrix as well as degrades proteins that fail to be imported and remain on the outer mitochondrial membrane (13, 163, 204). Left unattended these oxidized or misfolded proteins can aggregate and lead to organelle toxicity. The mitochondrial Lon protease is responsible for the specific degradation of these proteins and is highly inducible during increased mitochondrial stress (136, 186). Another recently discovered mitochondrial quality control mechanism involves mitochondrial-derived vesicles that engulf only select mitochondrial proteins and deliver them to lysosomes or peroxisomes for degradation (165, 271). Moreover the existence of lysosome-like organella within mitochondria have also been proposed to assist with the degradation of select mitochondrial proteins within the matrix (170). These

pathways collectively act as guardians of mitochondrial protein integrity under steady state conditions and can be further upregulated by metabolic and oxidative stressors. If these mechanisms fail to absolve mitochondria of damaged proteins and injury continues to snowball, the whole organelle can be targeted for degradation by selective autohpagy in order to ensure cellular survival in light of pending apoptotic death.

2.1.3.2 Mitochondrial Dynamics in quality control

Despite the long lasting dogma about the stationary nature and bean-like shape of mitochondria, in reality, these organelles are dynamic and constantly undergo cycles of fission and fusion. Moreover, the size and shape of the organelle greatly varies from punctate fragments, to long, branched, tubular networks. Rapid alterations in mitochondrial shape and trafficking are advantageous during sudden changes in local energy needs. Fission and fusion of mitochondria not only control organelle morphology and distribution, but also contribute to mitochondrial protein repair and mtDNA continuity along the network (21, 41). Mitochondrial dynamics are regulated by unique fission and fusion machinery composed of members of the dynamin-like GTPase family. The Dynamin-related protein 1 (DRP1) mediates mitochondrial fission by forming a multimeric ring-like structure around the mitochondrial outer membrane. This results in the division of the mitochondria into two daughter organelles. Mitochondrial fusion of inner and outer membranes occurs separately, where the fusion of mitochondrial outer membrane is mediated by the Mitofusins 1 and 2 (MFN1 and MFN2), while optic atrophy 1 (OPA1) mediates inner membrane fusion. Mitochondrial fission and fusion aid in quality control by allowing the complementation and exchange of mitochondrial contents and of mtDNA throughout the network. Interestingly, Twig et al proposed that fission is not a randomly occurring event, but is rather a targeted assymetrical division of the organelle resulting in daughter mitochondria with

uneven membrane potentials (293). This provides a major quality control benefit, as only the daughter mitochondrion that maintains a membrane potential will persist and be allowed to rejoin the healthy network. The depolarized daughter mitochondrion, on the other hand, will be segregated from the reticulum and targeted for degradation, unless it is able to restore its membrane potential (293). This allows for the selective maintenance of preferentially healthy and fully functional mitochondria. Interestingly, wholesale organelle removal requires fission as loss DRP1 or Fis1 or alternatively, overexpression of OPA1, prevents mitochondrial division and their subsequent autophagic degradation (140, 262). It has also been noted that during starvation, mitochondria elongate to survive and those that fail to join the network are eliminated (71). Therefore, organelle dynamics play a pivotal role not only in mitochondrial shaping and distribution but also in quality assurance, by selectively isolating damaged or nascent mitochondria for elimination.

2.1.4 Mitochondrial degradation: Mitophagy

Wholesale removal of mitochondria occurs through a selective process of autophagy termed mitophagy. During mitophagy, the organelle is engulfed within a double membrane vesicle known as the autophagosome, and it is subsequently delivered to the lysosome for proteolytic degradation. Mitophagy is ever-ongoing at low levels, and accounts for routine mitochondrial turnover which occurs approximately every 7-14 days, but may be accelerated during energetic imbalance or oxidative stress (168). Mitophagy also participates in the pruning of healthy mitochondria during various physiological processes such as erythrocyte maturation and the elimination of paternal mitochondria during oocyte fertilization (187, 218, 242, 249). Interestingly, the stimulation of mitochondrial oxidative phosphorylation has also been demonstrated to enhance mitophagy thorough the small GTPase Rheb (167). This acts as a pre-

emptive mechanism to facilitate mitochondrial renewal in response to enhanced metabolic demands.

During irreversible mitochondrial damage, when all other quality control mechanisms have failed to restore proper mitochondrial function, mitophagy is triggered (Fig. 1). During canonical mitophagy, mitochondrial membrane potential is lost which allows the stabilization of PINK1 (PTEN-induced putative kinase protein 1) on the mitochondrial outer membrane. PINK1 serves as a docking station for the arrival of the cytosolic E3 ubiquitin ligase parkin to damaged mitochondria. PINK1 was documented to regulated parkin action by phosphorylation (130, 179, 297). Together, PINK1 and parkin ensure the effective segregation of dysfunctional mitochondria into quarantine from the healthy network. To keep mitochondria stationary, PINK1 phosphorylates Miro, a component of the motor adaptor complex. Miro is responsible for anchoring the motor protein kinesin to the mitochondrial surface and facilitating mitochondrial movement along microtubule tracks. Miro phosphorylation predisposes it for degradation in a parkin-dependent manner (303) thus, keeping mitochondria at a standstill. Moreover, parkin also mediates the ubiquitination and degradation of factors belonging to the mitochondrial fusion task force, MFN1 and MFN2. This ensures the isolation of the damaged organelle. Parkin has also been documented to ubiqitinate a variety of other outer mitochondrial membrane proteins such as the voltage dependent anion channels (VDACs), translocases of the outer membrane (TOMs) as well as many others (245). The presence of ubiquitinated proteins on the mitochondrial outer membrane, act as signals for the arrival of adaptor proteins such as NBR1 and p62/SQSTM1 which contain both ubiquitin and LC3 interacting domains (68, 125). These proteins then act as middlemen to facilitate the formation of a double membrane around the targeted organelle. However, some new evidence suggests that p62 may not be required for mitochondrial

degradation by autophagy (178, 318). Alternative pathways that facilitate recognition of dysfunctional mitochondria by autophagosomes involve the mitochondrial BH3-only receptors BNIP3 and BNIP3-like (BNIP3L/NIX). These transmembrane proteins act as direct receptors for LC3 independently of PINK1/Parkin (83, 159, 187, 220). It is evident then, that mitophagy-specific mechanisms are beginning to emerge that separate mitochondrial degradation from non-specific bulk autophagy.

2.1.4.1 The PINK1/Parkin pathway

PINK1 and Parkin were first discovered for their role in parkinsonism, where mutations in either one of these factors result in early-onset of the disease (127, 295). Under steady state conditions, PINK1 is imported into the mitochondria and is rapidly cleaved by mitochondrial proteases such as presenilin-associated rhomboid-like (PARL) (111). This is followed by a rapid degradation of the protein. When the mitochondrial membrane potential is compromised, PINK1 can no longer be imported and it accumulates on the outer membrane. This recruits Parkin to the organelle. Parkin has been documented to ubiquitinate many proteins on the mitochondrial outer membrane including VDAC, the mitochondrial Rho GTPases, as well as components of mitochondrial translocase complex (TOMs) (179, 245). The presence of ubiquitinated proteins on the outer mitochondrial membrane engages the autophagic machinery to aid in the removal of the tagged organelle. However, Parkin can also promote the elimination of mitochondria without affecting protein ubiquitination, by directly associating with AMBRA1(97). AMBRA1, in turn, triggers mitophagy by interacting with the Beclin1-PI3K complex involved in autophagosome formation (63). These studies connect the mechanisms responsible for selective cargo recognition and targeting, with those responsible for the activation of core autophagic machinery (Fig. 1). Moreover, the cytoplasmic SMAD-specific E3 ubiquitin protein ligase 1(SMURF1) has

also been recently recognized by a genome-wide siRNA screen, and was deemed required for Parkin-dependent mitophagy (194). The exact role and mechanism of action of SMURF1 in mitophagy remains to be further elucidated. Interestingly, additional E3 ubiquitin ligases are beginning to emerge, suggesting some redundancy and overlap in this vital process. The mitochondrial ubiquitin protein ligase 1 (Mul1) has been recently documented to poly-ubiquitinate the Parkin target, MFN2. In skeletal muscle, Mul1 mediated FoxO3-dependent mitophagy during muscle atrophy (156).

In addition to the now fairly well documented role of Parkin in mitophagy, it has also been implicated in mitochondrial biogenesis. Parkin was found to target the trascriptional repressor PARIS (ZNF746) for proteasomal degradation. Uninhibited, PARIS represses PGC-1α transcriptional activity. Parkin-dependent elimination of PARIS results in the release of PGC-1α and an increase in mitochondrial biogenesis. Parkin has also been documented to ubiquitinate the proapototic factor Bax, thus playing a cytoprotective role. Therefore, the PINK1/Parkin pathway is emerging as a turn-key mechanism for the efficient turnover of mitochondria and cell death evasion.

2.1.4.2 BNIP3 and BNIP3L/NIX-mediated mitophagy

Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and BNIP3-like protein X (BNIP3L/NIX) are BH3-only proteins, originally described to participate in mitochondrially-induced apoptosis (40). These factors have since been recognized to play a role in selective mitochondrial autophagy. These proteins possess an LC3 interacting region (LIR) and act as receptors displayed on the mitochondrial outer membrane, to attract the autophagosomal isolation membrane. NIX is vital in mediating vigorous mitochondrial elimination during erythrocyte maturation (187, 242). Moreover, BNIP3 has been documented to

trigger mitochondrial fission by recruiting DRP1 to the mitochondria, which can then activate canonical Parkin-dependent mitophagy (140). BNIP3 and BNIP3L/NIX also promote autophagy under hypoxic conditions by competing for Bcl-2 binding with Beclin1. This results in the liberation of Beclin1 from its Bcl-2-mediated captivity, allowing for the initiation of autophagosome formation (16, 103, 201). The expression of BNIP3 and NIX during hypoxia has been attributed to the hypoxia-inducible factor 1 (HIF-1α), the master regulator of hypoxia-inducible genes (16). During hypoxia superfluous mitochondria can generate excess ROS thus further exacerbating oxygen deficits; the elimination of these organelles is therefore vital. Indeed, additional hypoxia-inducible mitophagy mediators are beginning to emerge. For instance, FUNDC1 was recently identified as an outer mitochondrial membrane protein, involved in mitochondrial clearance in response to hypoxic conditions (153). FUNDC1 acts in a similar fashion to NIX and BNIP3 in promoting mitophagy by interacting with LC3, as it too is endowed with an LIR. FUNDC1 expression was proposed to also be regulated by HIF-1α which forms the basis for an attractive model where NIX, BNIP3, and FUNDC1 may function in concert to eliminate mitochondria during hypoxic stress (146, 153).

The study of mitophagy, although still in its infancy, has already demonstrated the vital role of this process not only as a pillar of mitochondrial quality control, but also as an important mediator of programmed developmental elimination of mitochondria. Persistence of damaged or superfluous organelles due to deficiencies in mitophagy results in developmental defects and debilitating conditions such as Parkinsons Disease, cardiovascular disease, muscular dystrophies and countless others.

2.1.5 Co-ordination between mitochondrial biogenesis and degradation

Evidence for the coordinated regulation of mitochondrial sysnthesis and degradation are now beginning to emerge. Cooperation between these two opposing processes allows the fine-tuning of mitochondrial quantity and quality in response to cellular metabolic needs. During nutrient deprivation, the activation of cellular energy sensors such as AMPK and SIRT1 results in the mobilization of autophagic machinery. AMPK initiates autophagic signaling by activating ULK1, while SIRT1 deacetylates several essential autophagy components, including Atg5, Atg7, and Atg8 (also known as LC3) (55, 138). These cellular energy indicators are also well recognized to promote mitochondrial biogenesis through PGC-1α. Interestingly, cells lacking the acetyl transferase GCN5 experience an upregulation in both autophagy and mitochondrial biogenesis though a concomitant increase in the expression of the autophagy-lysosome master regulator TFEB, and the mitochondrial biogenesis co-ordinator PGC-1α (257). Moreover, TFEB and PGC-1α were also found to regulate the expression of one another in various cell types (259, 291), further hinting at the co-ordinated regulation of mitochondrial synthesis and degradation. PGC-1α overexpression, itself, was also documented to upregulate the expression of several autophagic proteins as well as enhance lysosomal biogenesis (280) (Fig. 1). The role of the co-activator in mitophagy specifically, however, has not been investigated as of yet. Moreover, protein kinase D1 (PKD) is activated during oxidative stress (300) and can directly phosphorylate VSP34, which is involved in autophagosome formation (57). Furthermore, PKD can also sense ROS levels and induce the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) to promote the expression of antioxidants and mitochondrial biogenesis through the transcriptional regulation of PGC-1β (14). As previously mentioned, Parkin also participates in both mitochondrial elimination and regeneration by regulating the transcriptional repressor

PARIS. In addition, Parkin can also promote mitochondrial biogenesis by enhancing mtDNA replication through interaction with Tfam (135). In summary, a great deal of coordination between mitophagy and mitochondrial biogenesis must occur in order to maintain mitochondrial content and vitality. A disturbance in either one of these processes will result in the loss of homeostasis and in impending energetic stress, which form the underlying causes of diverse pathologies. Moreover, concomitant decreases in mitochondrial biogenesis and autophagy flux have been documented with aging (154, 155, 189), resulting in an overall decrease in mitochondrial turnover, and therefore diminished function. This can certainly account for the progressive accumulation of dysfunctional and nascent mitochondria during aging (39). Furthermore, these dysfunctional mitochondria are strongly correlated with age-associated loss of muscle mass (sarcopenia), increased incidence of neurodegenerative disorders, as well as increased risk of cardiomyopathies and metabolic diseases (161, 195, 286). Thus, targeting the mitochondrial turnover circuit represents a viable and enticing option for the treatment of a plethora of pathologies.

2.1.6 Conclusions

Mitochondrial content hangs in the balance between organelle synthesis and degradation. While organelle synthesis occurs through mitochondrial biogenesis, a process that has been extensively studied, the selective degradation of wholesale mitochondria has only recently begun to be described. A selective form of autophagy known as mitophagy mediates last resort mitochondrial quality assurance and the removal of nascent or superfluous mitochondria. Mitochondrial distribution is likely to occur through mechanisms of mitochondrial dynamics such as fission, fusion and mitochondrial movement. All of these processes must work in unison in order to maintain proper mitochondrial health and cellular homeostasis.

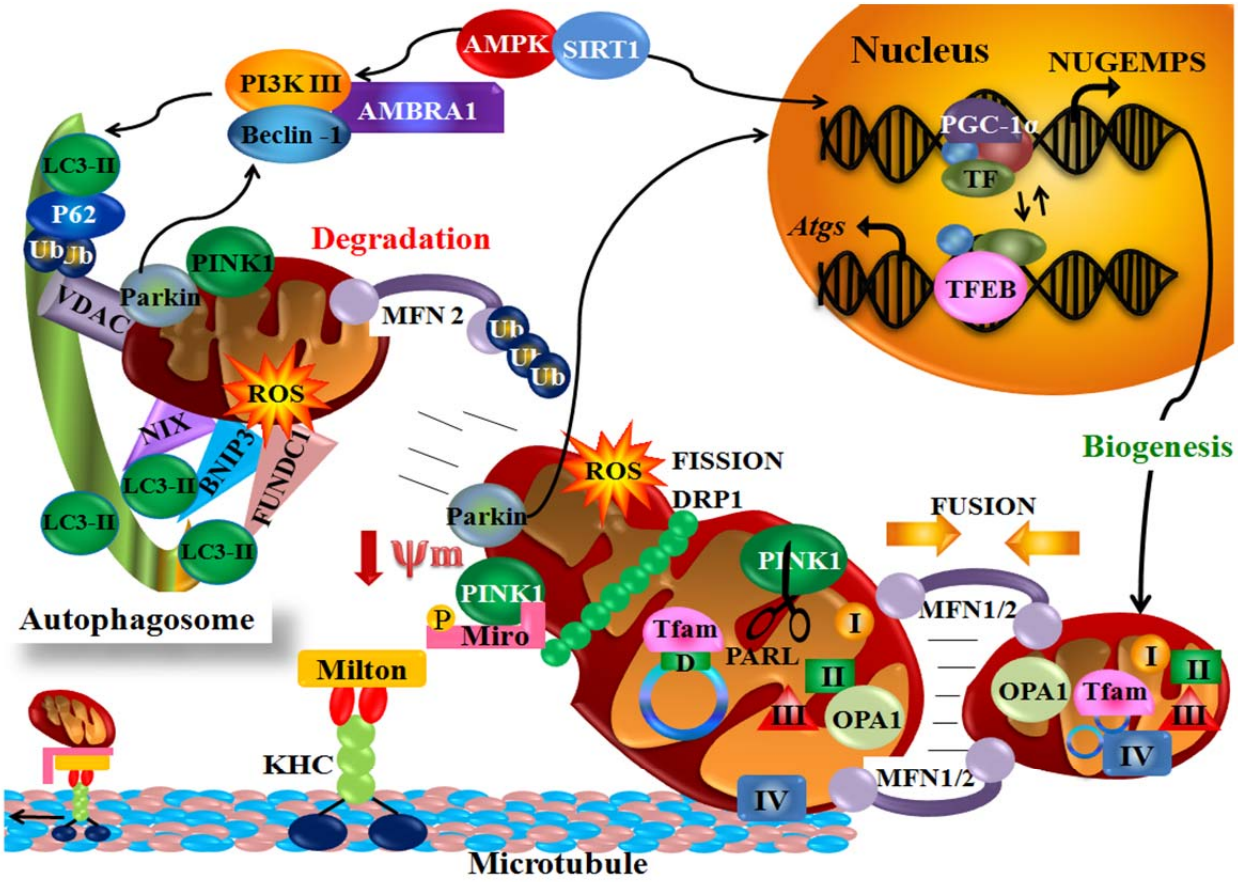

Figure 1. Coordinated regulation of mitochondrial biogenesis and degradation. Increases in metabolic demands result in the activation of energy sensors AMPK and SIRT1. Those activate PGC-1α -mediated transcription of NUGEMPS which can be incorporated in the ETC as well as enhance mtDNA replication by Tfam. Healthy mitochondria can join the larger network through mitochondrial fusion mediated by MFN1/2 and OPA1. Under these conditions mitochondrial membrane potential is intact thus resulting in the import and degradation of PINK1 by PARL. When mitochondria reach the end of their lifespan or become damaged they lose membrane potential and produce elevated levels of ROS. This disrupts PINK1 import resulting in its stabilization on the outer membrane and recruitment of Parkin. This results in mitochondrial fission by DRP1 and the segregation of the mitochondria from the network by both inhibition of trafficking through PINK1-mediated phosphorylation of Miro and block of mitochondrial fusion by Parkin-mediated ubiquitination (Ub) of MFN2. Miro then detaches from the motor complex (composed of Kinesin heavy chain (KHC) and milton) thus halting mitochondrial movement along microtubule tracks. Concomitantly autophagosome formation is induced by the formation of the PI3K-Beclin1-AMBRA1 complex. The presence of ubiquitinated proteins on the outer membrane as well as the translocation of specific receptors (NIX, BNIP3, FUNDC1) recruits the autophagosome to form around the nascent mitochondria thus targeting it for lysosomal degradation. Parkin can then contribute to the replacement of lost mitochondria by promoting PGC-1α –mediated biogenesis. PGC-1α can also mediate autophagy by enhancing TFEB expression, and vice versa.

2.2 Autophagy-lysosome System

2.2.1 Introduction

The term Autophagy literally means to self (auto) eat (phagy) in Greek. As suggested by its name, this is a process of self cannibalism, where cellular components are degraded by the lysosome. Autophagy is conserved on the evolutionary scale, which hints at the importance of this mechanism for cellular survival. However, the concept of self cannibalism is rather unsettling, and seems counter-intuitive to the main purpose of biological entities, which by and large is to survive. Why did then evolution favor this mechanism? Interestingly, autophagy appears to be cyto-protective, allowing for the recycling of dysfunctional and toxic proteins, as well as superfluous or damaged organelles into their basic building blocks. These can, in turn, be utilized for energy provision or biosynthesis. Autohpagy was first described to occur in the hepathocytes of starved rats, in the late 1950s by Clark and Novikoff (44, 188). However, it was not until 1963 that this process received its modern name “autophagy”, as coined by the visionary Christian de Duve (128). De Duve defined autophagy as vesicles containing material that is in various states of disintegration; he was also the first to suggest the involvement of lysosomes in this process. In 1974 de Duve received the Nobel Prize in physiology or medicine for his discovery of peroxisomes and lysosomes. Interestingly, the most significant breakthroughs in autophagy research occurred only in the last 20 years or so. Renewed interest in this process surfaced after novel evidence from yeast suggested autophagy can be genetically regulated. Indeed, pioneering work from Dr. Yoshinori Ohsumi's group indicated that the morphology of autophagy in yeast was similar to that documented in mammals (279). They then carried out a genetic screen in yeast to identify the first autophagy-related (Atg) genes (164). It was the identification of *ATG* genes in yeast that was the cornerstone upon which the field of

autophagy, as we know it today, was founded. This seminal finding along with major advancements in molecular biology and gene sequencing led to an autohpagy renaissance, giving rise to deeper molecular analysis of the process in higher eukaryotes.

Autophagy, is now well recognized as a highly conserved catabolic process responsible for the degradation of damaged or dysfunctional cellular components, as well as protein aggregates through the lysosomal machinery. A basal level of autophagy is ever-ongoing in all eukaryotic cells, and aids in cellular housekeeping, as it is responsible for the homeostatic turn-over of molecules and organelles (21). Indeed, in long-lived, post-mitotic tissues, such as striated muscle and neurons, autophagy presents the sole mechanism for whole-sale organelle removal, and degradation of aggregated proteins. Autophagy is of greater importance in these cells, due to their inability to dilute undigested materials by cellular division. It has been estimated that in the liver approximately 1%–1.5% of cellular proteins are catabolised by autophagy per hour, even under basal conditions (256). During metabolic stress, autophagy can be further up-regulated and acts as an adaptive response system, essential for cell survival and the maintenance of energetic balance (58, 256). Aberrant autophagy contributes to tissue dysfunction, and collapse of cellular homeostasis. While excessive autophagy may exacerbate protein catabolism, autophagic deficiency during development is often lethal, and if disturbed in adulthood leads to devastating cellular abnormalities. Undeniably, the pathogenesis of a myriad of conditions has been attributed to defective autophagy including: neurodegenerative disease, muscular dystrophies, metabolic disorders, cancer and cardiovascular disease (162, 182, 184, 213). Thus, autophagy is a tightly controlled process, the regulation of which is vital for the maintenance of cellular homeostasis and function.

Generally, three distinct types of autophagy exist, microautophagy; chaperone-mediated autophagy (CMA); and macroautophagy (Fig. 2). During microautophagy, the lysosmal membrane directly and indiscriminately engulfs cytosolic proteins that are in its vicinity, through invagination of the lysosomal membrane (145). During CMA, select cytosolic proteins containing a KFERQ motif are shuttled into the lysosome by chaperones, such as heat shock cognate protein of 70kDa (hsc70) (120). This substrate protein-chaperone complex then binds to lysosome-associated membrane protein type 2A (LAMP-2A) which facilitates the translocation of the complex into the lysosomal lumen, where it is rapidly degraded by lysosomal proteases (120). Macroautophagy (hereafter referred to as autophagy), is the most well studied of the three, and is characterized by the engulfment of materials to be degraded by a double membrane vesicle, known as the autophagosome. Autophagosomes, are subsequently delivered to the lysosome for hydrolytic degradation. Thus, autophagy is a vital cellular process involved in the catabolism and recycling of cytoplamic components by the lysosome.

2.2.2 Autophagosome formation

The recipe for making an autophagosome is complex and multi-factorial, and requires the tethering together of membranes from donor sources, much like quilting. The exact origin of the autophagosomal membranes is the subject of much debate, but mitochondria (78, 80), endoplasmic reticulum (67, 302), plasma membrane (217), and even the nuclear envelope (214) have all been documented to donate membranes to the growing phagophore, the immature precursor to the autophagosome. It is becoming more and more evident that not one size fits all, but rather the autophagosome membrane source may vary based on the autophaphagic stimulus and the cargo destined for degradation.

Autophagy is now recognized to occur in both a selective manner by targeting specific flagged proteins and organelles, as well as in a non-selective way, by haphazardly sequestering portions of the cytoplasm. A common overall mechanism directed by the core autophagy machinery governs both selective and non-selective autophagic degradation. Thus far, 38 autophagy related genes (*Atg*s) have been identified in fungi, but many additional proteins have been recognized to play a role in autophagy. These factors can be subdivided into categories based on the stage at which they are most relevant. The **induction** of phagophore formation is mediated by the unc-51-like kinase 1 (ULK1, the mammalian homologue of yeast Atg1) complex (Fig. 3) (164). This induction complex is composed of the core components: ULK1 which is a serine/threonine protein kinase, Atg13 a phosphoprotein, and focal adhesion kinase family–interacting protein of 200 kDa (FIP200; also known as RB1CC1) (84, 93, 94). Typically, under steady state conditions, when energetic supply and demand are in equilibrium, the metabolic sensors mammalian target of rapamycin (mTOR) and protein kinase A (PKA) negatively regulate autophagy through the phosphorylation and inhibition of the induction complex (93, 113, 274). When nutrients become scarce, the metabolic stress that ensues is recognized by energetic sensors. This leads to the activation of AMP dependant kinase (AMPK), and inhibition of mTOR activity, culminating in the induction of the autophagy process (93, 123). The next stage of autohpagy is the **nucleation** of the autophagosomal membrane. This involves the class III phosphatidylinositol 3-kinase (PI3K) complex, which is composed of PI3K vacuolar protein sorting 34 (VPS34), beclin 1, VPS15, activating molecule in BECN1 regulated autophagy protein 1 (AMBRA1), and ultraviolet irradiation resistance-associated gene (UVRAG). Bcl-2, on the other hand, can antagonise autophagy induction by sequestering beclin 1 and AMBRA1 (63, 103). This step is followed by vesicle **expansion.**

Phagophore expansion is a poorly defined event, but is thought to involve Atg9–mediated membrane recruitment (316). Indeed, Atg9 levels determine the number of autophagosomes. Moreover, there are two ubiquitin like (UBL) protein conjugation systems that act to promote the formation of the autophagosome precursor, the phagophore (193). These are involved in conjugation of Atg12, and ultimately, microtubule-associated protein light chain 3 (LC3) and its close relatives GABARAP and GATE16 (99, 117, 134). One pathway involves the covalent conjugation of Atg12 to Atg5. This occurs with the help of Atg7 and Atg10, E1- and E2-like enzymes, respectively. The Atg12–Atg5 heteromer then interacts with Atg16-like 1 (Atg16L1) (217). This complex acts as a *de facto* E3-like ligase to promote the second conjugation reaction, involving LC3. The conjugation of LC3 to phosphatidylethanolamine (PE) is essential for phagophore expansion and sealing. LC3 exists in its inactive form free in the cytosol, and must first be cleaved by the protease Atg4 giving rise to LC3-I (250). The E1-like enzyme Atg7 and the E2-like enzyme Atg3 are then tasked with priming LC3-I for its final conjugation to PE, by the E3-like complex of Atg12-Atg5. Lipid conjugation converts LC3-I, to the LC3-II form that is attached to either side of the growing phagophore membrane.

Aside from its role in autophagosome expansion, LC3-II also plays an essential role in cargo selection. In selective types of autophagy, cargo recognition occurs during the expansion step. During selective autophagy the receptors p62 (also known as Sqstm1) (178), neighbor of BRCA1 gene 1 (NBR-1) (125), nuclear dot protein 52 kDa (NDP52) (177), and optineurin (309) interact with ubiquitinated proteins on organelles or invading pathogens and recruit the autophagosome. These proteins act as unique adaptors, as they are endowed with both ubiquitin (Ub) and LC3 interacting regions (LIR). The cargo–receptor complex is then selectively encapsulated by the autophagosome through receptor-mediated interaction with LC3-II.

Organelle specific autophagy receptors that act independently of Ub have also been identified. Mitophagy specific receptors such as BNIP3 and BNIP3-like (BNIP3L; also known as NIX), or pexophagy specific receptor Atg36, interact directly with LC3-II on the phagophore (83, 175, 242).

Fusion; Once the autophagosome is complete it begins its journey along microtubule track to the lysosome. LC3, Rab7 and FYCO1, form an adaptor protein complex that promotes microtubule transport of autophagic vesicles. FYCO1, which contains both an LIR and a phosphatidylinositol 3-phosphate (PtdIns3P)-binding domain is important for autophagosome movement (198).The autophagosome and lysosme then fuse together to form the autolysosome, a process which requires the assistance of lysosomal-associated membrane proteins (LAMPs), as well as the tethering and fusion components, including Rab proteins, and SNAREs (171). However, these proteins are not specific to macroautophagy.

Degradation and efflux; The autophagosomal contents are subsequently broken down within the lysosomal acidic environment by a wide range of hydrolases. The macromolecules released can then be recycled to restore cellular energetic balance or be used for biosynthesis. Moreover, the amino acids generated from proteolysis within the lysosomal lumen can act in a retrograde manner to activate mTOR and inhibit autophay progression, in a negative feedback loop.

2.2.3 Selective autophagy

Once considered a non-selective, bulk degradation process, autophagy is now well recognized for its selectivity. It is clear that autohpagy is able to discriminate between healthy and toxic organelles and proteins as well as invading organisms, with the aid of various receptors and signaling components. Although, autophagy induced by severe nutrient depletion may not be

overly selective, as the sole goal of bulk autophagy is nutrient liberation. Constitutive autophagy, however, is extremely selective and provides a helping-hand during the turnover of cellular components. Various types of substrates have already been described for selective autophagy, and this number is continuously on the rise. These include: mitochondria (mitophagy), endoplasmic reticulum (reticulophagy), peroxisome (pexophagy), nucleus (nucleophagy), pathogens (xenophagy), lipids (lipophagy) and many more (219). During selective autophagy, dysfunctional or obsolete proteins and organelles are targeted for degradation by various receptors, which in turn, entice the arrival of the autophagosome.

The process of selective autophagy, as mentioned above, relies on core autophagy components, but also requires specific selective receptors. The most infamous of them is p62, which is ubiquitously expressed and mediates the removal of a wide range of ubiquitinated proteins. p62 contains both a ubiquitin binding domain and an LIR, thus assisting with the association of cargos with autophagosomes (199). p62 itself is often degraded alongside its cargo, as it accompanies the targeted components to their final destination. Indeed, conditions that are characterised by defective autophagy often display large accumulations of p62 along with ubuiquitinated proteins. Interestingly, in some cases elimination of p62 in autohpagy deficient models results in protein aggregate clearance (221), suggesting a role for p62 in the coalescing of materials for autophagic degradation. Many other adaptor proteins have been described such as NBR1 (125), SMURF1 (194), mitophagy specific FUNDC1, NIX and BNIP3 (153, 242), xenophagy specific NDP52 (177) and optineurin (309), and the number of receptors continues to grow as more types of selective autophagy are identified .

The benefits of selective autophagy are vast, as it allows for the surgical removal of targeted substrates, without disturbing any other cellular components. For instance, the selective

removal of mitochondria during hypoxia, or organelle damage, or even when the organelle becomes obsolete such as during erythrocyte maturation, all occur selectively through mitophagy (16, 112, 187) (for details see Mitophagy chapter 2.1.4). The ability to specifically target the elimination of desired cellular constituents presents great therapeutic potential for the treatment of many diseases characterised by the accumulation of toxic materials such as Huntington's disease, Alzheimer's disease, lysosomal storage diseases and many more.

2.2.4 The Lysosome

Lysosomes are acidic membrane-bound organelles, containing hydrolytic enzymes required for the degradation of a wide variety of substrates including proteins, lipids, carbohydrates, nucleic acids, and more. The long standing dogma depicting lysosomes as mere cellular repositories, has recently been dismissed by evidence that lysosomes play a key role in cellular homeostasis (261). By controlling both cellular clearance and energy production in response to environmental cues, the lysosome has reinvented itself as an essential metabolic organelle. Lysosomes act not only as the final degradative organelles but also possess the ability to sense nutrient status and initiate the appropriate long- and short-term signaling cascades required to restore equilibrium, if it is disturbed (259, 261, 329). Moreover, lysosomal hydrolytic enzyme activity increases during cellular insults, indicating lysosomes are malleable and responsive to cellular needs (131, 232, 261). These organelles contain a slew of hydrolases (e.g., proteases, nucleases, esterases, polysaccharidases, and glycosidases) capable of degrading a wide range of components. As such, lysosomes act as the terminal degradative compartment for macromolecules, derived from within the cell or from its surroundings (Fig. 2). Aside from their well documented role in autophagy, lysosomes also present a central hub for vesicular trafficking. Indeed, vesicles originating from the trans-golgi network (TGN) or the endocytotic

network often end up in the lysosome, implicating the involvement of this organelle in receptor recycling as well as intra- and extra-cellular communications.

2.2.4.1 Lysosomal biogenesis

Lysosmes are not formed *de novo,* but rather receive input from both the endocytic and biosynthetic pathways. In addition, lysosomes exist in all shapes and sizes due to variations in their contents and stages of material degradation. This peculiar diversity of lysosomal morphology and the existence of various maturation steps resulted in a complex terminology for lysosomes.

The lysosome is mainly composed of two functionally essential classes of proteins, acid hydrolases, and integral lysosomal membrane proteins. There are currently over 50 known lysosomal hydrolases which function to break down the various substrates and macromolecules. These allow the lysosome its potent catabolic abilities. Lysosomal membrane proteins have also been documented to be vital for lysosomal function and are active in organelle acidification, and import of functional proteins as well as those destined for degradation. The most well-studied membrane proteins are the lysosome associated membrane proteins 1 and 2 (LAMP1 and LAMP2) and the vacuolar ATPases. Both classes of lysosomal proteins are required for the lysosomal biogenesis.

Due to its role as a major vesicular trafficking hub, it is often difficult to distinguish vesicles targeted to the lysosome for degradation versus those delivering freshly synthesised hydrolases for lysosomal biogenesis and expansion. Indeed, proteins destined for the lysosome may arrive from either the endocytic or biosynthetic pathways (131, 232). Cargo that is endocytosed at the plasma membrane travels through various endosomal intermediates with the goal of ending up at its final destination, the lysosome. A large number of endosomal

intermediates exist and they vary in everything from morphology, content, makeup, pH and more. A multitude of steps involving exchange of membranes and fusion result in the maturation of an early endosome into a late endosome and eventually may be even to a fully functional lysosome.

Similarly, the pathway of lysosomal enzyme biosynthesis involves transport of the newly synthesized enzymes through multiple endosomal compartments before reaching their final destination. This ensures that once reaching the lysosome, these hydrolases are proteolytically processed (131). A wide majority of lysosomal hydrolases are manufactured in the endoplasmic reticulum and sorted in the trans-Golgi network (TGN). The transport of newly synthesized lysosomal enzymes to lysosomes is dependent on their specific recognition by receptors for mannose 6-phosphate (MPR) (62). MPRs bind the mannose 6-phosphate tagged constituents, and the receptor-ligand complexes are transported through the TGN to endosomes in clathrin-coated vesicles. The ligands dissociate from the MPRs once they have reached the acidic pH of the endosome. MPR-independent transport can also occur but this process is poorly understood.

The structural and molecular machinery required for the transport of membrane proteins is enigmatic but is known to involve TGN sorting.

Thus, lysosomes are the final degradative organelles upon which many cellular trafficking networks converge. With this in mind, very little is actually known about the biosynthesis of this important organelle, warranting further investigation.

2.2.5 Transcriptional regulation of the autophagy-lysosome system

Transcriptional regulation of autophagy is still in its infancy and only a handful of transcription factors have been recognized to have a role in the regulation of the autophagy-lysosome system. However, a growing body of evidence suggests that the autophagic response to

stress may be biphasic. Cellular metabolic distress first induces a rapid increase in autophagic flux, which occurs within minutes or hours of exposure to stressful stimulus, and is mediated by post-translational protein modifications. If prolonged, the initial phase is generally followed by a long-term potentiation that relies on the activation of a transcriptional program (206). Stress-responsive transcription factors including the FoxO family, p53, and TFEB, have all been identified to play a major role in the regulation of the secondary autophagic response.

A recent seminal discovery, by the group of Dr. Andrea Ballabio, of a lysosomal gene network — the coordinated lysosomal expression and regulation (CLEAR) network — and of its master regulator TFEB has lead to an increased interest in the transcriptional regulation of the autophagy-lysosome system (197, 260). TFEB is a basic helix-loop-helix, leucine zipper, transcription factor, belonging to the family of microphthalmia-associated transcription factors (MITF). TFEB was found to regulate lysosomal biogenesis, and clearance as well as autophagy and autophagy-lysosome fusion related proteins (260). These findings placed TFEB at the epicentre of cellular degradation pathways. This factor acts by recognizing a 10–base pair CLEAR motif, enriched in the promoter regions of numerous genes. Interestingly, TFEB transcriptional activity appears to be regulated by mTORC1. When mTORC1 is activated by amino acids arising from within the lysosomal lumen, it phosphorylates TFEB on several serine and threonine residues, including serine 211 (261). Phosphorylated TFEB is detained in the cytosol by the chaperone14-3-3. Additionally, TFEB can also be phosphorylated and negatively regulated by ERK. During starvation, TFEB is liberated and can translocate to the nucleus, where it stimulates the expression of autophagy-lysosome genes (261). Interestingly, TFEB also translocates into the nucleus during lysosomal stress that is pharmacologically induced by drugs that block lysosomal acidification or autophagosome-lysosome fusion (261). Therefore, TFEB

provides a coordinated transcriptional regulation of autophagosomes and lysosomes in response to cellular and lysosomal stress. A TFEB family member, TFE3, has also been recently recognized to have very similar regulation and function to TFEB (160). This type of redundancy further supports the importance of these transcriptional modulators. Similar to TFEB, TFE3 is regulated by mTORC1 at the lysosome, and after nutrient deprivation rapidly translocates to the nucleus, where it too activates the transcription of genes carrying the CLEAR motif (160). In contrast to TFEB and TFE3, ZKSCAN3, is a repressor of autophagy (43). Overexpression of ZKSCAN3 results in suppression of autophagy, whereas silencing this repressor promotes autophagy. In contrast to TFEB and TFE3, ZKSCAN3 negatively regulates the expression of genes involved in autophagy and lysosome biogenesis. ZKSCAN3 also appears to be regulated by mTORC1, where nutritional abundance promotes the nuclear localization of ZKSCAN3 in an mTOR-dependent manner. Starvation, on the other hand, results in cytoplasmic accumulation of this repressor. Thus, TFEB and TFE3 provide a turn-key mechanism for cellular clearance by coordinately regulating both autophagosome and lysosme arms involved in this process. These transcription factors appear to be antagonized by the transcriptional repressor ZKSCAN3. This presents an interesting tug-of-war model, where the regulation of cellular clearance may hang in the balance between these antagonists. Indeed, this is an enticing regulatory axis to target pharmacologically, for the fine-tuning of cellular clearance.

Additional transcription factors that have been recognized to regulate the expression of autophagy and lysosomal biogenesis include the evolutionarily conserved Forkhead box O (FoxO). The function and activity of FoxO proteins are mainly regulated by post translational modifications such as phosphorylation and acetylation which effects their cellular localization. The FoxO family is negatively regulated by the insulin-Akt axis, which is upstream of mTOR.

Both transcription-dependent and -independent functions have been described for the FoxO family in the regulation of autophagy. FoxO3 drives a transcriptional program that enhances the expression of numerous autophagy-related genes in various cell types. For instance, FoxO3 upregulates the transcription of core autophagy machinery, in response to cellular stress (298, 324, 325). FoxO1, another family member, also transactivates autophagy genes once in the nuclear compartment (86, 327). However, cytosolic FoxO1 can also regulate autophagy, independently of its transcriptional activity (326). In summary, these observations indicate that the FoxO family regulates autophagy, mainly via transcriptional events (159, 324). Interestingly, like TFEB, FoxO3 can also be regulated by ERK. Where ERK directly interacts with and phosphorylates FoxO3 rendering it inactive. The ERK-phosphorylated FoxO3 is then degraded in an MDM2-mediated ubiquitin-proteasome pathway (317). Therefore, ERK and mTOR negatively regulate autophagy-lysosome transcription by blocking nuclear translocation of both FoxO3 and TFEB.

The tumour suppressor p53, often endearingly referred to as "the guardian of the genome", has been documented to regulate an ever increasing number of biological processes. Indeed, this jack of all traits has been implicated in the transcriptional regulation of the autophagy-lysosome system. p53 activation has been documented to inhibit mTOR activity and promote transcriptional upregulation of autophagy (60, 206). Moreover, a global genomic profiling of the p53 transcriptome revealed a large number of autophagy proteins, involved in various steps of the process to be direct p53 target genes (121, 158). However, this regulation appears to be complex and is highly dependent on the subcellular localization of this protein, as cytosolic p53 appears to arrest autohpagy (18, 227, 283). Moreover, the p53 target genes Sesn1 and Sesn2 have also been documented to regulate autophagy through the modulation of AMPK-

mTORC1 axis (158). On the contrary, however, cytoplasmic p53 inhibits autophagy in a TIGAR dependant mechanism, providing a negative feedback loop for this process (18).

The transcriptional co-activator PGC-1α has also been recently documented to exert transcriptional regulation over autophagy in several cell types (257, 280, 291). TFEB promoter activity was enhanced in Huntington disease patient cells that were over-expressing PGC-1α, which ultimately resulted in increased clearance of toxic protein aggregates via autophagy (291). Interestingly, the reverse was also documented, where during conditions of overfeeding over-expression of TFEB enhanced PGC-1α levels in the liver (259). Moreover, PGC-1α overexpression in a mouse model of Pompe disease, a lysosomal storage disease, has been attempted as a therapeutic approach, albeit with limited success (280). Interestingly, cells lacking the acetyl transferase GCN5 display an upregulation in both PGC-1α and TFEB. In this context manipulation of either TFEB or PGC-1α resulted in the same magnitude change in the other, suggesting a potential regulatory loop between the two transcriptional regulators. Therefore, against all odds, PGC-1α and TFEB make the unlikeliest of friendships in order to transcriptionally co-ordinate the regulation of metabolic homeostasis.

Altogether, a number of transcriptional regulators of autophagy have emerged and appear to potentiate a nuclear-autophagic response to cellular metabolic stress. With this in mind, the transcriptional regulation of autophagy and lysosomal components has only recently begun to be elucidated, much more work is necessary to establish additional transcriptional regulators of this pathway as well as further investigate the physiological circumstances that modulate this regulation.

2.2.6 Autophagy-lysosome in health and disease

The importance of the autophagy-lysosome system in cellular health has been documented time and again. Countless disorders have been attributed to dysfunctions in various stages of this pathway. However, the importance of this mechanism is further highlighted by conditions that require enhanced cellular turnover or increased energetic supply such as development and aging.

2.2.6.1 Autophagy in health and disease

Autopahgy has been documented to play a major role during oocyte fertilization in both, the degradation of maternal mRNAs, and the elimination of paternal mitochondria (171, 218, 249, 290). Moreover, autophagy works to provide nutrients to the developing zygote until implantation, and to the neonate during the period of nutrient deprivation that occurs immediately after birth (133). Indeed, the importance of autophagy in development is recapitulated when essential autophagy genes are deleted early in this process. Loss of Beclin 1 (320), AMBRA1 (63), or TFEB (260) is embryonically lethal, whereas mice succumb shortly after birth when either Atg3, (270), Atg5 (133), Atg7 (129), Atg9 (233), or Atg16L1 are eliminated (234). The generation of conditional tissue-specific knockout models further elucidated the importance of autophagy in organogenesis, lineage commitment, and differentiation of various cell types. Skeletal muscle from animals with autophagic deficiencies, mediated by a conditional deletion of core autophagy proteins, such as Atg5 or Atg7, exhibits massive accumulations of dysfunctional mitochondria that are less efficient at energy provision, and produce harmful reactive oxygen species as a by-product of improper respiration. This muscle also accrues p62 and ubquitin positive protein aggregates, all of which contributes to muscle wasting, weakness and fatigue. (162). Surprisingly, genetic mutations in extracellular

matrix proteins such as collagen-VI (col6a1), corresponding to human Bethlem myopathy and Ullrich congenital muscular disorder, also result in autophagy mismanagement and accumulation of damaged and superfluous proteins and organelles that are toxic to the cellular milieu (75). Remarkably, the reactivation of autophagy in a mouse model of col6a1 knockout with a low-protein diet, rapamycin, or cyclosporin A, results in the clearance of abnormal organelles, which ameliorates the dystrophic phenotype. This suggests a potential therapeutic model for the treatment of these debilitating genetic diseases. These studies confirm the importance of an effective autophagy program to skeletal muscle health.

2.2.6.2 Lysosomes in health and disease

Several pathologies, as well as aging have been associated with lysosomal dysfunction. This may be due to a decline in cellular clearance and a progressive accumulation of toxic intracellular materials.

Indeed, dysfunctional lysosomal degradation is observed with aging, and likely contributes to cellular senesce. Moreover, various genetic abnormalities that impede the autophagic process lead to deleterious pleiotropic defects, collectively dubbed lysosomal storage diseases (LSDs). LSDs result from rare mutations to essential lysosomal genes. LSDs are characterized by the accumulation of undegraded autophagosomes, implicating autophagy in the ethiology of these conditions. Over 60 distinct LSDs have been identified (181), and are characterized by severe neurodegeneration, mental decline, cognitive problems, behavioural abnormalities, myopathies, cardiomyopathies and more. Mutations that impair lysosome-mediated degradation result in the accumulation of undegraded substrates within the lysosome, which ultimately result in a backlog of undegraded materials and in the accumulation of autophagosomes as well as toxic protein aggregates and dysfunctional organelles. This leads to a

myriad of symptoms in different cells and tissues. Indeed, acid alpha-glucosidase (GAA) deficiency in mice, an animal model of Pompe disease, results in glycogen accumulation, lysosomal expansion and rupture as well as autophagosome accumulation all of which lead to cardiomyopathy and debilitating muscular myopathy (213). Moreover, evidence implicating lysosome and autophagy dysfunction in neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's continue to accumulate. The currently available therapeutic strategy for LSDs is enzyme replacement therapy, aimed at reactivating the defective lysosomal enzyme, however, this therapeutic model leaves much to be desired. TFEB over-expression was recently successfully used to clear autophagy-lysosome backlog in Pompe mice by mediating lysosomal exocytosis (273).

The autophagy-lysosome system appears to have a pleiotropic role in tissue turnover and cellular clearance, which are compromised during cellular senescence (95, 229). In accordance with this, suppression of autophagy causes progressive dysfunction in various organs with age. Interestingly, multiple factors currently recognized to be involved in life extension also impinge on autophagy. For instance, rapamycin-mediated longevity is mediated by mTOR suppression which leads to activation of autophagy. Similarly, resveratrol mediated-sirtuin activation also leads to enhanced autophagy. Caloric restriction can inhibit mTOR and activate sirtuins as well as AMPK, all signals that converge on autophagy activation. Moreover, spermidine- and coffee-mediated life-span extension have been attributed to autophagy activation (56, 207). Exercise also mediates many metabolic benefits that could potentiate longevity and these also appear to, at least partially, depend on functional autophagy (87). The compilation of these findings indicates that autophagy may indeed be an important element of the water flowing down the fountain of youth. How autophagy may mediate life extension, is not fully elucidated yet, but an educated

guess may be that autophagy facilitates the disposal of noxious proteins and organelles, thus preventing their accumulation and promoting cellular rejuvenation.

2.2.7 Conclusions

Autophagy is important for the maintenance of cellular vigour, health and youth as it allows for proper cellular remodelling and organelle turnover. The identification of factors that regulate the autophagy-lysosome system, both transcriptionally and post-translationally, illuminate attractive pharmacological targets intended for the treatment of diseases in which this system is compromised, and maybe even to combat some age-dependant whole body derailments.

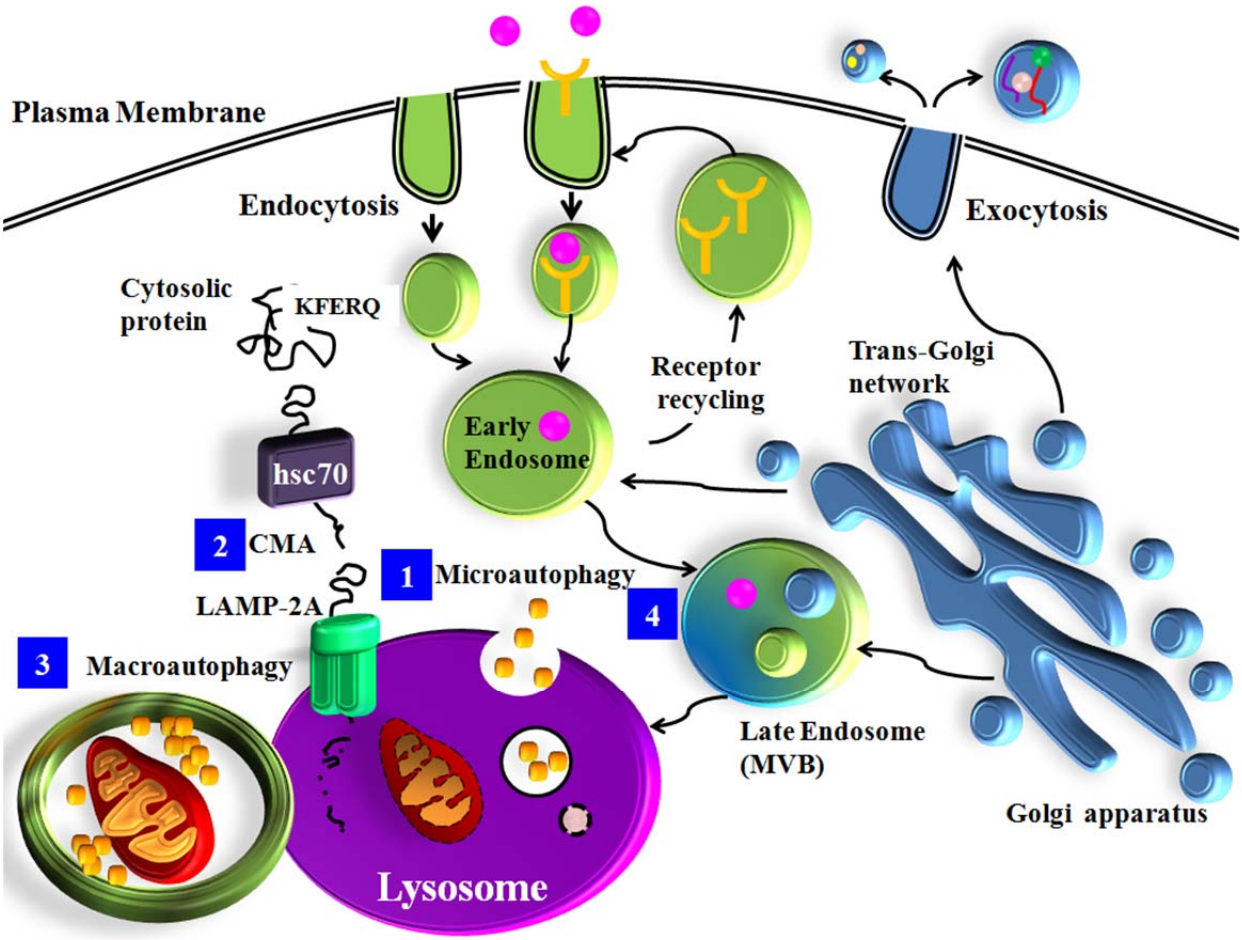

Figure 2. Lysosomal trafficking. 1) Microautophagy: the lysosmal membrane directly engulfs cytosolic proteins through the invagination of the lysosomal membrane. 2) CMA, (Chaperone -Mediated Autophagy) cytosolic proteins containing a KFERQ motif are shuttled into the lysosome by chaperones, such as heat shock cognate protein of 70kDa (hsc70). This substrate protein-chaperone complex then binds to lysosome-associated membrane protein type 2A (LAMP-2A) which facilitates the translocation of the complex into the lysosomal lumen, where it is rapidly degraded by lysosomal proteases. 3) Macroautophagy is characterized by the engulfment of materials to be degraded by a double membrane vesicle, known as the autophagosome. Autophagosomes are subsequently delivered to the lysosome for proteolytic degradation. 4) The lysosomal network of vesicular traficking is involved in lysosomal biogenesis. Vesicular trafficking from various sources such as endocytosis from the plasma membrane, through the endosomal pathway. Trans golgi network delivery of newly synthesized lysosomal hydrolases by direct endosomal trafficking or indirectly via plasma membrane.

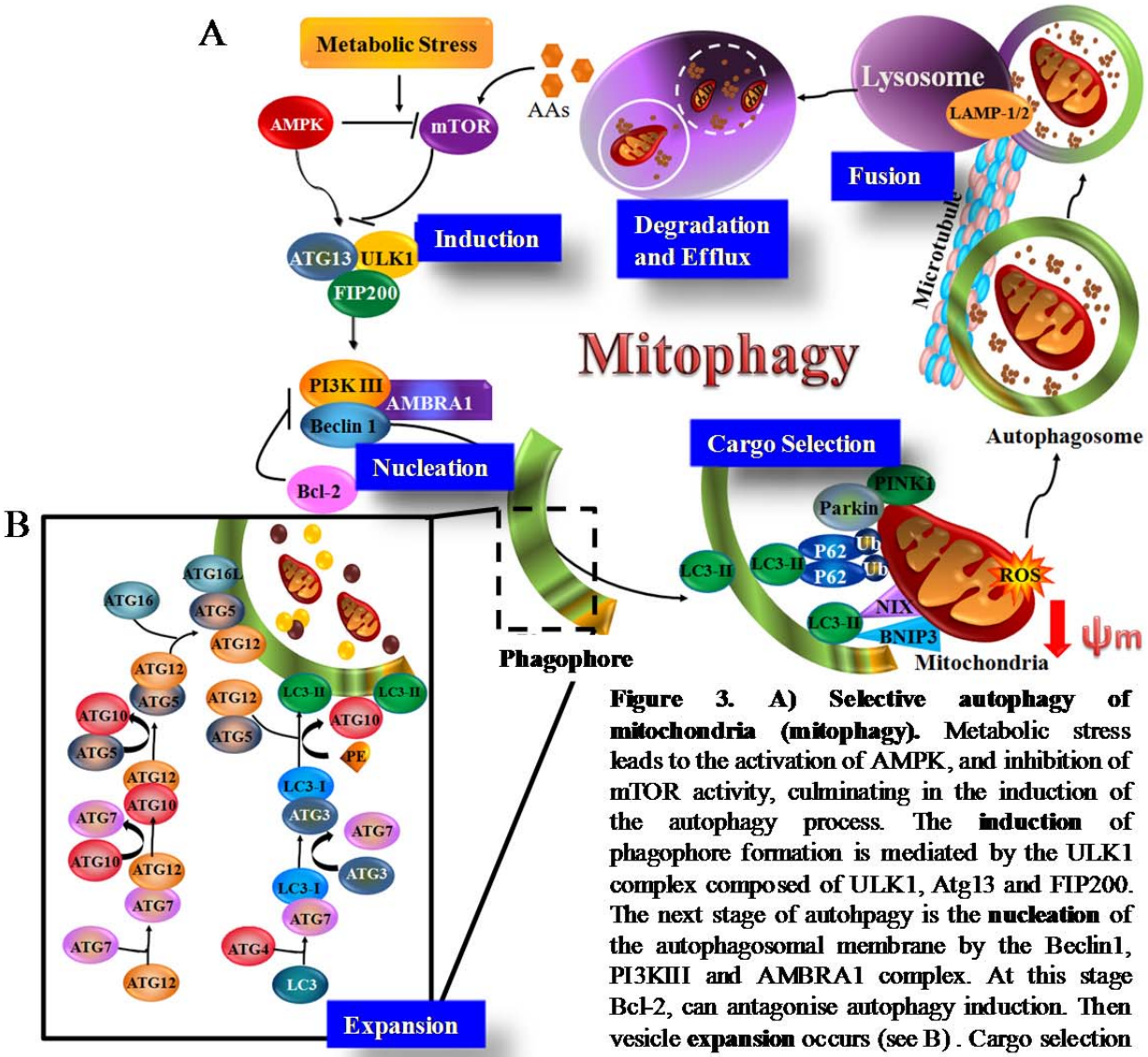

Figure 3. A) Selective autophagy of mitochondria (mitophagy). Metabolic stress leads to the activation of AMPK, and inhibition of mTOR activity, culminating in the induction of the autophagy process. The **induction** of phagophore formation is mediated by the ULK1 complex composed of ULK1, Atg13 and FIP200. The next stage of autohpagy is the **nucleation** of the autophagosomal membrane by the Beclin1, PI3KIII and AMBRA1 complex. At this stage Bcl-2, can antagonise autophagy induction. Then vesicle **expansion** occurs (see B) . Cargo selection occurs through LC3-mediated recognition of autophagy receptors. In the case of mitochondria, specific receptors such as NIX, and BNIP3 directly signal to LC3. Proteins ubiquitinated by PINK1 mediated Parkin recruitment attract the adaptor p62 which also binds LC3. Complete with cargo the autophagosome travels to the lysosome via microtubule tracks. Lysosome and autophagosome then fuse with the help of LAMP1/2 and the contents are degraded by hydrolases, resulting in the efflux of basic building blocks, such as amino acids back into the cytosol for reuse in biosynthesis or for energy provision.

B) Autophagosome expansion. Two ubiquitin-like conjugation systems are invovled in this process. One pathway involves the conjugation of Atg12 to Atg5. This occurs with the help of Atg7 and Atg10. The Atg12–Atg5 heteromer then interacts with Atg16L. This complex acts as a *de facto* E3-like ligase to promote the second conjugation reaction, involving LC3. The conjugation of LC3 to PE is essential for phagophore expansion and sealing. LC3 exists in its inactive state and is first cleaved by the protease Atg4 giving rise to LC3-I. Atg7 and Atg3 are then tasked with priming LC3-I. Then the E3-like complex of Atg12-Atg5 conjugates LC3-I to PE yielding LC3-II, the active form that is attached to either side of the growing phagophore membrane.

2. 3 Skeletal Muscle

2.3.1 Introduction

Skeletal muscle is the largest organ in the human body and can comprise 40-50% of the body mass of a lean healthy adult (108). Perhaps the most obvious role of skeletal muscle is locomotion. Throughout evolution, muscle allowed our ancestors to escape from whatever may have been chasing them and, in turn, hunt and capture pray to survive. However, skeletal muscle also serves as a protein and energy reservoir which is indispensible in times of nutritional deficits (20, 30, 59). Indeed, skeletal muscle is a highly adaptive tissue molded by its milieu, a property known as muscle plasticity (91). Physiological stimuli such as contractile activity, endocrine stimulation and nutritional status can all influence muscle homeostasis. Exercise, proper nutrition and growth factors help preserve muscle mass and health. Conversely, muscle disuse, loss of innervation, inflammation, nutritional deficit, loss of hormonal stimulation, and an underlying morbidity all lead to muscle atrophy (253). Moreover, muscle wasting is now being recognized as a co-morbidity accompanying a growing number of pathologies such as cardiovascular disease, diabetes, as well as cancer, and importantly, is associated with poor prognosis (11, 77, 119, 141, 277). Therefore, delving into the molecular mechanisms responsible for the regulation of muscle health will help shed light on potential targets for the treatment of debilitating muscle atrophy. Moreover, exercise is able to induce pleiotrophic metabolic benefits, as well as delay or even prevent the loss of muscle mass induced by various atrophic conditions (54, 223, 322). Indeed, physical activity is the only common remedy for a variety of ailments and metabolic abnormalities. Thus, illuminating proficient ways to harvest the benefits of exercise will lead to great breakthroughs in the treatment of disease.

Muscle mass, by and large, depends on the balance between protein synthesis and protein degradation, tilting the scale in one direction or another will result in muscle hypertrophy or atrophy. Interestingly, muscle mass also correlates with metabolic status. As mitochondria present the main source of energy in the form of ATP, organelle content and vitality also constitute an important avenue of muscle mass regulation. This chapter will focus on molecular mechanisms involved in the regulation of muscle mass and plasticity.

2.3.2 Muscle Atrophy

Atrophy is defined as a decrease in cellular or tissue size as a result of loss of organelles, cytoplasm and proteins. Prolonged muscle disuse, loss of innervation, starvation, diabetes, cardiovascular disease, and sarcopenia of aging all result in muscle atrophy. Moreover, various pathologies such as cancer, sepsis and AIDS are accompanied by muscle atrophy, known as cachexia. Loss of skeletal muscle mass and force generating capacity is debilitating and may lead to loss of independence. Thus, the prevention of muscle atrophy is of great importance and interest for public health.

Protein synthesis is largely regulated by the insulin and IGF-1 (Insulin-like Growth Factor 1) pathway acting through downstream kinases such as Akt (PKB) and mTOR (23, 47, 226). Various atrophic conditions perturb this axis, resulting in reduced protein synthesis (69). However, although lower protein synthesis contributes to the loss of muscle mass, increased protein degradation appears to play a more dominant role in muscle atrophy. In fact, during certain atrophic conditions, such as denervation, protein synthesis remains the same or can even increase slightly, but the robust induction in protein elimination predominates, resulting in a net loss of muscle protein.

Several proteolytic systems have been described in skeletal muscle, but not any one of those is an island, as these systems often work in concert to result in atrophy (278). The two major proteolytic pathways are the ubiquitin-proteasome and autophagy-lysosome systems which provide machinery for the degradation of a majority of proteins and organelles. Two additional systems, the calcium- dependent (calpain) and the caspase systems are responsible only for some restricted proteolysis (278, 287, 304). A small amount of atrophy can also be attributed to cell death (apoptosis <1%) although this topic is controversial (29, 192). Moreover, due to the dynamic nature of muscle atrophy, continuous transcriptional input is required to fuel proteolytic pathways (Fig. 4).

2.3.2.1 Ubiquitin-Proteasome system in muscle atrophy

The ubiquitin-proteasome system (UPS) is responsible for the degradation of a large number of individual proteins. Interestingly, components of the UPS are amongst the most commonly upregulated genes in a number of models of muscle atrophy including diabetes, denervation, cancer cachexia, fasting and renal failure (137). To be degraded, proteins must first be tagged by the addition of polyubiquitin chains. This allows the targeted protein to dock onto the proteasome, a large multisubunit proteolytic complex. The ubiquitination of a target substrate requires three distinct components E1, E2 and E3. E1 is a ubiquitin activating enzyme which must first prime ubiquitin (Ub) in an ATP-dependent process. The activated ubiquitin is then transferred to E2, a conjugating enzyme, and then transferred onto a specific substrate with the help of E3, a ubiquitin ligase enzyme. E3 enzymes catalyse the rate-limiting step in Ub conjugation and are extremely substrate-specific, providing selectivity to the UPS. Once tagged, the protein is delivered to the proteasome for digestion. Two muscle-specific ubiquitin ligases, muscle ring finger1 (MuRF1/Trim63) and muscle atrophy F-box (Atrogin1/MAFbx), are

dramatically upregulated in various atrophic models (22, 72, 137). Moreover, mice deficient in MuRF1 and MAFbx are partially protected against disuse atrophy (22). However, over 650 E3 ubiquitin ligases have been identified within the genome and additional E3 ligases involved in muscle atrophy continue to be recognized. For example, Trim32 has been recently documented to be responsible for the degradation of thin filaments by reducing PI3K-Akt-FoxO signaling in normal and atrophying muscle (45, 46). Inhibition or down-regulation of Trim32 during atrophic conditions protected against muscle loss (46). TRAF6 is another E3 ligase which acts upstream of NF-κB and is upregulated during atrophic conditions (202). TRAF6 is a peculiar ubiquitin ligase as it catalyzes the conjugation of ubiquitin to the Lys63 residue of its target proteins. Lys63-linked ubiquitination is more commonly associated with autophagic degradation as it is recognized by the adaptor protein p62, as compared to Lys48-linked polyubiquitin chains which signal proteasome-dependent degradation (126, 199). Mice lacking TRAF6 are protected against atrophy induced by denervation, cancer or starvation (202). An additional novel E3 ubiquitin ligase downstream of TGF-β /BMP signaling, named muscle ubiquitin ligase of the SCF complex in atrophy-1 (MUSA1/ *Fbxo30*), has only recently emerged and appears to contribute to muscle loss, however, its substrates remain to be defined (248). Interestingly, the ubiquitination process is reversible and targets can be untagged by de-ubiquitylating enzymes, which remove polyubiquitin chains from tagged proteins. This presents an enticing point of regulation however, to date, very little is known about these enzymes in skeletal muscle (48).

Although the UPS greatly contributes to muscle atrophy by degrading a large mass of proteins, this system cannot degrade intact myofibrils or organelles, and therefore cannot alone account for muscle atrophy (278). This suggests that proteolytic systems work in concert to result in muscular atrophy.

2.3.2.2 The Autophagy-Lysosomal system in muscle atrophy

The autophagy-lysosome system allows for the bulk degradation of long-lived organelles and protein aggregates. In this process (discussed in detail in chapter 2), tagged portions of the cytoplasm, dysfunctional organelles, and protein aggregates are sequestered into double membrane vesicles. These vesicles are known as autophagosomes and are delivered to the lysosome for proteolysis. Lysosomal involvement in cellular degradation, during nutrient depletion and denervation atrophy, was reported in muscle as far back as the 1970s, by the group of Dr. Stefano Schiaffino (254). However, the molecular basis for the regulation of this pathway remained largely unexamined until recent years. Autophagy was brought back into the limelight by the discovery of novel autophagy genes in yeast, and by the development of better tools allowing for the proper dissection of this intricate pathway (164, 279). The generation of a mouse line expressing green fluorescent protein tagged LC3 (an essential factor responsible for autophagosome formation), revealed muscle to have one of highest rates of autophagy during nutrient depletion (172). Interestingly, glycolytic type II muscles display a higher content of autophagosomes than slow oxidative type I muscles, and indeed autophagy flux is higher in these muscles both basally and in response to fasting (172, 173). Moreover, autophagy has been documented to contribute to muscle atrophy induced by constitutively active FoxO3, or by oxidative stress resulting from a mutation in superoxide dismutase ($SOD1^{G93A}$). Reducing autophagy by LC3 knockdown was protective of muscle mass in both of these atrophic models (52). These findings prompted a surge of interest into ways of blocking autophagy in an attempt to spare muscle mass during atrophic conditions. However, muscle-specific deficiencies in the essential autophagy factors Atg5 or Atg7 resulted in loss of force generating capacity, activation of unfolded protein response, accumulation of dysfunctional organelles and damaged proteins

culminating in myofiber degeneration (162, 213). These animals also exhibited an exacerbation in muscle wasting in response to atrophic stimuli such as starvation and denervation. Moreover, deficient autophagy results in a myriad of myopathies and muscular dystrophies (75, 196, 203, 213, 275) which can be rescued by reactivation of this process (75, 196, 203, 213). Thus, autophagy is required for muscle mass maintenance, and is critical for the quality control of skeletal muscle organelles, however, over-active autophagy can result in excessive catabolism and atrophy.

Interestingly, evidence implicating aberrant autophagy in aging-induced atrophy are beginning to emerge. Indeed, aged rats demonstrate higher expression of autophagy proteins basally, however, these are less able to further up-regulate autophagy in response to perturbations, indicating reduced plasticity (189). Moreover, the accumulation of undegraded cellular materials in the form of lipofuscin is also suggestive of impaired autophagy in aged animals (189). Indeed, a recent study demonstrated that the lack of autophagy in Atg7-null muscle resulted in the degeneration of the neuromuscular junction, and the loss of force generating capacity, leading to a premature aging phenotype. Interestingly, this phenotype is partially preventable by antioxidants, indicating the importance of autophagy in the elimination of oxidized proteins and dysfunctional mitochondria (37). Moreover, chronic caloric restriction with and without exercise, was found to activate autophagy and improve muscle health with aging (310).

Therefore, aside from its catabolic role in protein breakdown, autophagy is responsible for cellular quality control and proper organelle turnover, which is vital for muscle health and homeostasis. However, autopahgy must be kept in check, since, if gone rogue, autophagy can result in excessive catabolism and muscle wasting.

2.3.2.3 Apoptosis in muscle atrophy

Apoptosis is a type of programmed cell death, and is a rare phenomenon in healthy skeletal muscle. However, during cellular injury or prolonged metabolic distress apoptosis can be activated. In the context of muscle atrophy, apoptosis has been documented to contribute, albeit minimally. Since skeletal muscle is multinucleated, myonuclear decay results in the loss of transcriptional machinery in the specific region governed by the lost nucleus. This, in turn, results in the shrinkage of that particular domain (7). Increases in the expression of apoptotic proteins as well as in DNA fragmentation (3, 26, 61, 190, 264) have been noted under several atrophic conditions including denervation, microgravity and prolonged disuse. Interestingly, it is the mitochondrially-mediated apoptotic pathway that appears to be most at play during atrophy. Indeed, mitochondria isolated from atrophic muscle are more susceptible to permeability transition pore opening, and apoptosis (49). Moreover, mice with deletions in the pro-apoptotic proteins Bax and Bak, are partially protected from denervation-induced loss in muscle mass, further supporting a role for apoptotic cell death in atrophy (192, 265). However, this issue is controversial as a time lapse microscopy study of single fibers *in vivo* revealed no loss of murine myonuclei during prolonged muscle atrophy induced by denervation or unloading (29).

2.3.2.4 Transcriptional regulation of muscle atrophy

Although the transcriptional regulation of atrophy is complex and largely stimulus-dependent comparative gene expression profiling of a number of atrophy models resulted in the discovery of several genes that are coordinately regulated under the various atrophic conditions (22, 137). These genes are now known as atrophy related genes (atrogenes) and include molecules involved in both the ubiquitin-proteasome, and the autophagy-lysosome proteolytic systems. Interestingly, several cellular signaling pathways have been documented to converge on

the transcription of these key atrophic components. Forkhead family of transcription factors (FoxO) (159, 244, 324), NF-κB (31, 116) and factors downstream of TGF-β (166, 246, 248) have all been demonstrated to impinge on the atrophic transcriptional program, culminating in the induction of atrogenes.

The FoxO family of transcriptional regulators consists of FoxO1, 3, 4 and 6 which are involved in a wide range of biological processes including metabolism, cellular proliferation and differentiation, apoptosis, longevity and muscle mass regulation (1, 239). In skeletal muscle, FoxO1 and FoxO3 have both been documented to induce muscle atrophy in response to disuse and metabolic distress by coordinating the expression of factors involved in both of the cells major proteolytic systems, the UPS and ALP (137, 159, 244). Overexpression of FoxO3 is sufficient to induce muscle atrophy (159, 244, 324) through the up-regulation of atrogin-1 and MuRF1(244), as well as the autophagy factors LC3 and BNIP3 (159, 324). Moreover, FoxO3 knock down, or the expression of dominant negative FoxO3, blocks the induction of atrogin-1 and MuRF1 under some atrophic conditions (244, 258, 267). Similar to FoxO3, FoxO1 can also induce muscle atrophy by upregulating atrogin-1 and MuRF1 (239, 267). Furthermore, genetic deletion of three FoxO isoforms (FoxO1/3/4) induces muscle hypertrophy and prevents denervation-induced atrophy (282). FoxO activity is all about location, and these transcription factors must be de-phosphorylated before they are allowed entry into the nucleus. During atrophy, decreased levels of Akt leave FoxO3 uninhibited and free to enter the nucleus. Moreover, AMPK can phosphorylate FoxO3 on numerous residues which positively influences its activity (19, 74, 240). The capacity of FoxO3 to be post-translationally modified allows for the rapid potentiation of the atrophy program, and it also presents an attractive target for

pharmacological manipulation. Interestingly, PGC-1α and β can inhibit FoxO3 activity on the promoters of atrogenes, thus rescuing muscle mass during denervation and fasting (27, 243).

Inflammation often accompanies various models of atrophy and in itself can activate an atrophic program under the command of NF-κB. Levels of cytokines such as tumor necrosis factor α (TNFα) are increased in the circulation during inflammation. The binding of TNFα to its receptor facilitates the translocation of NF-κB to the nucleus where it induces the transcription of atrogenes. Blocking NF-κB action through the overexpression of a negative regulator of this system (inhibitor of NF-κβalpha, Iκβα) is sufficient to prevent disuse atrophy (116, 176). Moreover, the cytokine TNF-like weak inducer of apoptosis (TWEAK) and its receptor Fn14 are also up-regulated during muscle disuse (169, 311) and mice lacking TWEAK are resistant to denervation. TWEAK-Fn14 regulate TRAF6 action, which coordinates the degradation of proteins and organelles via the UPS and ALP by activating NF-κβ, AMPK and FoxO catabolic signaling pathways (202).

The role of the transforming growth factor beta (TGF-β) family in the regulation of muscle mass continues to grow and develop as novel downstream effectors emerge. The TGF-β family is composed of numerous secreted proteins which rule a wide variety of cellular processes. The TGF-β family can be subdivided into two ligand subfamilies: the TGF-β/activin subfamily and the bone morphogenetic protein (BMP) subfamily, which appear to have opposite roles in skeletal muscle. Myostatin, which is perhaps the most notorious TGF-β family member, is a potent negative regulator of muscle growth (139, 166). Mice, cattle, sheep and even humans with mutations in the myostatin gene are hyper muscular (139, 246). Myostatin and its TGF-β/activin family members negatively regulate muscle mass by activating SMAD2/3 mediated atrophic program (247, 284, 289). SMAD 2/3 can inhibit the transcription factor JunB which

normally promotes muscle growth and blocks atrophy by blocking FoxO3 (215, 247). On the other hand, BMPs act to prevent muscle atrophy by activating SMAD1/5/8 and repressing MUSA1, a novel E3 ligase downstream of TGF-β (246, 248). Indeed, RNA interference of MUSA1 was protective against denervation-induced atrophy. This pathway appears to be particularly promising for pharmacological intervention as blocking TGF-β signaling through administration of a soluble receptor (ActRIIB) was able to rescue muscle mass under several atrophic models. Even more importantly, blocking the ActRIIB was sufficient not only to reverse cancer cachexia, but also to delay mortality independently of tumor progression (328).

During atrophic conditions, reduced signaling through the IGF-1-Akt-mTOR axis contributes to atrophy not only through reduced protein synthesis, but also through increased degradation by releasing FoxO inhibition (23, 28, 266). Moreover, reduced mTORC1 activity can also result in the activation of autophagy, as mTORC1-mediated phosphorylation inhibits the autophagy induction complex composed of Atg13 and ULK1 (23, 93, 123). To add to the complexity, during early days of denervation the increase in cellular amino acids released from enhanced proteasomal activity can constitutively activate mTOR, resulting in feedback suppression of Akt, and thus the activation of FoxO-dependent protein breakdown, counter-intuitively promoting catabolism (212, 282). However, it is conceivable that mTORC1 is activated during denervation in order to support the synthesis of proteins needed for catabolism as the cell adapts to a new homeostasis. More research into the role mTOR during atrophy is warranted.

Finally, microRNAs (miRs) have also recently been documented to regulate muscle mass during disuse. MiRs are endogenous, noncoding, short (20-22 nucleotides) RNAs that are involved in a wide variety of cellular processes. These miRs recognize the 3'-untranslated

regions of their target substrates and silence their expression by either inducing transcript degradation, or by blocking translation. Atrophic conditions such as denervation, starvation, cancer cachexia or aging result in altered miR expression profiles. For instance miR-206 and -21 are induced with denervation and are both sufficient and required for atrophy (268). miR-182, on the other hand, targets FoxO3, thus attenuating atrophy related gene expression (96). Indeed, miR-182 levels are reduced in response to some atrophic stimuli. Additional miRs associated with muscle disuse include miR-1 and miR-133a, both of which are reduced with bed rest and denervation (109, 222).

Altogether, it is evident that the transcriptional regulation of muscle atrophy depends on the interplay between a wide range of pathways which often converge on the transcription of common atrophic factors known as atrogenes.

2.3.2.5 Mitochondrial involvement in atrophy

Muscle atrophy is also accompanied by alterations in cellular bioenergetics. Reductions in mitochondrial content and changes in organelle morphology are common among various types of atrophy. Indeed, atrophying muscle is characterised by both reduced mitochondrial biogenesis and increased mitochondrial elimination. For instance, 7 days of denervation result in a ~50% drop in mitochondrial density which correlates closely with the loss in muscle mass (308). Interestingly, the decrease in mitochondrial content precedes atrophy, suggesting that organelle loss contributes to the observed decline in muscle mass. However, causality has not been directly determined.

The level of the mitochondrial master regulator PGC-1α plummets under various atrophic conditions including diabetes, renal failure, cancer cachexia and denervation, and with it so does organelle biogenesis (3, 230, 243, 307). Moreover, levels of another PGC-1α family

member, PGC-1β, are also reduced rapidly upon cessation of contractile activity. The decrease in both of these coactivators results in diminished mitochondrial synthesis though decreased expression of ERRα, NRF-1/2 and Tfam. Moreover, transcript levels of mitochondrial electron transport chain proteins such as cytochrome c and COXIV also decrease with muscle disuse, further indicating diminished biogenesis (3, 25, 118, 230, 299). Overexpression of either PGC-1α or -β is sufficient to block protein degradation and spare muscle mass during denervation (27, 243). Interestingly, TWEAK was noted to induce atrophy by repressing PGC-1α. In this context the overexpression of PGC-1α was sufficient to block TWEAK-induced atrophy, as well as decrease the levels Fn14 in denervated skeletal muscle (89).

Muscle atrophy is also accompanied by alterations in mitochondrial morphology (100, 189). Indeed, reduced levels of fusion proteins MFN2 and OPA1 with denervation result in fragmented mitochondria (100). Small punctate mitochondria are less efficient at ATP production, more prone to ROS generation as well as apoptosis, and are therefore predisposed for elimination by autophagy, all of which add to atrophy. Interestingly, the overexpression of fission protein Fis1 is sufficient to induce muscle atrophy (224), which can be reversed if the mitochondrial network is restored (224). Importantly, PGC-1α/β regulate the expression of the mitochondrial morphology factor MFN2 through interaction with ERRα (148, 269). Indeed, animals overexpressing PGC-1α in muscle maintain mitochondrial integrity and are protected from the loss of muscle mass and function with aging (307).

2.3.2.6 Contribution of mitophagy to muscle atrophy

Although historically autophagy was considered to be a non-selective degradation pathway, the significance of selective autophagy is becoming increasingly evident. Indeed, autophagy can trigger the selective removal of specific organelles, such as mitochondria via

mitophagy. The contribution of this process to muscle atrophy is highlighted by models of chronic muscle disuse such as denervation. The expression of autophagy factors and the targeting of mitochondria for removal increase in muscle in response to disuse and denervation. The localization of Parkin, p62 and LC3-II to the mitochondrial subfraction (189, 191, 192) were all enhanced with denervation, supporting the role of mitophagy in the robust elimination of organelles observed with this condition. Mitochondrial degradation is likely triggered by organelle dysfunction and fragmentation. Indeed, mitochondria isolated from denervated muscle are smaller, consume less oxygen, produce more ROS, and are more susceptible to apoptosis, this is sufficient grounds for elimination by mitophagy (3, 263). ROS produced by dysfunctional mitochondria can directly activate autophagy through the induction of AMPK, and ROS scavenging decreases autophagic flux (216). Interestingly, mitochondrial morphology and dynamics also play a key role in sealing mitochondrial fate, since organelle fission alone is both sufficient and required to activate mitophagy (17, 224). Conversely, inhibition of mitochondrial fragmentation attenuates autophagy, as well as muscle atrophy (224, 225). Indeed, mitochondrial fusion proteins mitofusin1 and 2 are targeted for degradation by the E3 ubiquitin ligases Parkin and Mul1 (156, 281, 321) thus halting mitochondrial fusion, and facilitating fission under atrophic conditions. Mitochondrial E3 ligases preferentially ubiquitinate proteins on the mitochondrial outer membrane, this acts as a signal to entice the arrival of autophagosomes. Mice lacking Parkin were partially protected against the loss of organelle density and muscle atrophy in denervated slow-twitch muscle (64). Moreover, FoxO1/3 enhance Mul1 expression during denervation and Mul1 appears to be necessary for mitophagy in skeletal muscle (156). The importance of this process is highlighted by the existence of redundancy, where E3 ligases compensate one for the loss of another. For instance Mul1 steps in to help preserve

mitochondrial integrity when PINK1/Parkin are lacking (321). Furthermore, the targeting of mitochondria for degradation can also be achieved through the display of various receptors on the outer membrane of the organelle. BNIP3, and close relative BNIP3-like (BNIP3L) protein, also known as NIX, are two such receptors. These factors anchor to the mitochondrial membrane and directly signal to LC3 as they both possess an LC3 interacting region. BNIP3 and NIX are sufficient to induce muscle wasting and constitute another avenue of the atrophic program regulated by FoxO3 (159).

Despite its contribution to muscle wasting, mitophagy actually plays a vital role in proper organelle turnover and mitigation/ rehabilitation of damaged organelles. The importance of this process is particularly evident in myopathies induced by autophagy deficiency. In models of lysosomal storage disease, or in mice lacking fundamental autophagy factors Atg5 or Atg7, the burden of dysfunctional and swollen mitochondria contributes to, and exacerbates pathogenesis (75, 162). Although several mechanisms of mitochondrial quality control exist (discussed in chapter 1), mitophagy is the only known mechanism for the wholesale removal of mitochondria from post-mitotic tissues, such as skeletal muscle. This process is required for the proper progression of the mitochondrial life cycle and is therefore vital for mitochondrial vigor (292).

Thus, mitochondria are intimately involved in muscle wasting under a variety of atrophic conditions. Reduced mitochondrial biogenesis, increased mitochondrial fragmentation and mitophagy all contribute to muscle atrophy induced by disuse and denervation. However, a basal level of mitophagy is vital for routine mitochondrial turnover and housekeeping, as insufficient mitophagy results in mitochondrial dysfunction and oxidative stress which serve to exacerbate muscle wasting. However, it is not yet clear if mitochondrial alterations are the cause or consequence of muscle atrophy and further research is required in order to unravel this mystery.

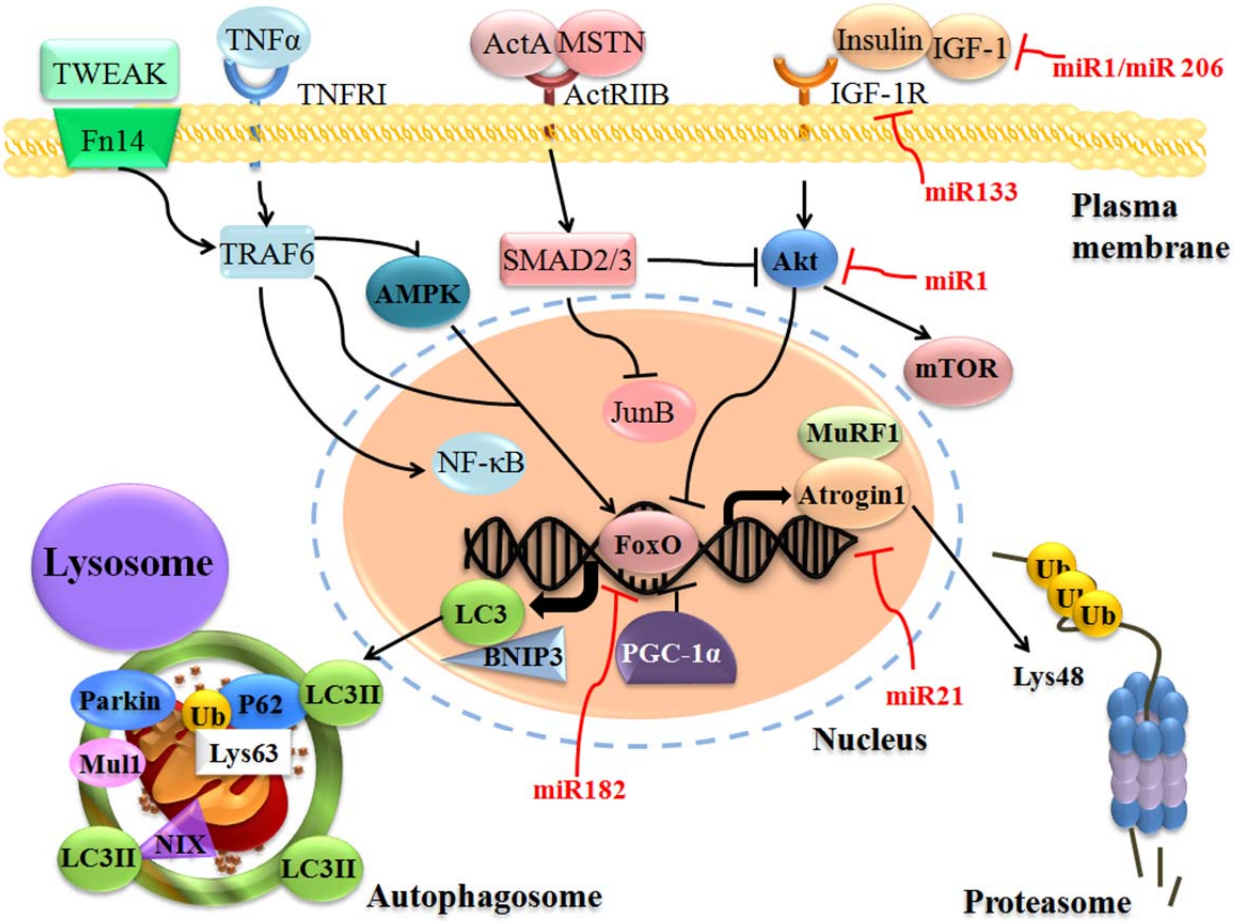

Figure 4. Proteolytic pathways involved in muscle atrophy. During muscle atrophy decreased binding of Insulin/IGF-1 to the IGF-1 receptor (IGF-1R) results in the loss of Akt/ mTOR activity. This releases FoxO and allows its entry to the nucleus, where it activates an atrophic program by increasing the expression of the E3 ubiquitin ligases MuRF1 and Atrogin1 and autophagy lysosome genes such as LC3 and BNIP3. This helps sustain the increased proteolytic activity of the proteasome and autophagy-lysosome systems. Increased binding of activins (ActA) and myostatin to the ActIIB receptor contributes to atrophy through the activation of SMAD2/3 and the inhibition of both Akt and transcription factor JunB. Inflammation also contributes to atrophy by increasing the levels of TNFα and TWEAK binding to the TNF-RI and FN14 receptors, respectively. This leads to the activation of TRAF6 which, in turn, activates AMPK, NF-κB and FoxO thus enhancing the expression of atrogenes. FoxO-induced atrophic program can be blocked by PGC-1α.Various miRs (in red) can be involved in many steps along the way. Mitophagy is also upregulated as evident by the localization of E3 ligases Mul1 and Parkin and the mitophagy receptor NIX to the mitochondria and its encapsulation within the autophagosome with the aid of adaptor protein p62 and the autophagy marker LC3.

2.3.3 Muscle metabolic plasticity with exercise

The numerous merits of regular physical activity on whole body metabolism and general health have been known for decades, making the field of exercise physiology busy exploring the how, and the why. Regular exercise has been reported to mediate widespread protective benefits ranging from glucose homeostasis during metabolic stress, to preservation of muscle mass during atrophic stimuli, and even improved mood and cognition with aging. Thus, exercise exerts health benefits for the body and the mind. Indeed, regular exercise can attenuate loss of muscle mass, health and function with disuse, ageing and cachexia. Exercise was also recently reported to reduce depression in stressed mice through a PGC-1α-mediated modulation of kynurenine metabolism (5). The molecular mechanisms underlying the merits of exercise have been under vigorous investigation, and although significant breakthroughs have been made, we are far from fully understanding the vast reaching consequences of exercise. Pioneering studies by Dr. John Holloszy and colleagues in the 1960s demonstrated that muscle oxidative capacity is induced with aerobic exercise training (90), and the implication of the transcriptional co-activator PGC-1α in this process by the same group in the early 2000s indicated a true inflection point in exercise physiology (10). PGC-1α is strongly induced by an acute bout of exercise and is in itself sufficient to induce mitochondrial biogenesis in cells and tissues (10, 208, 251, 315). Although the need for PGC-1α in exercise-induced benefits is debated (208, 228, 294), its role in organelle biogenesis is a soundly supported conclusion (Fig. 5).

2.3.3.1 Mitochondrial biogenesis during exercise

During exercise, molecular messengers induce downstream signaling cascades that activate a transcriptional program aimed at increasing oxidative capacity. This transcriptional program is largely governed by the master regulator of mitochondrial biogenesis, PGC-1α.

Indeed, at times it seems as though all upstream signals impinge on this co-activator. Increased energy demands induced by contractile activity result in elevated levels of AMP relative to ATP which activates the metabolically sensitive AMP-activated kinase (AMPK) (85). AMPK, in turn, phosphorylates and activates PGC-1α (104). AMPK deletion in skeletal muscle results in diminished mitochondrial content and impaired performance (114), whereas constitutively active AMPK results in mitochondrial enrichment and fatigue resistance in mice (15, 66). Moreover, administration of AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) a synthetic activator of AMPK, recapitulates some of the metabolic benefits induced by exercise (180). AMPK can also induce an increase in cellular NAD^+ which activates the NAD-dependent deacetylase sirtuin-1 (SIRT1). SIRT1 can further activate PGC-1α through de-acetylation (36). Calcium transients are also typical during muscle contraction, and the temporary increases in intracellular calcium signal downstream to calcium/calmodulin phosphatase (Calcineurin) and kinase (CaMK) (42). Both Calcineurin and CaMK can upregulate the transcription of PGC-1α via NFAT and MEF-2, respectively (9, 42, 81). Indeed, deficient calcineurin or its pharmacological inhibition attenuates exercise-induced adaptations, whereas over-expression of the phosphatase promotes the formation of slow-oxidative muscle fibers (183, 200). Similarly, CaMK overepression promotes the formation of slow-oxidative fibers, whereas reduced CaMK activity results in lower expression of oxidative genes (132, 312). Increased ROS production as a result of higher ETC action and NADPH oxidase activity also impinges on PGC-1α. Indeed, p38 MAPK is activated by ROS and enhances PGC-1α transcription (6, 209). Genetic deletion of the p38γ isoform in muscle has revealed that it is required for endurance exercise-induced mitochondrial biogenesis and PGC-1α transcription (209). Finally, some recent data suggest a role for p53 in exercise induced-mitochondrial biogenesis. Indeed, PGC-1α protein levels are

reduced in muscle from p53 knockout animals (236). Moreover, in the absence of p53, exercise-induced p38 activation and the translocation of PGC-1α to the nucleus were abolished, as were the increases in the transcript levels of its downstream targets COXIV and cytochrome c (237). Once PGC-1α is active, it enters the nucleus and enhances the activity of various transcription factors such as NRF-1/2 and ERRα, thus augmenting the expression of nuclear genes that encode mitochondrial proteins (NUGEMPS) (92). NUGEMPs, in turn, contribute to the growth and expansion of the mitochondrial reticulum either by becoming incorporated into the existing mitochondrial network, or by enhancing mitochondrial DNA transcription and replication (70, 231). Exercise-induced increases in the expression of ETC components as well as mitochondrial transcription factor A (Tfam) ensure the co-ordinated up regulation of both mitochondrial and nuclear encoded genes. Indeed, mitochondrial DNA copy number as well as the protein import machinery are all induced with chronic contractile activity (115). Interestingly, proteins involved in the regulation of mitochondrial morphology are also induced with contractile activity. Indeed, chronic exercise results in more elongated reticular mitochondria and an increase in the fusion factors OPA1 and MFN2 (38, 100). This effect could be mediated by PGC-1α, as the coactivator induces the expression of both MFN1 and MFN2 through ERRα (38). However, the issue of mitochondrial dynamics with exercise warrants further investigation.

In summary, increases in energetic demands, ROS production and calcium signaling during muscle contraction all converge on PGC-1α activation, and thus the stimulation of mitochondrial biogenesis.

2.3.3.2 Autophagy and mitophagy with exercise

Chronic contractile activity induces a cellular remodeling in an attempt to satisfy newly elevated energy requirements. Inefficient, nascent, and dysfunctional organelles, as well as

oxidized and damaged proteins must be recycled to allow for the synthesis of new and improved machinery. This cellular renovation process results in superior myofiber health and improved oxidative capacity, which contributes to whole body metabolic improvements, and is therefore a major benefit of chronic exercise. Indeed, autophagy is activated with an acute bout of endurance exercise in various organs and tissues including skeletal muscle of humans and rodents (76, 87, 105, 106, 237). It was first hypothesized that autophagy is required during exercise in order to provide energy for contraction, and more chronically to increase turnover of damaged organelles.

Enhanced lysosomal degradation with strenuous exercise has been observed in liver and skeletal muscle as far back as 1980 (235, 238). However, more recently Grumati et al. demonstrated that autophagy is induced immediately following an acute bout of exercise, and that autophagy is required for proper muscle function during physical activity (76). Indeed, the dystrophic phenotype of animals lacking collagen-VI, a model where autophagy is compromised, was exacerbated by acute and chronic exercise (75, 76) suggesting that functional autophagy is required for a healthy response to exercise. Autophagy has also been shown to be activated with an acute bout of low intensity exercise, but this activation was enhanced if combined with nutrient deprivation (107). An increase in the expression of autophagy genes as well as induction in autophagy proteins has also been noted in humans following ultra-endurance exercise, which consisted of 24-28h of treadmill running (105, 106).

Seminal work by the group of Dr. Beth Levine also demonstrated the induction of autophagy with an acute bout of exercise in heart, skeletal muscle, as well as other peripheral metabolic tissues such as liver, pancreas, and adipose tissue (87). This study revealed that exercise-induced autophagy engages the Bcl-2–beclin1 complex. Bcl-2 is an anti-apoptotic protein that can also inhibit autophagy by sequestering beclin1. When autophagy is induced,

phosphorylation of Bcl-2 results in the release of beclin1 from its captivity, thus promoting the formation of autophagosomes. To study the role of autophagy in exercise-mediated metabolic adaptations, Bcl-2 AAA knock-in ($Bcl2^{AAA}$) mice were generated by mutagenesis of three conserved phosphorylation residues of Bcl-2 (Thr 69, Ser 70 and Ser 84). These mice exhibited normal basal autophagy, but were deficient in stimulus-induced autophagy. $Bcl2^{AAA}$ had a lower maximal endurance capacity, as well as impaired exercise-induced increases in insulin sensitivity, which was attributed to the lower activation of AMPK. These findings were further confirmed in mice heterozygous for beclin 1 (Beclin1+/- mice), as well as mice hypomorphic for Atg16L1, an essential autophagy protein vital for autophagosomal membrane synthesis. This study also revealed that autophagy is required for exercise-mediated protection against high fat diet (HFD)-induced metabolic derailment. $Bcl2^{AAA}$ mice were more susceptible to HFD-induced obesity, and failed to exhibit exercise-training mediated protection against HFD-induced impairments in glucose tolerance. These findings point to the involvement of autophagy in metabolic renovation chronically (87). However, the perturbations in autophagy described above were global and the importance of autophagy in skeletal muscle specifically revealed certain differences. Mice deficient in the essential autophagy protein Atg7 ($Atg7^{-/-}$) specifically in skeletal muscle do not indicate alterations in endurance performance. In fact, these mice show an improved metabolic profile as well as protection from obesity, all stemming from mitochondrial stress and the release of the mitokine FGF21 (124, 157). These contrasting findings bring into question the requirement of autophagy as an energy source for muscle contraction. Repeated bouts of damaging downhill running exercise, however, revealed a progressive drop in performance as well as diminished organelle membrane potential in muscle-specific $Atg7^{-/-}$ females, a finding which was not evident in WT exercised animals. This suggests that autophagy

is needed for organelle turnover post-exercise. This autophagy activation is, in part, dependent on ROS, as treatment with general or mitochondria-specific ROS scavengers attenuated autophagy induction in WT mice (157). Moreover, aerobic exercise also enhanced mitochondrial LC3II, p62, and ubiquitin content with exercise in mice suggesting the induction of mitophagy with exercise (237). These findings indicate autophagy is important for cellular turnover post-exercise, thus contributing to exercise-induced adaptations. With this in mind, chronic voluntary exercise-training results in increased basal autophagy and mitophagy protein expression (152). However, this increase is compromised in mice that are heterozygous for beclin1 (beclin1-/+), as expected. Beclin1-/+ mice also showed attenuated mitochondrial biogenesis, and angiogenesis in skeletal muscle, along with impaired improvement of endurance capacity. These results demonstrate that increased basal autophagy is required for exercise training-induced skeletal muscle adaptations and improvement of physical performance (152). Due to the involvement of the autophagy-lysosome system in exosome release and in vesicular trafficking (12), it is conceivable that deficient autophagy may alter the release of muscle-derived myokines and miRs. This could potentially dampen whole body exercise-induced benefits, as these messengers play an important role in relaying information from muscle to other metabolic tissues.

Long-term exercise training was also demonstrated to restore autophagy flux in skeletal muscle of animals treated with the lysosomal inhibitor chloroquine (110). Indeed, lifelong exercise, in combination with caloric restriction, was found to improve the decline in autophagy with aging, as well as dampen age-related increase in oxidative damage and apoptosis (310). This suggests exercise as potential form of therapy for myopathies characterized by deficient autophagy. However, exercise should be prescribed with caution, as in certain autophagy deficient conditions, such as collegen VI-deficiency, it may be detrimental (76).

Although the early events leading to autophagy activation with exercise have not been thoroughly examined, several possibilities exist since muscle contraction constitutes a form of energetic stress, much like nutrient deprivation due to caloric restriction or fasting. First, the increased metabolic demands of contracting muscle and elevated ROS production activate the cellular stress responders comprised of AMPK, SIRT1 and p38. These molecules can lead to the induction of the autophagic machinery as well as potentiate the autophagic transcription program by transcription factors such as p53 and FoxOs. An acute bout of exercise also leads to reduced Akt phosphorylation, which would result in mTOR inhibition and FoxO activation, thus inducing the transcription of autophagy related genes (76, 87). Indeed, FoxO1 and 3 gene expression along with certain autophagic and proteasomal markers were all increased following exhaustive exercise in humans (105, 106). Exercise-mediated increases in AMPK and SIRT11 also activate FoxO1 and 3 through post-translational modifications (36).

Another possible mechanism at play may be the unfolded protein response (UPR), which is activated in response to sarcoplasmic reticulum stress induced by contractile activity (122, 313). Certain UPR factors have been implicated in the activation of autophagy in various cell types (144, 186, 319). Interestingly, PGC-1α was shown to contribute to UPR induction with exercise acting through the UPR activating transcription factor 6 (ATF6), and mice lacking ATF6 are exercise-intolerant (313) (Fig. 5).

Therefore, although several studies indicate that autophagy is activated during an acute bout of exercise, the mechanisms underlying this activation remain to be elucidated. It is also becoming increasingly evident that autophagy is involved in chronic physical activity-induced adaptations through increased organelle turnover. It would therefore be interesting to investigate the kinetics of autophagy activation during and following an exercise bout.

2.3.4 Conclusions

Given the magnitude and importance of skeletal muscle in whole body metabolism and the intrinsic capacity of this tissue to rapidly and effectively adapt to its milieu, it is a prime candidate for pharmacological manipulation. Indeed, the molecular mechanisms responsible for muscle plasticity are much sought after. When it comes to muscle mass however the mechanism is simple, and the old adjunct "use it or lose it", is definitely applicable. Under conditions of disuse, muscle atrophies and is characterised by a decrease in strength and oxidative capacity, whereas physical activity increases muscle oxidative capacity and is protective of muscle mass and health. Skeletal muscle mass depends on the balance between protein synthesis and protein degradation. During atrophy, a rise in the activity of cellular proteolytic pathways, regulated by a transcriptional atrophy program leads to muscle wasting. The cellular energetic status dictated by mitochondria also plays a key role in cellular destiny. Decreases in mitochondrial biogenesis, along with increased organelle fragmentation and elimination by mitophagy, lead to a net loss in mitochondrial volume and contribute to cellular shrinkage. On the other hand, restoration of mitochondrial health by inducing the expression of PGC-1α co-activators is protective of muscle mass. This can be achieved via chronic contractile activity, resulting in enhanced oxidative capacity, increased organelle efficiency and overall improved muscle health. This is largely due to pathways mediating an increase in organelle density, reticulum formation and the elimination of nascent and dysfunctional organelles by mitophagy. Although some studies suggest a role for autophagy in exercise-induced adaptations, very few have actually examined mitophagy regulation during an acute bout of exercise, and even fewer have evaluated the mechanisms involved in this activation. Moreover, the interplay between autophagy and PGC-1α during exercise, and its related adaptations, has yet to be evaluated.

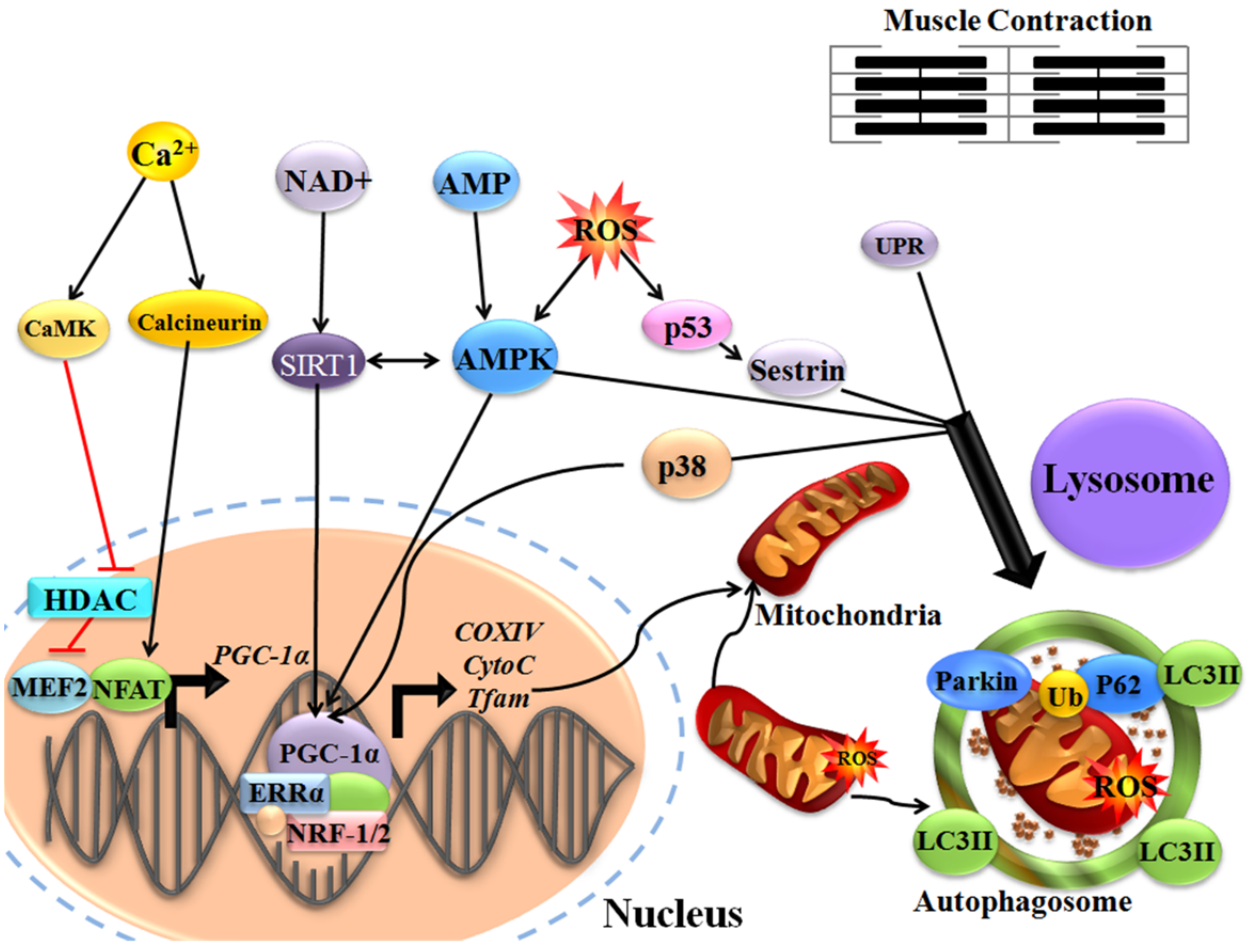

Figure 5. Exercise induced signaling . During muscle contraction levels of calcium, NAD+, AMP and ROS increase. These molecular messengers, in turn, activate their respective downstream effectors, CaMK/Calcineurin and unfolded protein response (UPR) , SIRT1/AMPK, p38 and p53. CaMK can enhance PGC-1α transcription via inhibition of HDAC and thus activation of MEF2. Calcineurin also induces PGC-1α expression via activation of NFAT. SIRT1, AMPK and p38 upregulate PGC-1α activity by de-acetylation and phosphorylation, respectivley. Once active in the nucleus PGC-1α co-activates transcription factors such as NRF-1/2 and ERRα resulting in the increased expression of nuclear genes that encode for mitochondrial proteins such as COXIV, cytochrome c (CytoC) and mitochondrial transcription factor A (Tfam) leading to the expansion of the mitochondrial reticulum. Autophagy is also activated during muscle contraction. Although it is not exactly clear how, this activation could be mediated by increased unfolded protein response factors (UPR), sestrins, AMPK or p38. In this context, autophagy mediates the degradation of dysfunctional mitochondria as well as proteins that were damaged or oxidised during contraction.

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CHAPTER 3:

PhD Dissertation Objectives and Hypotheses

Based on the review of the literature, it can be concluded that the process of autophagy and the activity of the transcriptional co-activator PGC-1α remain two important, but distinct entities in the regulation of muscle health and metabolism. Both appear to be involved under various metabolic alterations in muscle such as nutrient deprivation, contractile activity, and disuse. However, it is not clear if PGC-1α plays a role in the process of autophagy in skeletal muscle throughout the continuum of muscle contractile and metabolic states. Due to the inherent importance of both autophagy and PGC-1α for muscle and mitochondrial health, we were interested in evaluating the role of PGC-1α in the regulation of autophagy under divergent muscle metabolic and contractile states, namely exercise, and denervation. To this end, the purpose of this dissertation was to examine skeletal muscle mitochondrial remodeling within the spectrum of changes in muscle contractility. We investigated the interplay between the master regulator of mitochondrial biogenesis PGC-1α and the mitochondrial degradation machinery with the hopes of uncovering any potential cross-talk between these two opposing processes. Therefore, the objectives of my dissertation were three-fold:

Objective 1 (Chapter 4): Investigate the role of PGC-1α in autophagic signaling, basally and during chronic muscle disuse, utilizing a loss of function model.

Hypotheses:

1) We hypothesized that autophagy and mitophagy would be induced with denervation;
2) We expected that the lack of PGC-1α would attenuate autophagy/mitophagy induction as well as autophagy flux by transcriptionally modulating key autophagy and lysosomal genes.

Objective 2 (Chapter 4): Elucidate the effect of PGC-1α overexpression on muscle health as well as autophagy/mitophagy basally and following denervation.

Hypotheses:

1) We posited that PGC-1α gain-of-function would result in enhanced muscle oxidative capacity as well as protection of muscle mass and mitochondrial content during denervation-induced atrophy;
2) We anticipated that enhanced PGC-1α levels would result in greater basal and denervation-induced autophagy and mitophagy signaling and flux through enhanced expression of autophagy and lysosomal genes.

Objective 3 (Chapter 5): Delineate autophagy and mitophagy activation and flux following an acute bout of contractile activity, and establish the involvement of PGC-1α in this process.

Hypotheses:

1) We estimated that autophagy and mitophagy would be activated with an acute bout of exercise and contribute to mitochondrial turnover;
2) We speculated that the lack of PGC-1α would dampen mitochondrial biogenesis and autophagy/mitophagy in response to exercise, resulting in reduced organelle turnover.

CHAPTER 4:

PGC-1α modulates denervation-induced mitophagy in skeletal muscle

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Abstract

Background: Alterations in skeletal muscle contractile activity necessitate an efficient remodeling mechanism. In particular, mitochondrial turnover is essential for tissue homeostasis during muscle adaptations to chronic use and disuse. While mitochondrial biogenesis appears to be largely governed by the transcriptional co-activator PGC-1α, selective mitochondrial autophagy (mitophagy) is thought to mediate organelle degradation. However, whether PGC-1α plays a direct role in autophagy is currently unclear.

Methods: To investigate the role of the co-activator in autophagy and mitophagy during skeletal muscle remodeling, PGC-1α knockout (KO) and overexpressing (Tg) animals were unilaterally denervated, a common model of chronic muscle disuse.

Results: Animals lacking PGC-1α exhibited diminished mitochondrial density alongside myopathic characteristics reminiscent of autophagy-deficient muscle. Denervation promoted an induction in autophagy and lysosomal protein expression in wild type (WT) animals, which was partially attenuated in KO animals, resulting in reduced autophagy and mitophagy flux. PGC-1α overexpression led to an increase in lysosomal capacity as well as indicators of autophagy flux, but exhibited reduced localization of LC3II and p62 to mitochondria, compared to WT animals. A correlation was observed between the levels of the autophagy-lysosome master regulator TFEB and PGC-1α in muscle, supporting their co-ordinated regulation.

Conclusions: Our investigation has uncovered a regulatory role for PGC-1α in mitochondrial turnover, not only through biogenesis, but also via degradation using the autophagy-lysosome machinery. This implies a PGC-1α-mediated cross-talk between these two opposing processes, working to ensure mitochondrial homeostasis during muscle adaptation to chronic disuse.

Background

Skeletal muscle is the largest organ of the body, and as such is recognized for essential roles that extend beyond locomotion. Muscle is an indispensable metabolic center that possesses a remarkable capacity to adapt to alterations in its milieu, a property known as muscle plasticity. This type of malleability to cues such as contraction, nutrient availability or hormonal stimuli, requires efficient cellular remodeling and a rapid shift in metabolic profile. Since mitochondria are central to muscle metabolism, these types of alterations require amendments in organelle content and its network. Mitochondrial density depends on the intricate balance between biogenesis and degradation. Biogenesis is largely regulated transcriptionally through the coordinate expression of nuclear and mitochondrial genes, governed by the transcriptional co-activator peroxisome proliferator gamma coactivator-1α (PGC-1α) [1]. On the other hand, mitochondrial degradation is achieved through a selective form of macroautophagy (hereafter autophagy) termed mitophagy [2]. This process is of particular importance for long-lived post-mitotic tissues such as striated muscle and neurons, as this represents the sole mechanism for these cells to rid themselves of dysfunctional organelles. During mitophagy, defective mitochondria are first segregated from the network and are then engulfed into double membrane vesicles termed autophagosomes [3], which are subsequently delivered to the lysosome for proteolytic degradation.

Mitochondrial health is vital not only for proficient energy provision but also for proper cellular signaling and homeostasis, as mitochondria are often found at the fulcrum of cellular life-and-death decisions[4]. Thus, it is not surprising that mitochondrial abnormalities have been implicated in a plethora of muscle wasting conditions such as the sarcopenia of aging[5], pathology-related cachexia[6] and various muscular dystrophies[7–9]. Interestingly, skeletal muscle from animals with autophagic deficiencies closely resembles that of sarcopenic and

atrophic patients[10]. Thus, the intricacies underlying mitochondrial remodeling in skeletal muscle have great therapeutic potential for a myriad of debilitating conditions. While mitophagy is important for proper tissue remodelling and organelle turnover, the regulation of this process in skeletal muscle remains largely elusive.

PGC-1α, which is most well recognized for its role in mitochondrial biogenesis, has been documented to spare muscle mass and improve endurance in atrophic muscle induced by senescence [11], chronic heart failure [12], and a variety of additional muscle wasting conditions [13, 14]. More recently, PGC-1α has been implicated in the autophagy-lysosome pathway, and its over-expression was demonstrated to induce lysosomal biogenesis, possibly through the upregulation of TFEB [15–17], a transcription factor that is a master regulator of the lysosomal system. However, the role of PGC-1α in mitochondrial removal and autophagy in skeletal muscle has not been thoroughly examined. To this end, the purpose of this study was to examine the possibility of a co-ordinated regulation of mitochondrial remodeling by the metabolic master regulator PGC-1α in skeletal muscle. Here we investigate the involvement of PGC-1α in basal and denervation-induced autophagy using both gain- and loss-of-function approaches. Our results implicate PGC-1α in the regulation of the mitochondrial network, not only via biogenesis, but also through degradation.

Methods

Animal generation, procedures and treatment

The generation and characterization of PGC-1α KO and PGC-1α Tg mice have been described in detail elsewhere[13, 18–20]. PGC-1α whole body KO animals were generated by Lin J wt al as described previously[18]. For PGC-1α Tg mice, the transgene was expressed specifically in muscle under the control of muscle creatine kinase (MCK) promoter. All mice were housed in a

12:12-h light-to-dark cycle, and given food and water *ad libitum*. Where indicated, animals were unilaterally denervated by severing the sciatic nerve as previously described [21], while the contralateral limb served as an internal control. To assess autophagy flux, animals were treated with either colchicine or an equal volume of vehicle (water) through an intraperitoneal injection every 24 hours at a dose of 0.4mg/kg/day [22] for the last 4 days of denervation, with the final injection taking place 24h prior to sacrifice. Following 7 days of denervation, the muscles were harvested and either immediately frozen for histology, protein, and gene expression analysis, or used for cellular fractionation. EDL muscles were fixed for single fiber analysis or electron microscopy. All procedures involving PGC-1α KO and corresponding WT animals were approved by and conducted in accordance with the regulations of the York University Animal Care Committee in compliance with the guidelines set forth by the Canadian Council on Animal Care. All PGC-1α Tg and corresponding WT procedures were approved and authorized by the Italian Ministry of Health.

Histology and cross sectional area

Cytochrome oxidase (COX) and succinate dehydrogenase (SDH) staining was performed on 10-μm cross sections of digitorum longus (EDL) and tibialis anterior (TA) muscles as previously described [23]. Fiber cross-sectional area (CSA) of individual muscle fibers was determined using Image J software (NIH, Bethesda, MD, USA) by a blinded investigator. Fiber sizes were expressed in micrometers squared.

COX activity

Cytochrome c oxidase (COX) enzyme activity was measured as previously detailed [24] by determining the maximal rate of oxidation of fully reduced cytochrome *c*, evaluated as a change in absorbance at 550 nm using a microplate reader (Bio-Tek Synergy HT).

Electron microscopy

Tissue preparation for electron microscopy (EM) was performed as previously described [25]. Briefly, sections of EDL muscles from WT and KO animals were fixed for 1h in 3.0% glutaraldehyde followed by a 1h fixation in 1% osmium tetroxide diluted in 0.1 M sodium cacodylate at room temperature. Muscle sections were dehydrated and embedded in Epon resin, sliced into Ultrathin (60-nm) sections and stained with uranyl acetate and lead citrate. Electron micrographs were obtained using a Philips EM201 electron microscope.

Gene expression analysis

Quantitative real-time PCR was performed to determine mRNA expression levels. Total RNA was isolated using TRIzol reagent (Invitrogen, 15596-026). RNA was reverse transcribed into cDNA using a Superscript III first strand synthesis kit (Invitrogen, 18080-044) according to manufacturer instructions. The primers used for gene expression analysis are listed in Table S1 and were designed based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Analyses were performed with SYBR® Green chemistry (PerfeC$_T$a SYBR® Green Supermix, ROX, Quanta BioSciences, 95055-500) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). *Gapdh* and *Actb* were used in combination as housekeeping genes.

Immunoblotting

Protein extracts from frozen TA cryosections[26], isolated mitochondria, or nuclear extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were blocked with 5% skim milk or 5% BSA solution. Membranes were incubated overnight at 4°C with the appropriate concentration of primary antibody (see Table S2 for a full list of antibodies). Membranes were subsequently washed and incubated with the suitable HRP-conjugated

secondary antibody for 1h at room temperature, and visualized with enhanced chemiluminescence. Quantification was performed with Image J Software (NIH, Bethesda, MD, USA) and values were normalized to the appropriate loading control.

Autophagy and Mitophagy flux calculation

Autophagy flux was calculated by the Δ change in LC3II and p62 protein levels in whole muscle extracts, between colchicine treated and untreated animals of the same condition and genotype. Where Δ change = (colchicine treated-Vehicle treated)/Vehicle treated.

Mitophagy flux was calculated by the Δ change in LC3II and p62 protein levels in isolated mitochondria, between colchicine treated and untreated animals of the same condition and genotype.

Single fiber Immunofluorescence

Immunofluorescence staining was performed on isolated fixed EDL fibers [27] and imaged using a confocal microscope. Briefly, freshly excised EDL muscles were anchored at both ends, and fixed with 2% paraformaldehyde in phosphate buffer for 1h at room temperature. Muscles were then washed with PBS, kept in 50% glycerol at 4˚C overnight, and were subsequently transferred to -20˚C and stored until further use. Muscles were gradually transitioned through diminishing concentrations of glycerol and individual fibers were then mechanically teased apart in a puddle of 0.04% saponin. Fibers were mounted onto glass slides and permeabilized with 0.2% Triton X-100 in 10% goat serum in PBS blocking solution. Fibers were then co-incubated overnight at 4 °C with the appropriate primary antibodies (Table S2) diluted in blocking solution. Fibers were washed with PBS and then co-incubated with the suitable fluorescent secondary antibodies for 2 h at room temperature. Fibers were subsequently washed three times with PBS and DAPI was added to the first wash at a 0.5 μg/ml concentration in order to visualize the myonuclei. Glass

cover slips were mounted onto the slides with DPX Mountant for histology (Fluka, 44581) and sealed. Images were acquired with an Olympus Fluoview confocal microscope equipped with a 60x objective (Olympus Corporation, Shinjuku, Tokyo, Japan).

Cellular Fractionation

Enriched mitochondrial and nuclear cellular subfractions were isolated by differential centrifugation, as previously described[24]. Briefly, muscles were minced on ice and homogenized using a Teflon pestle and mortar, and suspended in mitochondrial isolation buffer (MIB; 250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) supplemented with protease (Complete, Roche, 1169749801) and phosphatase inhibitor cocktails (Cocktail 2 and 3, Sigma, P5726 and P0044). The homogenates were then centrifuged at 1,000 g for 10 min at 4 °C to pellet the nuclei while mitochondrial and cytosolic fractions were contained within the supernate. The supernate fraction was re-centrifuged at 16,000 g for 20 min at 4 °C to pellet the mitochondria. The mitochondrial pellet was washed twice and resuspended in a one-fold dilution of MIB. Mitochondria were subsequently sonicated 3x3sec to yield the enriched mitochondrial fraction. Pellets containing nuclei were re-suspended in nuclear lysis buffer (1.5 mM $MgCl_2$, 0.2 mM EDTA, 20mM HEPES, 0.5M NaCl, 20% glycerol, 1% Triton-X-100), incubated on ice for 30min, and then sonicated 3x10 sec followed by a final centrifugation step of 15min at 16,000g. The supernate was collected to obtain the enriched nuclear fraction. Protein concentrations within the samples were determined using the Bradford method. Fraction purity was determined by western blot analysis (Fig. S3)

Statistics

Comparisons between WT and KO or TG, Con and Den animals were evaluated using two-way analyses of variance (ANOVA) on each of the treatment conditions. Bonferroni post-tests were

performed when applicable. All values represent the mean ± S.E. Data were considered statistically different if $P < 0.05$.

Results

Lack of PGC-1α results in diminished mitochondrial content, reduced muscle mass, and a myopathic phenotype. In order to ascertain the role of PGC-1α in skeletal muscle autophagy, 8 month old whole body PGC-1α knockout (KO) and wild type (WT) animals were unilaterally denervated for 7 days by severing the sciatic nerve of one hindlimb, with the contralateral limb serving as an internal control (Con). Denervation resulted in similar reductions in muscle mass in both WT and KO animals (Fig. 1A). When corrected for body weight, muscle mass of KO animals was greater than that of WT littermates, however this was a result of their reduced body weight and not due to muscle hypertrophy, as fiber cross-sectional areas were smaller in mice lacking PGC-1α (Supp Fig.1A-C). Basal mitochondrial content was significantly diminished in KO animals, as evident from the reduced intensity of COX and SDH staining, as well as a lower COX activity measured biochemically (Fig. 1B-D). Denervation resulted in 33% and 42% decrease in mitochondrial content in WT and KO mice, respectively (Fig.1B). Electron microscopy images revealed myopathic features in PGC-1α KO mice, evident from the accumulation of multivesicular bodies, aberrant mitochondria as well as tubular aggregates (Fig. 1E). These features are suggestive of autophagic deficiency in animals lacking PGC-1α.

Attenuated autophagic signaling, lower lysosomal abundance and decreased denervation-induced autophagy flux in mice lacking PGC-1α. We next set out to assess the role of the co-activator in the transcriptional regulation of key autophagy genes and in autophagy signaling, both basally and in response to denervation. Denervation resulted in increased expression of various genes that are involved in a variety of aspects of autophagy and mitophagy. Significant

increases in *Park2*, *Sqstm1*, *Maplc3b*, *Atg7*, *Lamp2* and *Ctsd* mRNA expression in response to denervation (Fig.2A) were observed. We did not find a significant difference in the mRNA expression of autophagic factors between WT and KO animals, other than an attenuated denervation-induced increase in *Atg7*. We also assessed autophagy signaling using western blot analysis. In accordance with our mRNA data, we found significant increases in autophagy proteins in response to denervation (Fig.2B). Beclin1, Atg7, Cathepsin D and Lamp-2 all increased, while parkin tended to increase in WT animals (Fig.2B-G). Although there was no significant difference in the content of autophagy proteins between WT and KO animals basally, the denervation-induced increases in Beclin1 and Lamp-2 levels were significantly attenuated in KO muscle, while Cathepsin D induction tended to be lower in KO animals.

In order to ascertain the influence of PGC-1α on autophagy flux, we treated the animals with the microtubule de-stabilizing drug colchicine, previously reported to effectively block autophagic degradation [22] (Fig.3). Denervation resulted in increased accumulation of LC3B-II, p62 and Nix (Bnip3l) proteins in WT muscle. Colchicine treatment resulted in further accumulations of LC3B-II and p62 in denervated WT muscle, indicating a successful block in autophagic degradation with the drug (Fig.3A-C). In contrast, PGC-1α KO animals exhibited an attenuated LC3B-lipidation and expression of the mitophagy specific receptor Nix both basally and in response to denervation (Fig.3 A-B, D). No significant difference in p62 expression was observed basally between the genotypes, and p62 levels did not change in KO animals with either Den or Col treatment (Fig.3C). Importantly, both basal and denervation-induced p62 and LC3B-II flux were lower in KO animals, as evident by a smaller accumulation of LC3BII and p62 in KO animals treated with colchicine (Fig. 3E-F). We further examined lysosomal abundance and autophagy flux in KO and WT animals using confocal microscopy (Fig.3G). We

did this by isolating single fibers from fixed EDL muscles and co-immunostaining them for LC3B and Lamp-2. KO animals had lower lysosomal abundance, as indicated by a diminished intensity and frequency of red fluorescence, and this was especially evident following denervation (Fig.3G and Fig.S2). We also noted an increase in the colocalization (yellow) of autophagosomes and lysosomes and their aggregation in the perinuclear region of denervated WT muscles (Fig.3G Merge). This was not as evident in denervated KO muscle, as indicated by decreased yellow flourescence. Taken together, these results indicate that the lack of PGC-1α results in lower lysosomal abundance and reduced autophagy flux in response to denervation.

Mitophagy is attenuated in PGC-1α-deficient muscle. Since PGC-1α is a major metabolic regulator that plays a key role in mitochondrial homeostasis, we were interested in elucidating the role of this co-activator in mitochondrial removal by mitophagy. During denervation, the mitochondrial pool within skeletal muscle undergoes a substantial reduction (33-42% Fig.1B). To determine the involvement of PGC-1α in mitophagy, we isolated mitochondria from both denervated and control gastrocnemius muscle of WT and KO animals that were treated with either vehicle or colchicine (Fig.4). Denervation resulted in an increase in the localization of LC3BII, p62 and parkin to the mitochondria, as well as an elevated overall ubiquitination of mitochondrial substrates (Fig.4A-E), culminating in increased mitophagy flux (Fig.4H). These findings indicate that mitophagy is involved in the removal of mitochondria from muscle basally, and during denervation. This response was reduced in the KO animals (Fig 4G-I). Muscle from KO animals exhibited similar basal localization of autophagic proteins to the mitochondria (Fig. 4A-F), but had an attenuated increase in mitochondrial localization of these proteins with denervation. Importantly, KO animals exhibited lower basal and denervation-induced mitophagy flux (Fig.4G-H). To further investigate mitophagy flux, we co-stained isolated single EDL fibers

with mitochondrial cytochrome c (Cyto C; green) as well as with Lamp-2 (red) and assessed their co-localization (Merge; yellow) using confocal microscopy (Fig. 4I). The images suggest a decrease in the co-localization of mitochondria with lysosomes in denervated KO animals compared to WT animals, further supporting a decrease in mitophagy flux in the KO animals in response to denervation.

TFEB protein levels are induced with denervation and may mediate PGC-1α action on autophagy. To examine the role of PGC-1α in the expression of autophagy genes we examined TFEB, the master transcriptional regulator of the autophagy-lysosome system, as several recent studies have indicated an interplay between these two factors[16, 17, 28, 29]. TFEB protein levels were increased with denervation in WT animals (Fig. 5A-C), and this tended to impact the levels of TFEB in the nucleus. In the KO animals, TFEB levels and nuclear localization were lower under basal conditions ($p<0.05$), and did not change in response to denervation (Fig. 5A-C).

Overexpression of PGC-1α results in increased mitochondrial content and protection from denervation-induced mitochondrial loss and muscle atrophy. Since our results indicate a potential role for PGC-1α in regulating autophagy flux in response to denervation, we investigated whether PGC-1α alone was sufficient to induce autophagy. Thus, we compared WT to muscle-specific PGC-1α overexpressing animals (Tg). TA muscle from Tg animals displayed a much more oxidative phenotype (Fig. 6C-D) and they were protected from denervation-induced muscle atrophy (Fig.6A). We confirmed PGC-1α over-expression in Tg animals by immunoblotting for PGC-1α (Fig. 6B) and COXIV (Fig.6C), a downstream target of the co-activator, as well as with histochemical staining for SDH (Fig. 6D). TA muscles of Tg animals were significantly richer in mitochondria and exhibited a reduced loss of mitochondrial content

in response to denervation, as indicated by greater COXIV protein (Fig. 6C) and darker SDH staining (Fig. 6D), compared to WT controls.

PGC-1α overexpression increases lysosomal and mitophagy receptor expression. We examined the effect of PGC-1α overexpression on the levels of autophagy genes and proteins basally, and in response to denervation. Real-time PCR analyses confirmed that 7 days of denervation resulted in increased expression of various autophagy and mitophagy genes, such as *Park2*, *Sqstm1*, *Maplc3b*, *Atg7*, and *Ctsd* (Fig.7A) consistent with our earlier results (Fig. 2A). We found a decrease ($p<0.05$) in the basal expression of *Sqstm1, Bnip3l and Atg7* in Tg animals. In addition, we also observed attenuated denervation-induced increases in *Sqstm1, Maplc3b, and Atg7.* We also assessed autophagy signaling by western blot analysis. Parkin, Beclin1 and Atg7 protein levels were similar in WT and Tg animals, both basally and in response to denervation (Fig. 7B-E). However, the levels of the lysosomal proteins Cathepsin D and Lamp-2 were significantly higher in Tg as compared to WT animals, and these were markedly increased in denervated Tg muscle (Fig. 7F-G). Tg animals also displayed significantly higher levels of both LC3I and II than WT controls, and these were augmented to a greater extent by denervation compared to WT animals (Fig.8A-C). Similarly, Nix protein content was higher in Tg, as compared to WT animals, both basally and in response to denervation (Fig, 8D), while p62 levels were similar in both genotypes (Fig. 8E). Taken together, these data suggest that PGC-1α selectively induces the expression of specific autophagy and lysosomal proteins.

PGC-1α overexpression resulted in reduced autophagy markers in the mitochondrial subfraction. Since we found a protective effect of PGC-1α overexpression on mitochondria following denervation (Fig. 6) we wanted to investigate whether mitophagy was affected. Our results indicate that PGC-1α overexpression leads to a decreased localization of LC3BII and p62

to the mitochondrial fraction in Tg muscle, both basally and in response to denervation (Fig. 9 A-C).

TFEB is induced with denervation and may mediate PGC-1α action on autophagy. Since we found a decrease in TFEB protein levels in animals lacking PGC-1α (Fig. 5), we further investigated the effects of PGC-1α over-expression on this transcription factor. TFEB protein levels were significantly higher in PGC-1α Tg animals (Fig. 10A-B). Similar to our earlier findings, TFEB increased with denervation in WT animals, to a level that was similar to the higher basal level evident in Tg animals (Fig. 10A-B). TFEB protein was not further induced by denervation in Tg animals.

Discussion

Metabolic plasticity is a unique property which permits the fine tuning of energy production to meet energy demands in skeletal muscle, allowing for adaptations in response to alterations in nutrient availability, hormonal stimuli and contractile activity. This property makes muscle a pillar of whole body homeostasis, in particular during energetic distress. Interestingly, both the autophagy-lysosome system and the transcriptional co-activator PGC-1α have been separately documented to contribute to whole body metabolic homeostasis, as well as to muscle plasticity, in response to alterations in nutrient availability and contractile activity[10, 15, 29–34]. However, the role of PGC-1α in autophagy and mitophagy has not been dissected thus far. In this study, we illuminate a role for PGC-1α in autophagy and mitophagy in skeletal muscle in response to chronic muscle disuse. Moreover, we identify the transcription factor TFEB to be a potential target of PGC-1α in the regulation of autophagy in this tissue.

Our results have confirmed the myopathic phenotype evident in muscles of animals lacking PGC-1α. Muscle of KO animals was characterized by a diminished mitochondrial

content, smaller cross-sectional area, and an accumulation of damaged organelles and multivesicular bodies as evident by the appearance of abnormal structures in EM images. Some of the myopathic features of PGC-1α KO muscle are reminiscent of those found in autophagy-deficient animals which also exhibit deficient mitochondria and increased apoptosis [10, 35, 36]. We did not note a basal difference between WT and KO animals in the mRNA or protein expression of various autophagy markers. However, it is possible that an earlier time point following the onset of denervation, when autophagy gene expression may have peaked, could have revealed some endogenous expression differences between the two genotypes in response to this muscle atrophy stimulus. Importantly, we did observe an attenuated induction in LC3B lipidation and protein expression of lysosomal factor Lamp-2 with denervation in KO animals. A lack of PGC-1α also resulted in reduced basal as well as denervation-induced autophagy flux, suggesting that the presence of PGC-1α has a significant impact on the maintenance of autophagy in muscle.

In contrast to the mitochondrial phenotype observed in KO animals, over-expression of the co-activator resulted in a highly oxidative muscle that was protected from denervation-induced loss of mitochondria and muscle mass. This was evident from the much darker SDH staining, as well as the increased COXIV protein expression in Tg animals that did not decrease with denervation. This protection has been previously documented to be a result of improved mitochondrial function [37], cellular oxidative status [38], and suppression of FoxO3-mediated catabolism [13]. Similar to our observations with KO animals, overexpression of the coactivator did not result in dramatic alterations in autophagy protein expression. However, levels of the lysosomal marker Lamp-2, and the protease cathepsin D were significantly induced in Tg animals, and were further increased with denervation. Moreover, LC3B protein levels, as well as

LC3B lipidation were both enhanced in the Tg animals, suggesting an increased autophagy flux mediated by PGC-1α. In another study involving the use of fasting as an inducer of autophagy, we have confirmed the increase in autophagy flux in Tg animals (Fig. S4)

To investigate the role of PGC-1α in mitophagy, we examined the expression of the mitophagy receptor Nix (Bnip3L) as well as the localization of autophagy factors to isolated mitochondria. The expression of Nix was significantly reduced in KO, and strongly induced in Tg, when compared to WT animals, indicating that Nix may be under the control of PGC-1α. This could be mediated by the PGC-1α-HIF-1α axis, as Nix was found to be under HIF-1α control during hypoxia [39], and HIF-1α was documented to be stabilized by PGC-1α in muscle cells [40]. However, further research is required to confirm this interaction in muscle. We also noted an enhanced localization of LC3B-II, p62, parkin as well as ubiquitin to isolated mitochondria with denervation in WT animals, but this effect was attenuated in KO animals. Indeed, both basal and denervation-induced mitophagy flux were reduced, indicating impaired mitophagy in the absence of PGC-1α. We have previously documented oxygen consumption deficits and enhanced susceptibility to apoptosis in mitochondria of PGC-1α KO muscle [18]. These findings can now likely be attributed to deficient mitochondrial turnover, resulting from a combination of an impairment in mitochondrial biogenesis [1, 19, 41] as well as mitophagy in the absence of PGC-1α.

Interestingly, we also noted reduced localization of autophagic markers p62 and LC3B-II to the mitochondrial fraction of Tg animals, both basally and in response to denervation. This corresponded to the enhanced protection of mitochondrial content during denervation in these animals, and it occurred despite increases in LC3B and the mitophagy receptor Nix in whole muscle extracts. We also confirmed reduced mitophagy flux under fasting conditions in PGC-1α

Tg animals compared to WT littermates (Fig. S5). These data suggest that PGC-1α mediates a reduced targeting of mitochondria for mitophagy when mitochondrial function and content are high. Based on our data, we contend that physiological levels of PGC-1α are required for the proper propagation of mitophagy in muscle, as a lack of PGC-1α results in reduced mitochondrial turnover, culminating in the accumulation of defective mitochondria, increased susceptibility to cell death [18] and overall muscle atrophy. In contrast, PGC-1α overexpression is protective of mitochondrial mass via several mechanisms (Fig. 11A and B). First, PGC-1α has been shown to improve mitochondrial function [42, 43] and enhance cellular antioxidant capability [38] which can result in reduced mitochondrial targeting for degradation. Second, mitochondrial fragmentation is required for mitophagy to occur, and PGC-1α augments the expression of factors involved in mitochondrial fusion such as mitofusin1 and 2[44], resulting in a more reticular mitochondrial phenotype that can better evade mitophagy. Third, PGC-1α overexpression has been documented to block FoxO3-mediated transcriptional activity [13], and since FoxO3 drives the expression of multiple mitophagy factors such as Mul1 and Bnip3 [45, 46], repression of FoxO3activity may be sufficient to protect mitochondria from elimination.

The involvement of autophagy during denervation has recently been brought into question. Some evidence has indicated a block in autophagy early in denervation [47, 48], while other data point to the contrary at later time points [10, 21, 22, 46, 49–51]. Here we demonstrate that autophagy and mitophagy flux were both elevated at 7 days of denervation, with mitophagy contributing to mitochondrial loss during disuse. Indeed, localization of LC3B-II, p62 and parkin to the mitochondria were all induced during denervation resulting in enhanced mitophagy flux in WT animals.

Our study has also revealed key evidence supporting a role for PGC-1α in lysosomal biogenesis. Lack of PGC-1α resulted in reduced denervation-induced lysosomal protein expression and overall lysosomal abundance, while PGC-1α overexpression provoked an increase in basal and denervation-induced levels of the lysosomal proteins Lamp-2 and cathepsin D. Furthermore, we found a correlation (Pearson r=0.84; data not shown) between levels of PGC-1α and the lysosomal master regulator TFEB. This further supports findings by Scott et al [17] on the co-ordinated regulation of PGC-1α and TFEB, which works to ensure the proper matching between mitochondrial removal and biogenesis. Thus, we highlight a role for PGC-1α in lysosomal biogenesis which could be mediated, at least in part, by TFEB.

Conclusions

Our results suggest a role for the transcriptional co-activator PGC-1α in the regulation of autophagy-lysosomal machinery and mitophagy in skeletal muscle. A lack of PGC-1α results in reduced disuse-induced autophagy and mitophagy signaling and flux, whereas PGC-1α overexpression increased lysosomal abundance and bulk autophagy flux while suppressing mitophagy (Fig. 11B) Therefore, our findings elucidate a previously unidentified role for PGC-1α in the fine tuning of autophagy and mitophagy in skeletal muscle, and the identification of pharmacological targets along the PGC-1α-autophagy axis could be of therapeutic benefit to those suffering from metabolic or muscle wasting myopathies.

List of Abbreviations:

Atg7, autophagy related 7; Con, control; COX, cytochrome oxidase; Den, denervated; EDL, extensor digitorum longus; FOXO3, Forkhead box O3; KO, Knockout; Lamp-2, lysosome associated membrane protein 2; LC3/Maplc3, microtubule-associated protein 1 light chain 3;

MCK, muscle creatine kinase; Nix/Bnip3l, Bcl-2/adenovirusE1B 19kDa interacting protein 3-like; p62/Sqstm1, sequestosome 1; Parkin/park2, RBR E3 ubiquitin protein ligase; PGC-1α, Peroxisome Proliferator co-activator 1 alpha; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TA, tibialis anterior; TFEB, Transcription factor EB; Tg, transgenic; WT, wild type.

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Authors' contributions

AV participated in the design of the study, collected the data, analyzed the data, performed statistical analysis, helped write and edit the manuscript. EMD and AA collected data and provided technical assistance. MS participated in study design, provided guidance and assisted with data interpretation. DAH conceived of the study, participated in its design and coordination, provided guidance and assistance with data interpretation, and helped write and edit the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Fig.1 PGC-1α KO animals have lower mitochondrial content and display myopathic features. A. TA muscle mass corrected for body weight B. Cytochrome C oxidase activity. C. Representative images of COX staining of EDL muscle from Con and Den WT and KO animals. D. Representative images of SDH staining of EDL muscle from Con and Den WT and KO animals. E. EM images of control WT (top left panel) and PGC-1α KO muscle (top right and bottom two panels). PGC-1α KO animals display a myopathic phenotype characterised by the accumulation of multivesicular bodies (top right), aberrant mitochondria (bottom left) as well as tubular aggregates (bottom right). *P<0.05, significant effect of denervation. †P<0.05, significant effect of genotype (n=4-8 for all groups).

Fig.2 Lack of PGC-1α results in attenuation of autophagic signaling induced by denervation. A. Autophagy gene expression measured by real time PCR. mRNA fold change between wild type (WT) and PGC-1α KO (KO) control (Con) and denervated (Den). All groups were compared to WT Con, and *Gapdh* and *Actb* were used as housekeeping genes. B-G; Blots and quantification of autophagic proteins in TA muscle of control (C; Con), denervated (D; Den) WT and KO animals. B. Representative blots. Quantification of C. Parkin; D. Beclin 1; E. Atg7; F. Cathepsin D; G. Lamp-2. *P<0.05 significant difference between Con and Den. GAPDH was used as a loading control (n=4-8 for all groups).

Fig.3 Lack of PGC-1α results in reduced lysososmal abundance and denervation-induced autophagy flux. A-C. Blots and quantification of autophagic proteins in TA muscle of control (Con), denervated (Den) WT and PGC-1α KO (KO) animals; treated with vehicle (water) or 0.4mg/kg/day colchicine (col) for 4 days. A. Representative blots. Quantification of B. LC3BII; C. p62; D. Representative blot and quantification of Nix; E. Basal autophagy flux; F.

Denervation-induced autophagy flux; G. Confocal images of fixed single fibers immuno-stained for LC3 (green) and lysosomal Lamp-2 (red) and colocalization is shown in yellow (Merge) and represents autophagosomes within lysosomes. Nuclei are in blue (DAPI). *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH was used as a loading control (n=3-5 for all groups).

Fig.4 Lack of PGC-1α results in attenuated mitophagy flux. A-D. Blots and quantification of autophagic proteins in isolated mitochondrial fractions in control (Con), denervated (Den) WT and PGC-1α KO (KO) animals treated with vehicle (water) or colchicine (col) 0.4mg/kg/day for 4 days. A. Representative blots. Quantification of B. LC3BII; C. p62; D. Representative blots. Quantification of mitochondrial E. Ubiquitin; F. Parkin. G. Basal mitophagy flux. H. Denervation-induced mitophagy flux. I. Confocal images of fixed single fibers immuno-stained for cytochrome c (a mitochondrial marker, green) and lysosomal Lamp-2 (red), their colocalization (yellow) represents mitochondria within lysosomes. *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. VDAC was used as loading control. (n=3-5 for all groups).

Fig.5 Lack of PGC-1α results in lower TFEB protein levels and nuclear localization. A-C Blots and quantification TFEB protein levels between wild type (WT) and PGC-1α KO (KO) control (Con) and denervated (Den). A. Representative blots. Quantification of B. whole muscle TFEB protein; C. Nuclear localization of TFEB in TA muscle*P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH was used as loading control and Histone 2 B (H2B) was used as nuclear loading control (n=4-8 for all groups).

Fig.6 PGC-1α over-expressing animals have higher mitochondrial content and are protected from denervation-induced muscle atrophy A. TA muscle mass corrected for body

weight. B. PGC-1α blot confirming overexpression in TA muscle C. Representative blot and quantification of COXIV as a mitochondrial content marker. D. Representative images of SDH staining of TA muscle from Con and Den WT and Tg animals. *P<0.05, significant effect of denervation .†P<0.05, significant effect of genotype (n=3-5 for all groups) .

Fig.7 Over-expression of PGC-1α results in enhanced lysosomal protein expression induced by denervation. A. Autophagy gene expression. mRNA fold changes between wild type (WT) and PGC-1α Tg (Tg) control (Con) and denervated (Den). All groups are compared to WT con, *Gapdh* and *Actb* were used as housekeeping genes. B-G Blots and quantification of autophagic proteins in Con, Den WT and Tg animals. B. Representative blots. Quantification of C. Parkin; D. Beclin 1; E. Atg7; F. Cathepsin D; G. Lamp-2. *P<0.05 significant difference between Con and Den. †P<0.05, significant effect of genotype. GAPDH was used as a loading control. (n=3-5 for all groups).

Fig.8 Elevated PGC-1α results in greater basal and denervation-induced autophagy protein expression. A-E. Blots and quantification of autophagic proteins in control (Con), denervated (Den) WT and PGC-1α Tg (Tg) animals. A. Representative blots. Quantification of B. LC3BI; C. LC3BII; D. Nix; E. p62; *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH was used as a loading control. (n=3-5 for all groups)

Fig.9 PGC-1α Tg animals demonstrate lower presence of autophagy markers in isolated mitochondria. A-C. Blots and quantification of autophagic proteins on isolated mitochondria in control (Con), denervated (Den) WT and PGC-1α Tg (Tg). A. Representative blots. Quantification of B. LC3BII; and C.p62. †P<0.05 significant effect of genotype. VDAC was used as loading control. (n=3 for all groups).

Fig.10 Elevated PGC-1α results in increased TFEB protein levels. A-B. Blots and quantification TFEB protein level in TA muscle and in nuclear fraction of wild type (WT) and PGC-1α Tg (Tg) control (Con) and denervated (Den). A. representative blots. B. Quantification of TFEB protein in TA muscle. *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH was used as loading control for whole muscle and Histone 2 B (H2B) was used as nuclear loading control (n=3-5 for all groups).

Fig.11 Proposed relationship between PGC-1α expression, mitochondrial function and mitophagy during cellular stress. A-B Upon cellular metabolic stress such as denervation or nutrient deprivation lack of PGC-1α results in diminished mitochondrial function, biogenesis and impaired autophagy and mitophagy, whereas its overexpression results in superior mitochondrial function and biogenesis as well as enhanced autophagy, but reduced mitophagy. A. Hypothetical graphical representation of the relationship between levels of PGC-1α, mitochondrial function and mitophagy. B. Schematic outlining mitochondrial turnover during denervation-induced metabolic stress in muscle in light of variations in PGC-1α expression. Steady state mitochondrial content is represented in the center of each panel. In the presence of endogenous PGC-1α levels (middle panel), biogenesis is active at a low level during denervation, but both mitophagy and autophagy flux are enhanced leading to a reduced steady state mitochondrial content. When the expression of PGC-1α is abolished (upper panel), biogenesis is further reduced during denervation, as are autophagy and mitophagy flux, leading to a smaller, dysfunctional pool of mitochondria. When PGC-1α levels are elevated (lower panel), biogenesis is higher while mitophagy is lower than normal during denervation, leading to a maintained organelle content. Arrow thickness provides an indication of the magnitude of the pathway.

Fig.1

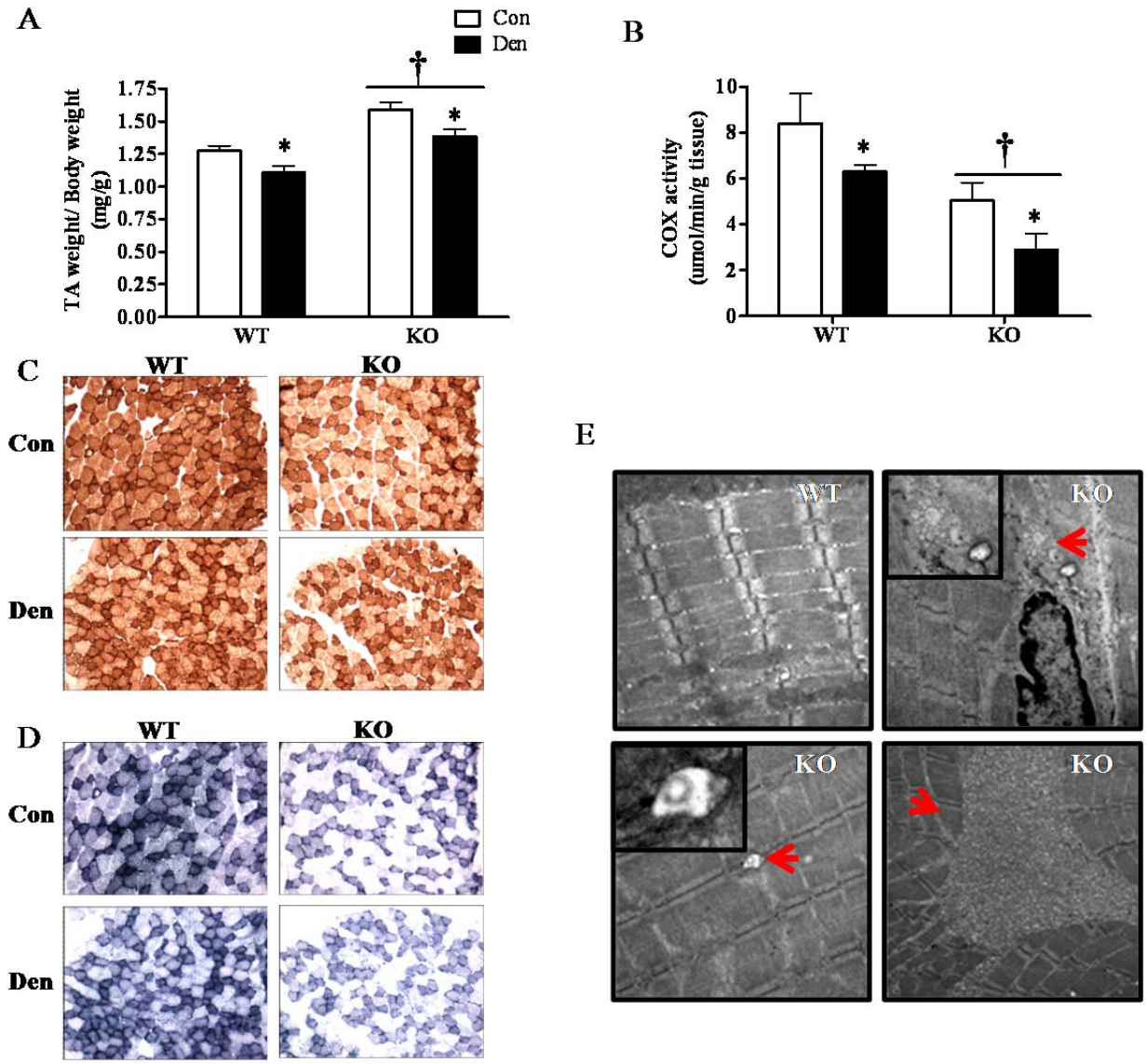
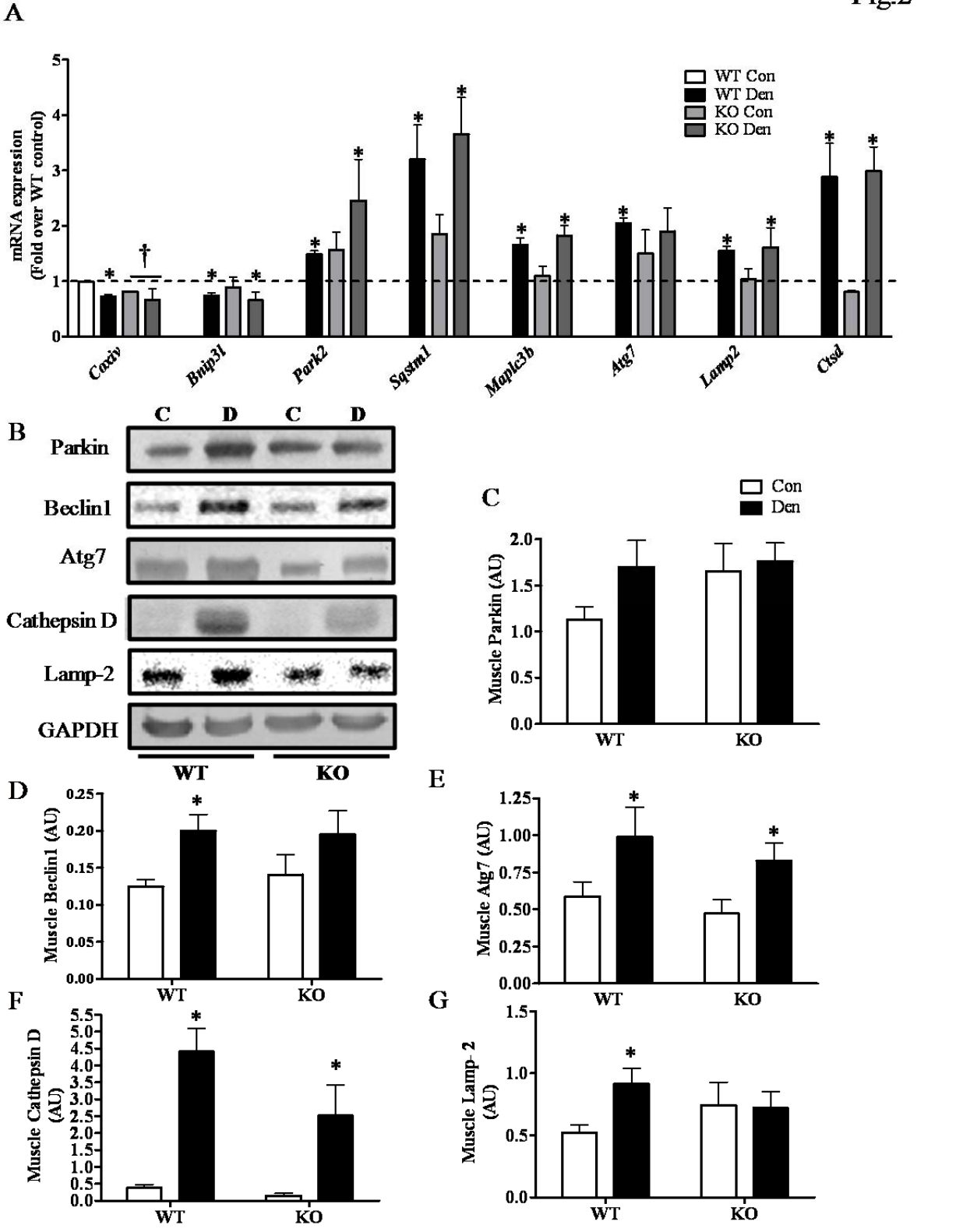

A
Con
Den
TA weight/ Body weight (mg/g)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
WT
KO
B
COX activity (umol/min/g tissue)
10
8
6
4
2
0
WT
KO
C
WT
KO
Con
Den
D
WT
KO
Con
Den
E
WT
KO
KO
KO

Fig.2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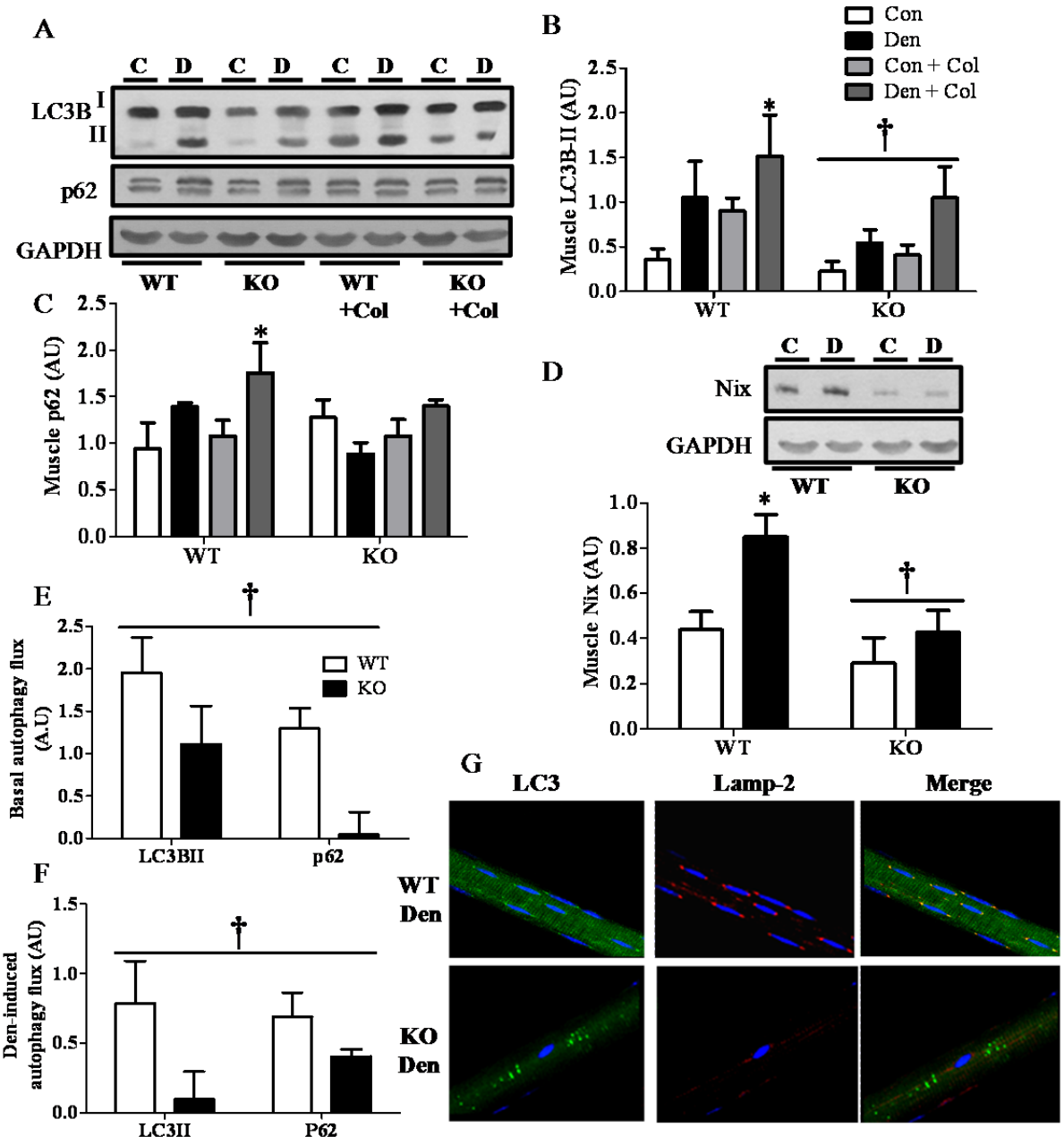
mRNA expression (Fold over WT control)
WT Con
WT Den
KO Con
KO Den
Cox4i
Bnip3l
Park2
Sqstm1
Maplc3b
Atg7
Lamp2
Ctsd
B
C
D
Parkin
Beclin1
Atg7
Cathepsin D
Lamp-2
GAPDH
WT
KO
C
Con
Den
Muscle Parkin (AU)
D
Muscle Beclin1 (AU)
E
Muscle Atg7 (AU)
F
Muscle Cathepsin D (AU)
G
Muscle Lamp-2 (AU)

Fig.3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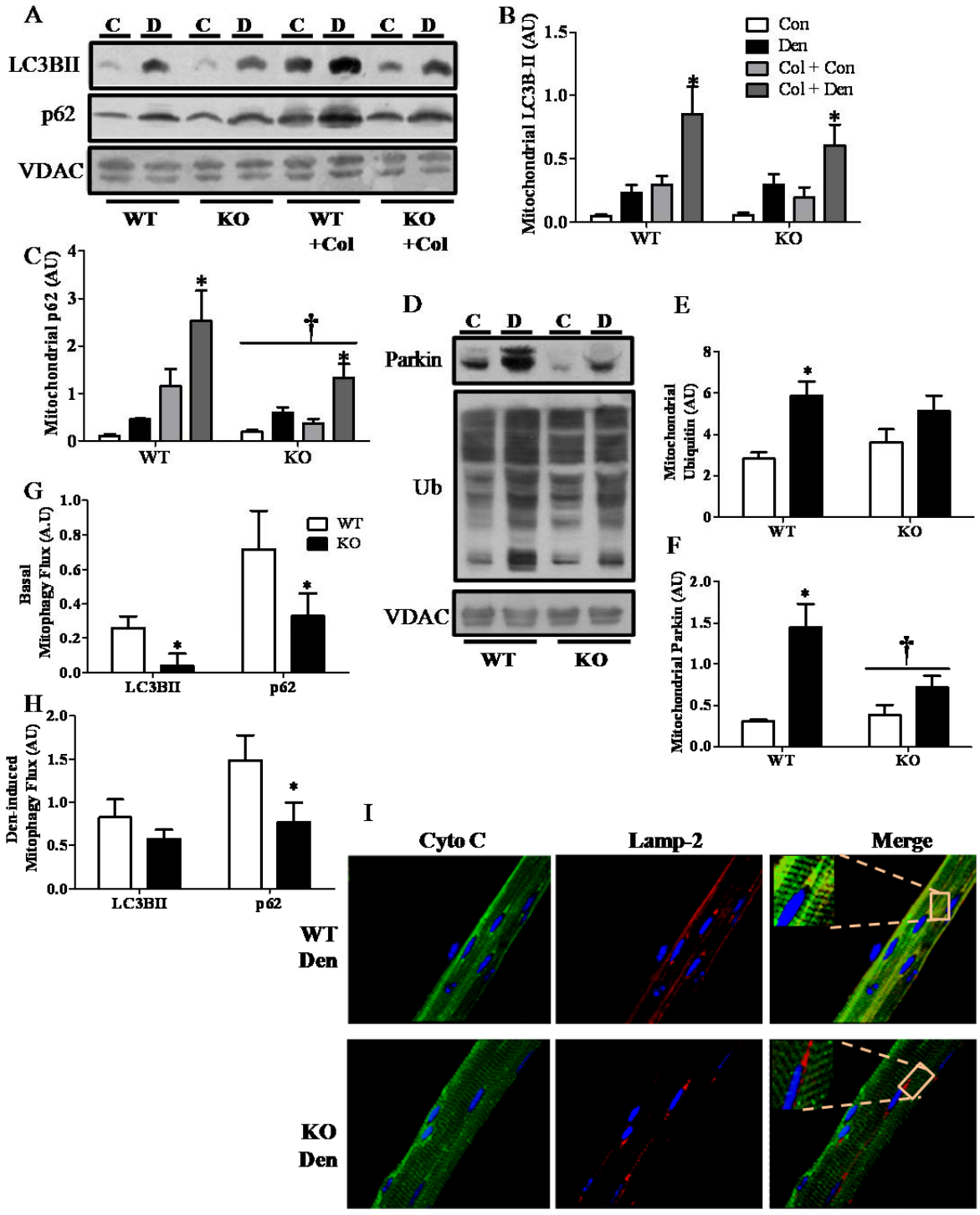
C D C D C D C D
LC3B I
II
p62
GAPDH
WT KO WT KO
+Col +Col
B
Con
Den
Con + Col
Den + Col
Muscle LC3B-II (AU)
WT KO
C
Muscle p62 (AU)
WT KO
D
C D C D
Nix
GAPDH
WT KO
Muscle Nix (AU)
WT KO
E
Basal autophagy flux (A.U)
WT
KO
LC3BII p62
F
Den-induced autophagy flux (AU)
LC3II P62
G
LC3 Lamp-2 Merge
WT Den
KO Den

Fig.4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LC3BII
p62
VDAC
WT KO WT +Col KO +Col
B
Mitochondrial LC3B-II (AU)
Con
Den
Col + Con
Col + Den
WT KO
C
Mitochondrial p62 (AU)
WT KO
D
C D C D
Parkin
Ub
VDAC
WT KO
E
Mitochondrial Ubiquitin (AU)
WT KO
F
Mitochondrial Parkin (AU)
WT KO
G
Basal Mitophagy Flux (A.U)
WT
KO
LC3BII p62
H
Den-induced Mitophagy Flux (AU)
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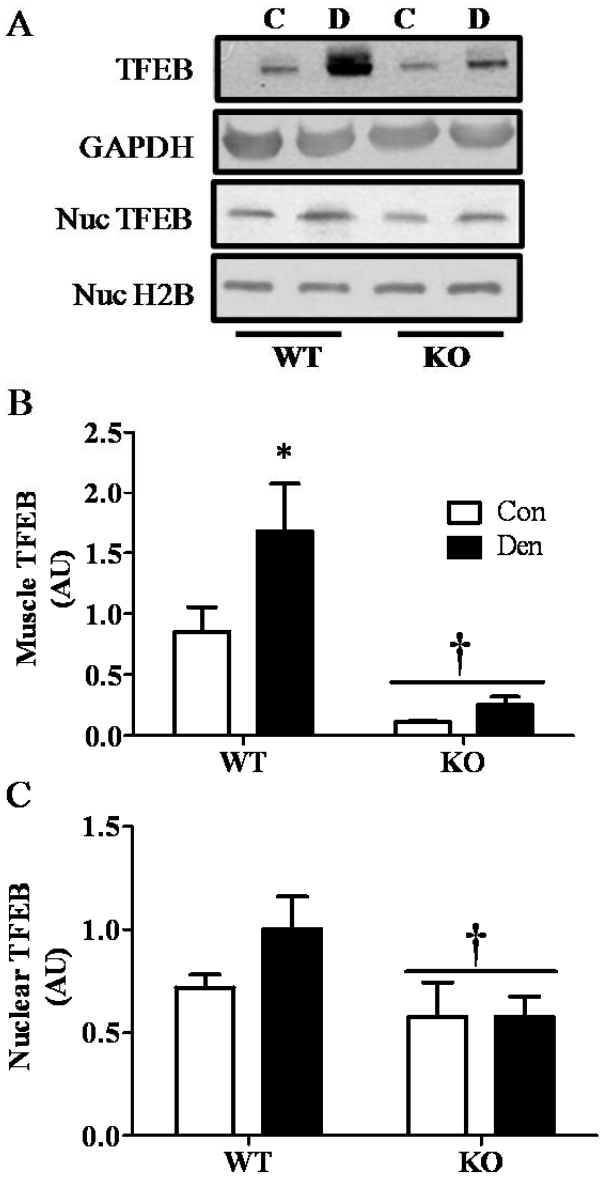
I
Cyto C
Lamp-2
Merge
WT Den
KO Den

Fig.5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C
D
C
D
TFEB
GAPDH
Nuc TFEB
Nuc H2B
WT
KO
B
Muscle TFEB (AU)
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2.0
1.5
1.0
0.5
0.0
*
Con
Den
†
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C
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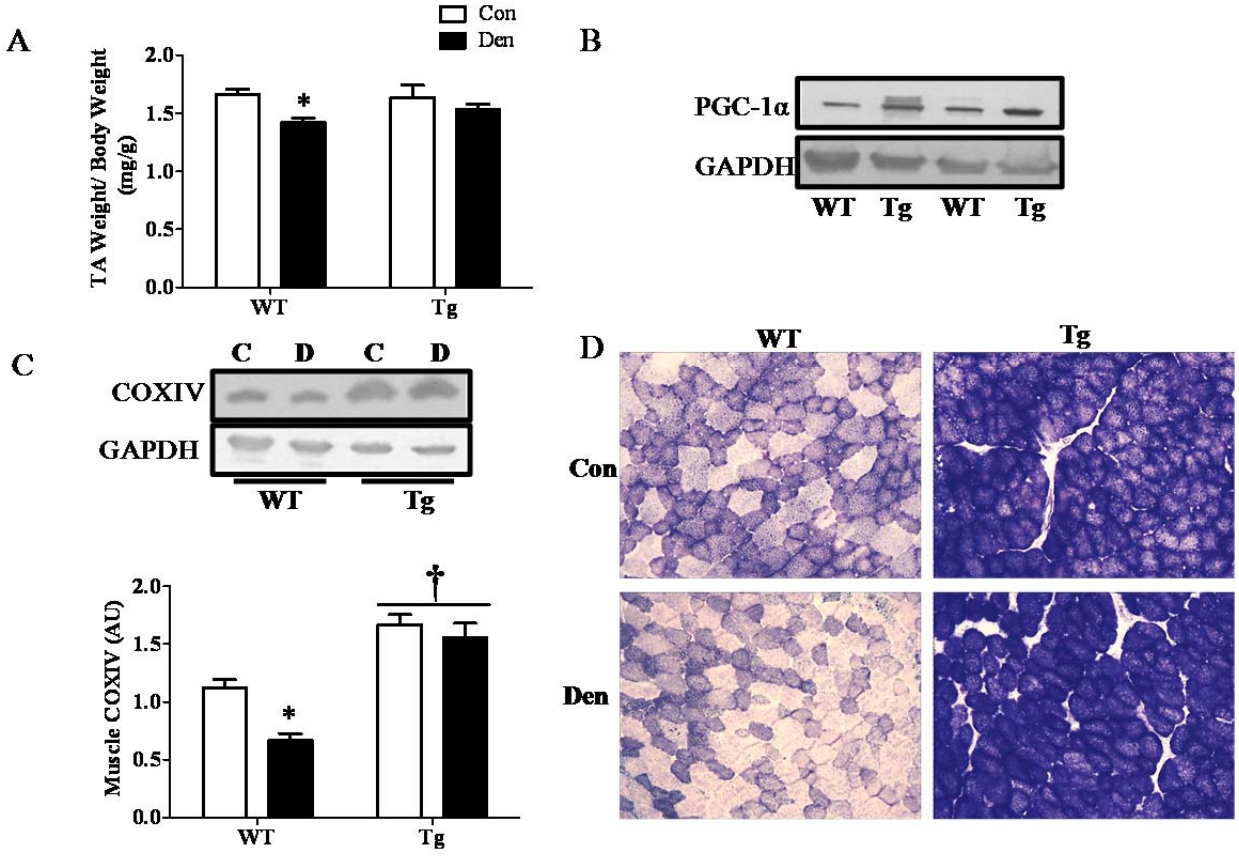
†
WT
KO

Fig.6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Con
Den
TA Weight/ Body Weight (mg/g)
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1.5
1.0
0.5
0.0
*
WT
Tg
B
PGC-1α
GAPDH
WT
Tg
WT
Tg
C
C
D
C
D
COXIV
GAPDH
WT
Tg
Muscle COXIV (AU)
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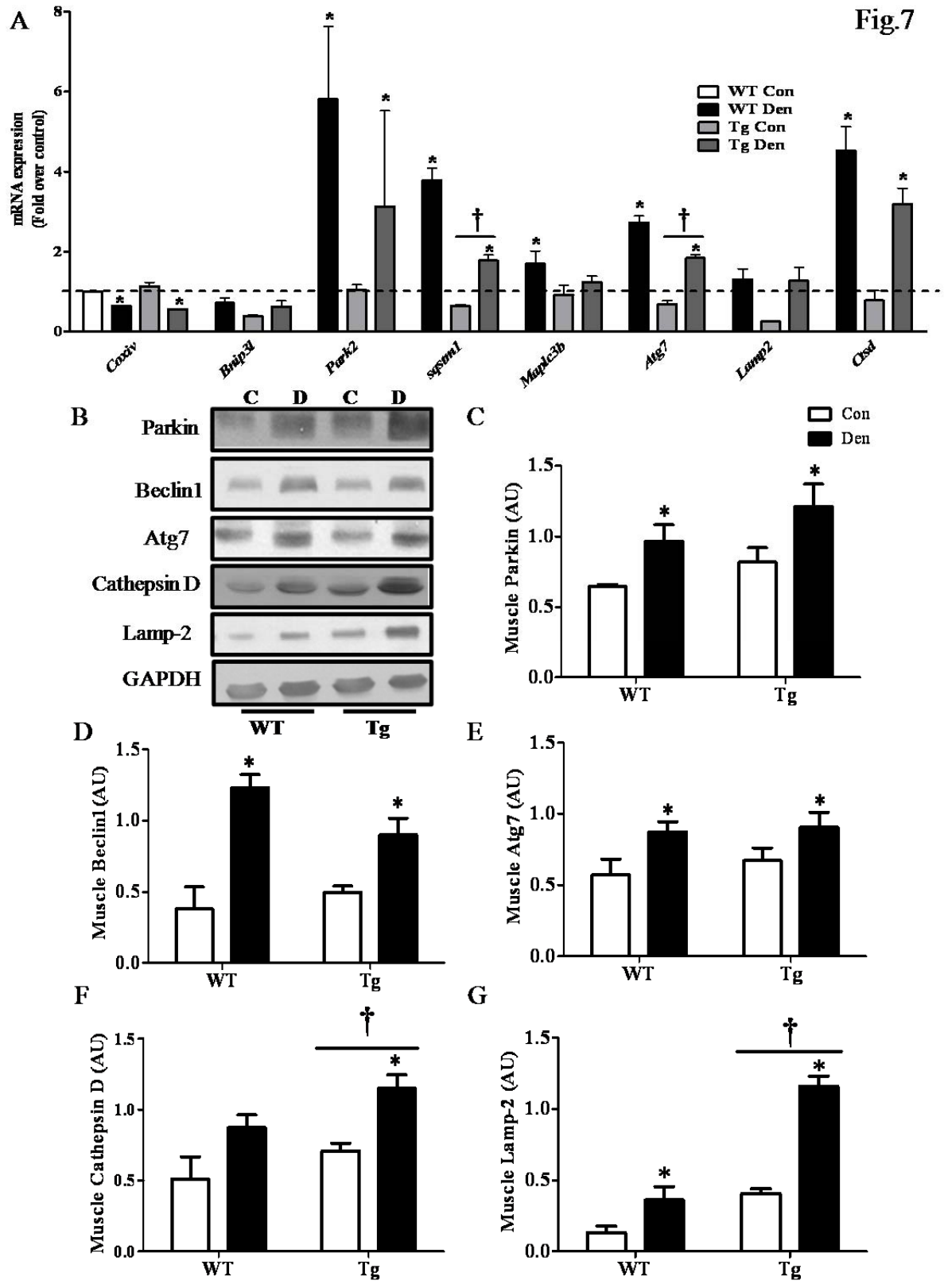
1.5
1.0
0.5
0.0
*
†
WT
Tg
D
WT
Tg
Con
Den


A
Fig.7
mRNA expression (Fold over control)
WT Con
WT Den
Tg Con
Tg Den
Cox4v
Bnip3l
Park2
sqstm1
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Atg7
Lamp2
Ctsd
B
C D C D
Parkin
Beclin1
Atg7
Cathepsin D
Lamp-2
GAPDH
WT
Tg
C
Con
Den
Muscle Parkin (AU)
D
Muscle Beclin1 (AU)
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Muscle Atg7 (AU)
F
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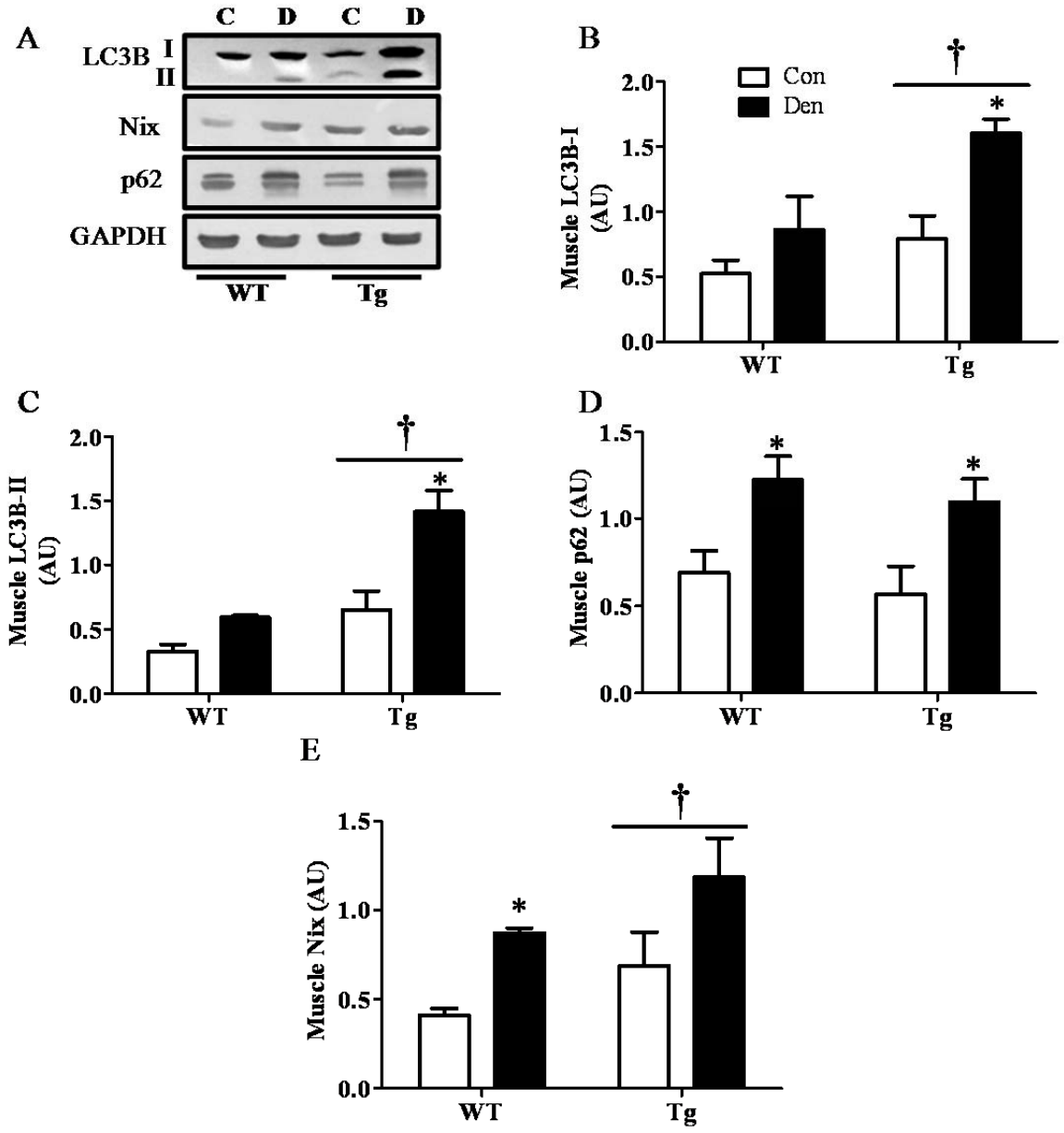
G
Muscle Lamp-2 (AU)

Fig.8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D
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D
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p62
GAPDH
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Tg
B
Con
Den
Muscle LC3B-I (AU)
2.0
1.5
1.0
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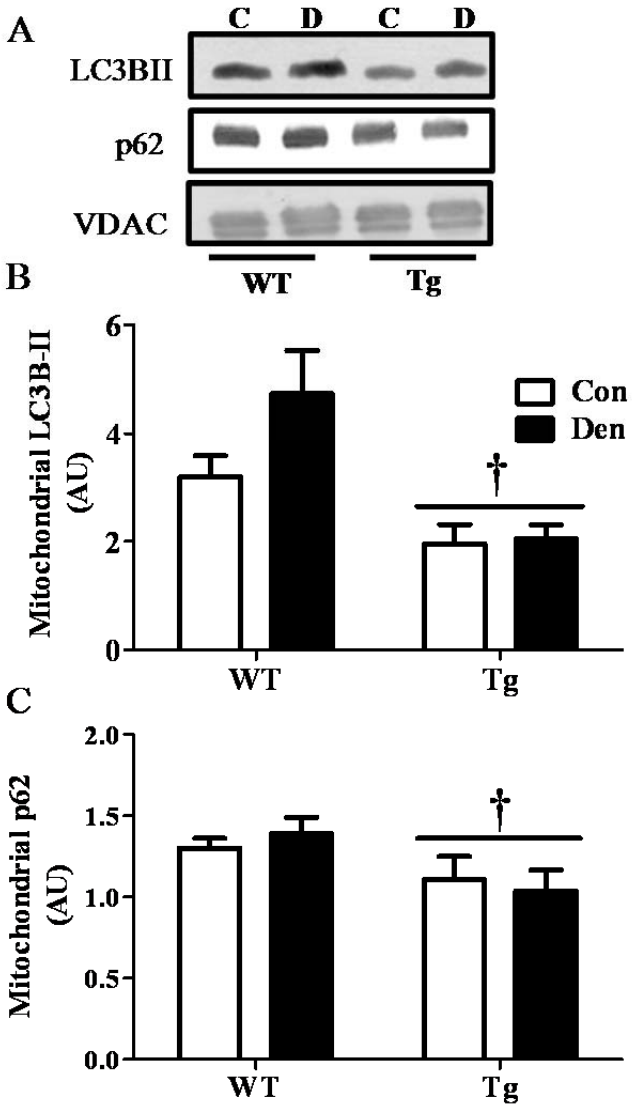
D
Muscle p62 (AU)
E
Muscle Nix (AU)

Fig.9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D
C
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LC3BII
p62
VDAC
WT
Tg
B
Mitochondrial LC3B-II (AU)
6
4
2
0
Con
Den
†
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1.5
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0.5
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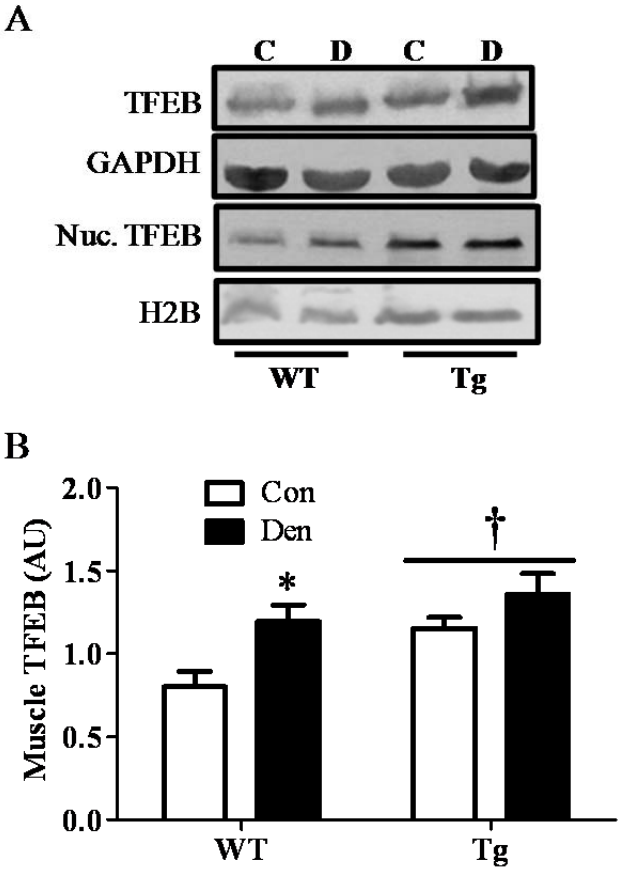
†
WT
Tg

Fig.10


A
C
D
C
D
TFEB
GAPDH
Nuc. TFEB
H2B
WT
Tg
B
Muscle TFEB (AU)
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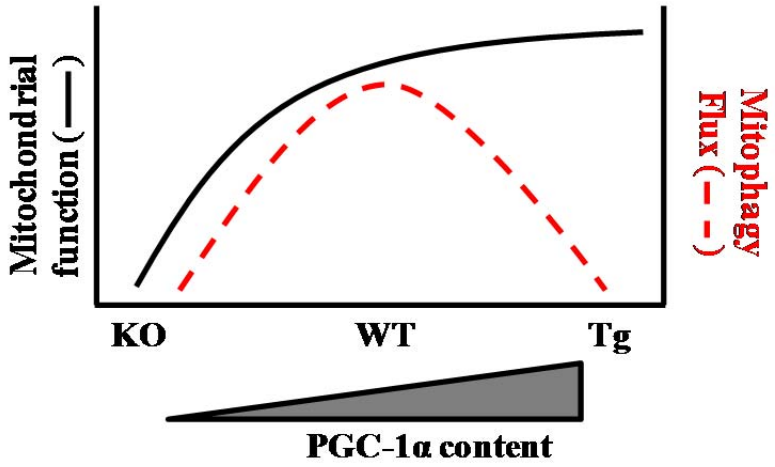
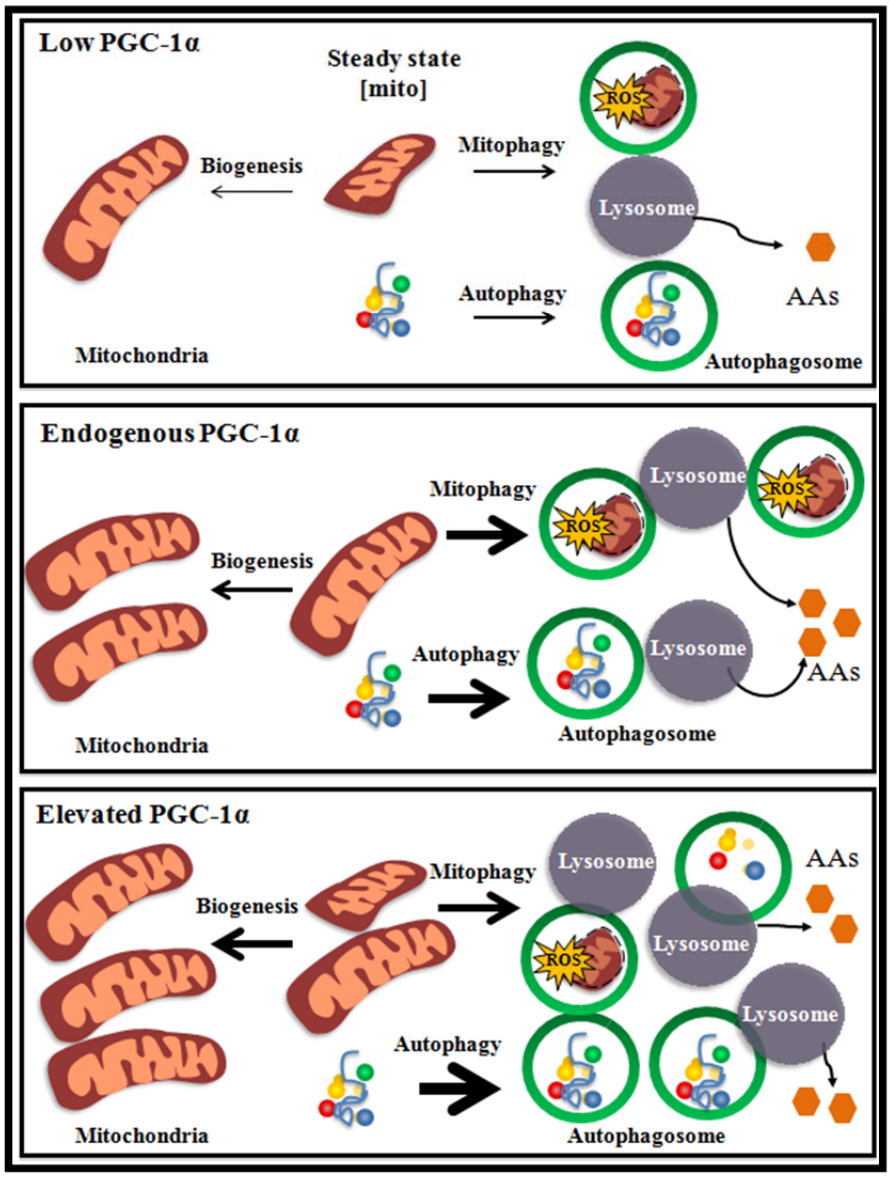
1.5
1.0
0.5
0.0
Con
Den
*
†
WT
Tg

Fig.11

A


Mitochondrial function (——)
Mitophagy Flux (– – –)
KO
WT
Tg
PGC-1α content

B


Low PGC-1α
Steady state [mito]
ROS
Biogenesis
Mitophagy
Lysosome
Autophagy
AAs
Mitochondria
Autophagosome
Endogenous PGC-1α
Mitophagy
Lysosome
ROS
Biogenesis
Autophagy
AAs
Mitochondria
Autophagosome
Elevated PGC-1α
Mitophagy
Lysosome
AAs
Biogenesis
ROS
Autophagy
Mitochondria
Autophagosome

Supplementary Figures For

PGC-1α contributes to denervation-induced mitophagy in skeletal muscle

Anna Vainshtein[1], Eric M. Desjardins[1], Andrea Armani[2], Marco Sandri[2,3,4], and David A. Hood[1]

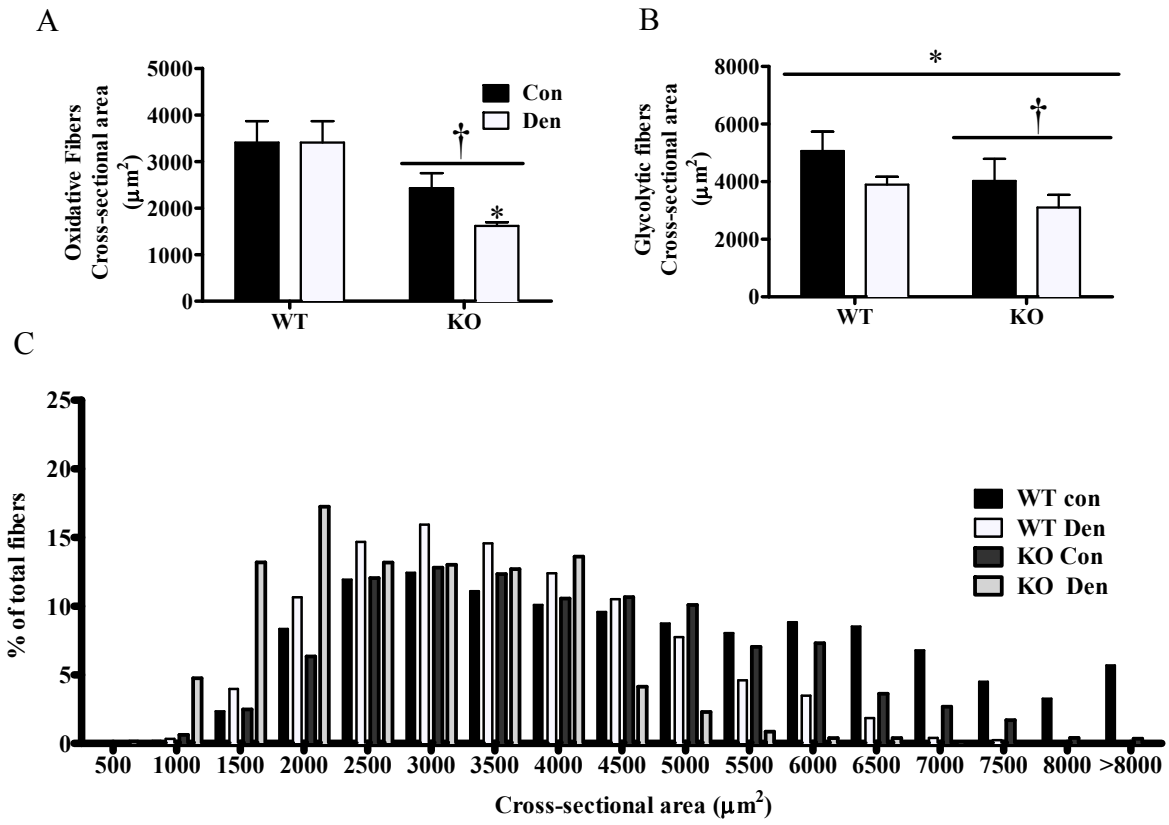


Fig.S1 PGC-1α KO animals have lower muscle cross-sectional area . A-C. TA muscle fiber cross sectional area of WT KO Con and Den was measured from serial cross sections stained with SDH. A. Average size of oxidative fibers. B. Average size of glycolytic fibers. C. Fiber size distribution . *P<0.05, significant effect of denervation .† P<0.05, significant effect of genotype (n=4 for all groups).

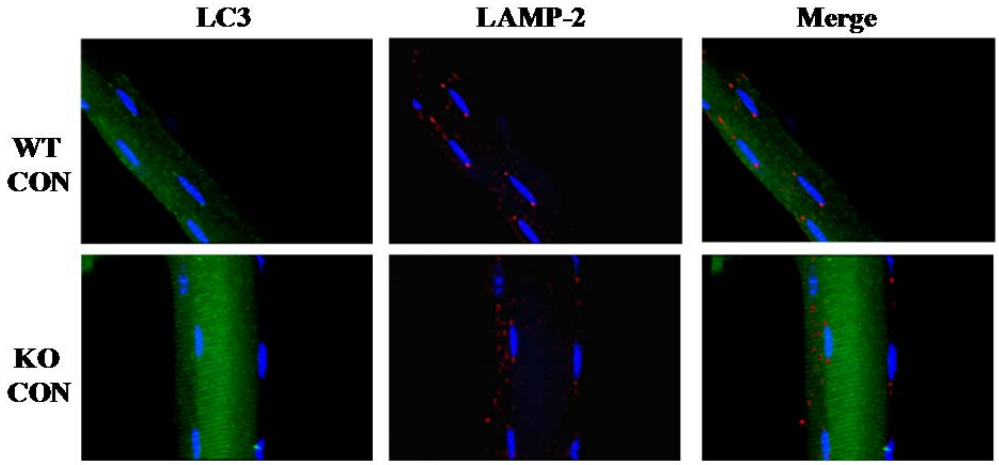


Fig.S2 Confocal images of fixed single fibers immuno-stained for LC3 (green) and lysosomal Lamp-2 (red). Colocalization is shown in yellow (Merge) which represents autophagosomes within lysosomes. Nuclei are in blue (Dapi).

Fig.S3

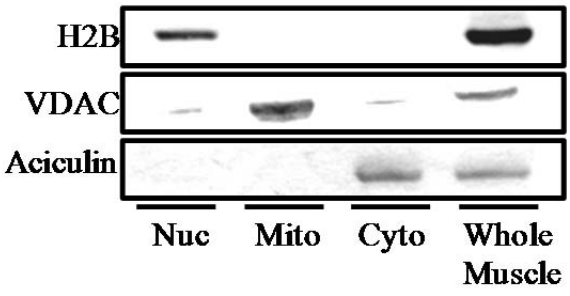


Fig S3. Fraction purity. Representative blots demonstrating the quality of cellular fractionation in nuclear (Nuc), mitochondrial (Mito), cytosolic (Cyto) cellular subfractions as well as whole muscle lysates, for positive control. H2B is a nuclear factor, VDAC is a mitochondrial protein and Aciculin is a cytosolic protein. Equal amounts of protein were loaded for each.

Fig.S4

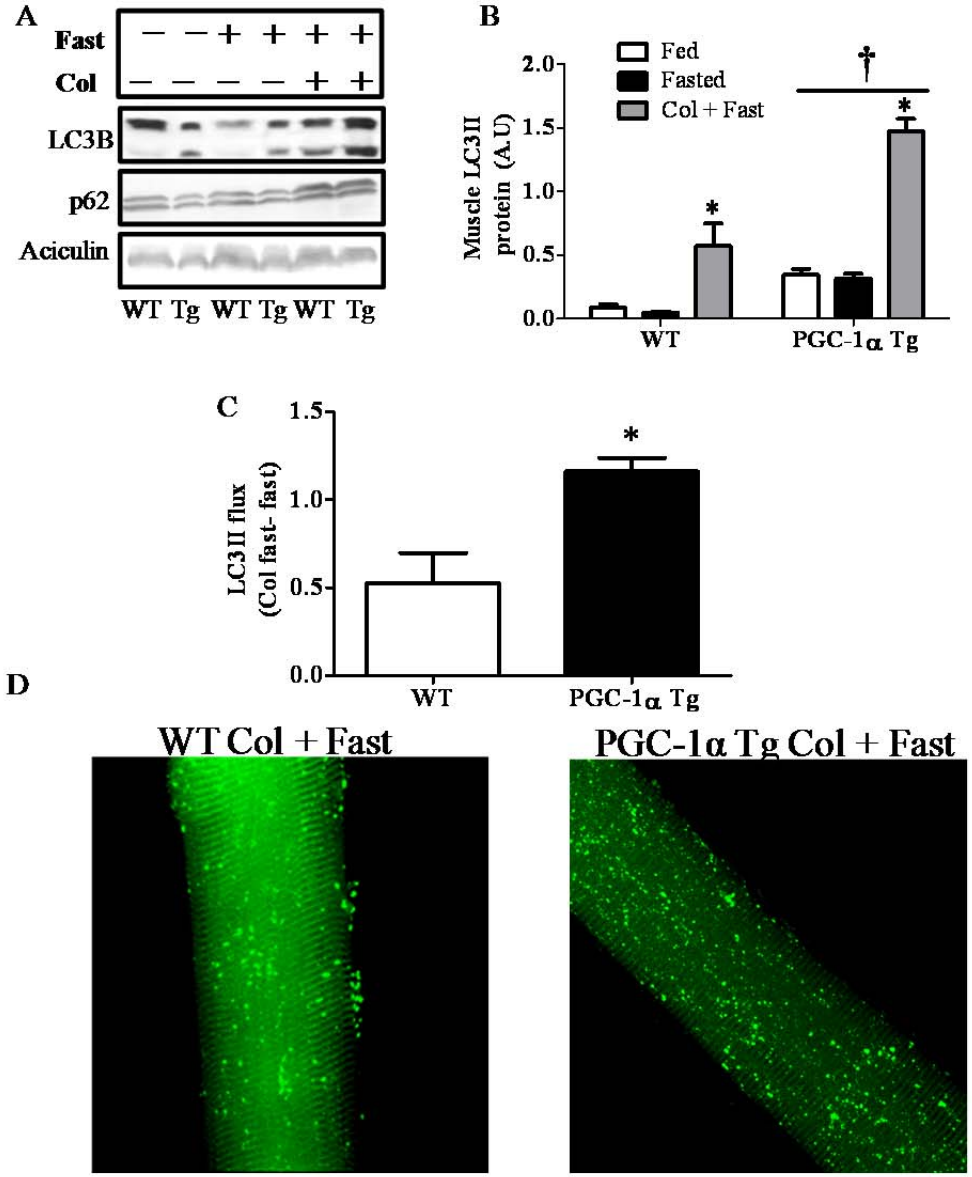


Fig. S4: Autophagy protein expression and flux. A) Representative blots of autophagic proteins and B-C) quantification of protein expression and autophagy flux in WT and PGC-1αTg animals that were fed (Control) or fasted (24h), with or without colchicine (Col) treatment; B) LC3II; C) autophagy flux. GAPDH was used as loading control. D) Representative images of single fibers isolated from FDB muscle transfected with LC3-YFP (Green) showing increased fasting-induced autophagsome accumulation in PGC-1αTg treated with colchicine as compared to WT. *P<0.05 significant effect of treatment. †P<0.05 significant effect of genotype. Aciculin was used as a loading control (n=3 for all groups).

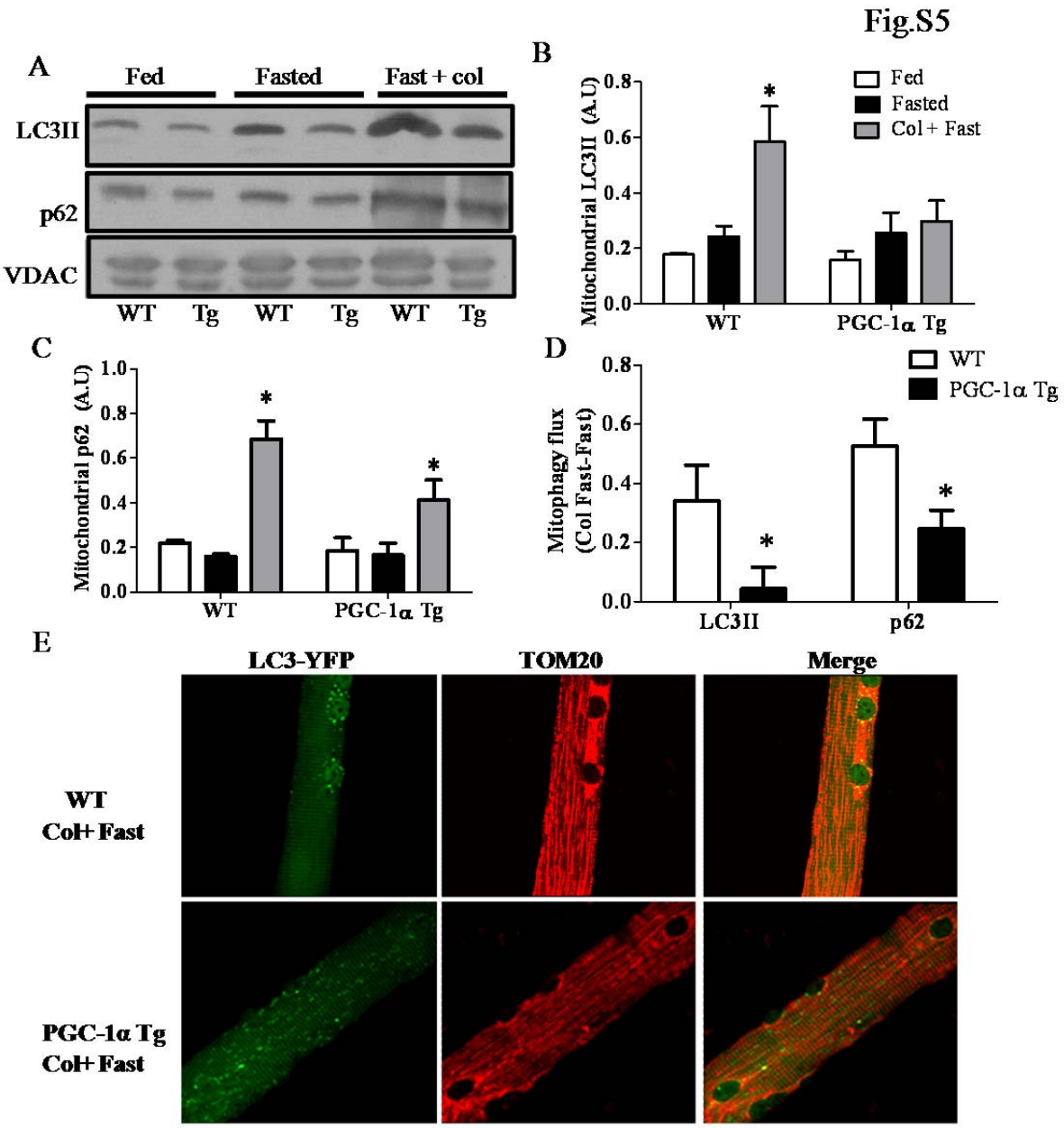


Fig. S5: Nutrient deprivation-induced Mitophagy flux is reduced in Tg animals A) Representative blots of autophagic proteins on isolated mitochondria in WT and PGC-1αTg animals that were fed or fasted, with or without colchicine (Col) treatment; B-C) quantification of protein expression and autophagy flux; B)LC3II; C)p62; D) mitophagy flux. VDAC was used as loading control. E) Single fibers isolated from the FDB muscle of animals that were fasted and treated with colchicine. Muscle was transfected with LC3-YFP (green) and immunostained for mitochondrial marker TOM20 (Red). Colocalization of LC3 with TOM20 (yellow) represents mitochondria within autophagosomes, which are more prevalent in WT animals when compared to Tg. *P<0.05 significant effect of treatment. †P<0.05 significant effect of genotype (n=3 for all groups).

Table S1. Primer sequences based on gene transcripts available in GenBank.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Coxiv	CTCCAACGAATGGAAGACAG	TGACAACCTTCTTAGGGAAC
Mul1	GCCTTGCTGATGTAGTTGTCTG	CAGGCCCAGCTTATGATAGAA
Bnip3l	GGAAAGCGGCACAGAGAA	GAATGACGCCAGTGCTGAT
Park2	GTCTGCAATTTGGTTTGGAGTA	GCATCATGGGATTGTCTCTTAAA
Sqstm1	TGTGGTGGGAACTCGCTATAA	CAGCGGCTATGAGAGAAGCTAT
Maplc3b	GCTTGCAGCTCAATGCTAAC	CCTGCGAGGCATAAACCATGTA
Atg7	TTTCTGTCACGGTTCGATAATG	TGAATCCTTCTCGCTCGTACT
Lamp2	GCTGAACAACAGCCAAATTA	CTGAGCCATTAGCCAAATACAT
Catsd	TTTGCCAATGCTGTCGTACT	AGCGAGTGTGACTATGTGTGAG
Foxo3	ATGGACGACCTGCTGGATAAC	GGAGCTCTTGGCGGTATATG
Actb	TGTGACGTTGACATCCGTAA	GCTAGGAGCCAGAGCAGTAA
Gapdh	AACACTGAGCATCTCCCTCA	GTGGGTGCAGCGAACTTTAT

Table S2. Antibodies list

Antibody	Manufacturer	Product number
Goat anti-Rat Alexa Flour® 647	Life Technologies	A-21247
Goat anti-Rabbit Alexa Fluor® 488	Life Technologies	A-11034
Atg7	Sigma-Aldrich	A2856
Beclin1	Cell Signaling Technology	3738
Cathepsin D	Santa Cruz Biotechnology	Sc6486
COXIV	Abcam	Ab14744
GAPDH	Abcam	Ab8245
Histone-2B/H2B	Cell Signaling Technology	2934
Lamp-2	Abcam	Ab13524
LC3B/Maplc3b	Cell Signaling Technology	2775
NIX/Bnip3l	Abcam	Ab109414
p62/Sqstm1	Sigma-Aldrich	P0067
Parkin	Cell Signaling Technology	4211
PGC-1α/Ppargc1a	Millipore Corporation	AB3242
T-eIF2α	Cell Signaling Technoogy	9722
TFEB	MyBioSource Inc	MBS120432
Ubiquitin (Ub)	Enzo Life Sciences	ADI-SPA-203
VDAC/Porin	Abcam	14734

Fig.S6

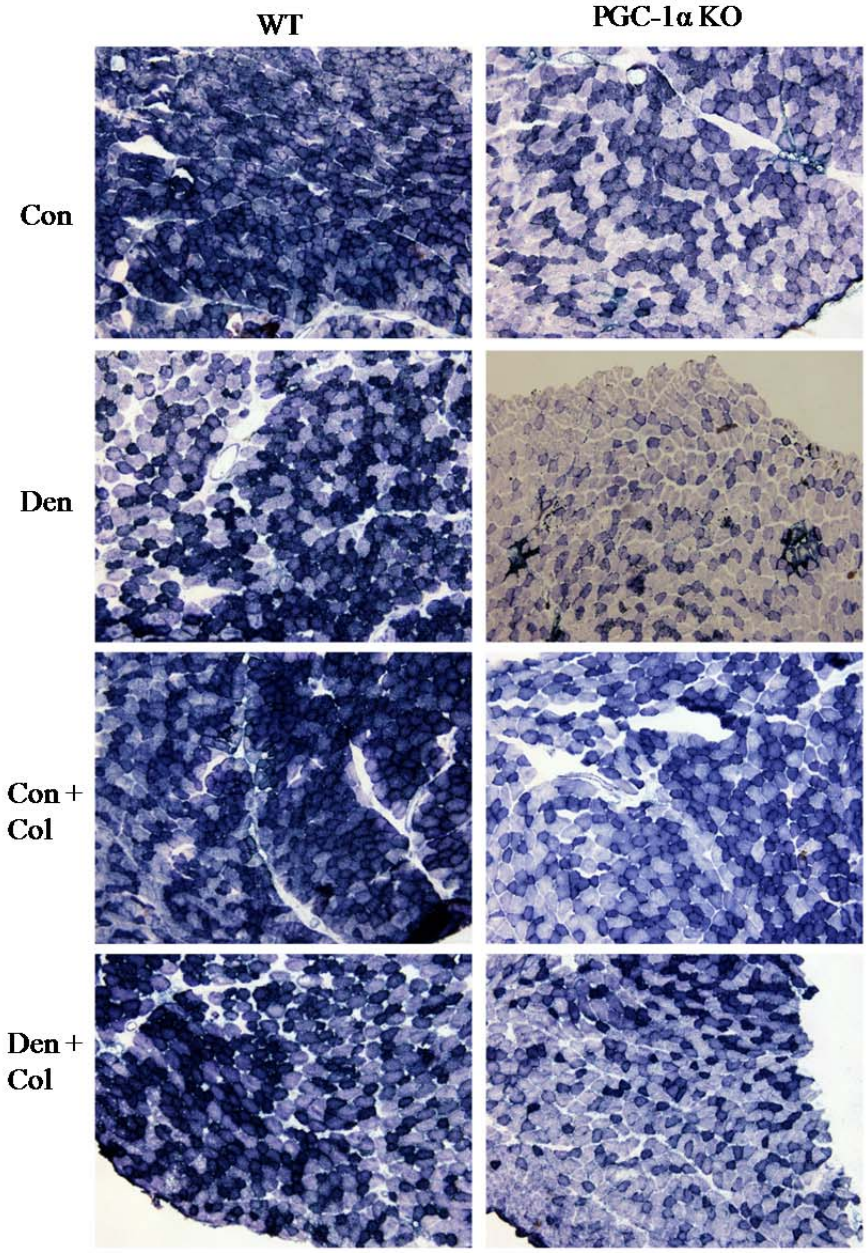


Fig. S6: Animal model characteristics. SDH staining of TA muscle cross sections from WT PGC-1α KO Control (Con) and Denervated (Den) both treated vehicle with Colchicine (Col).

Fig.S7

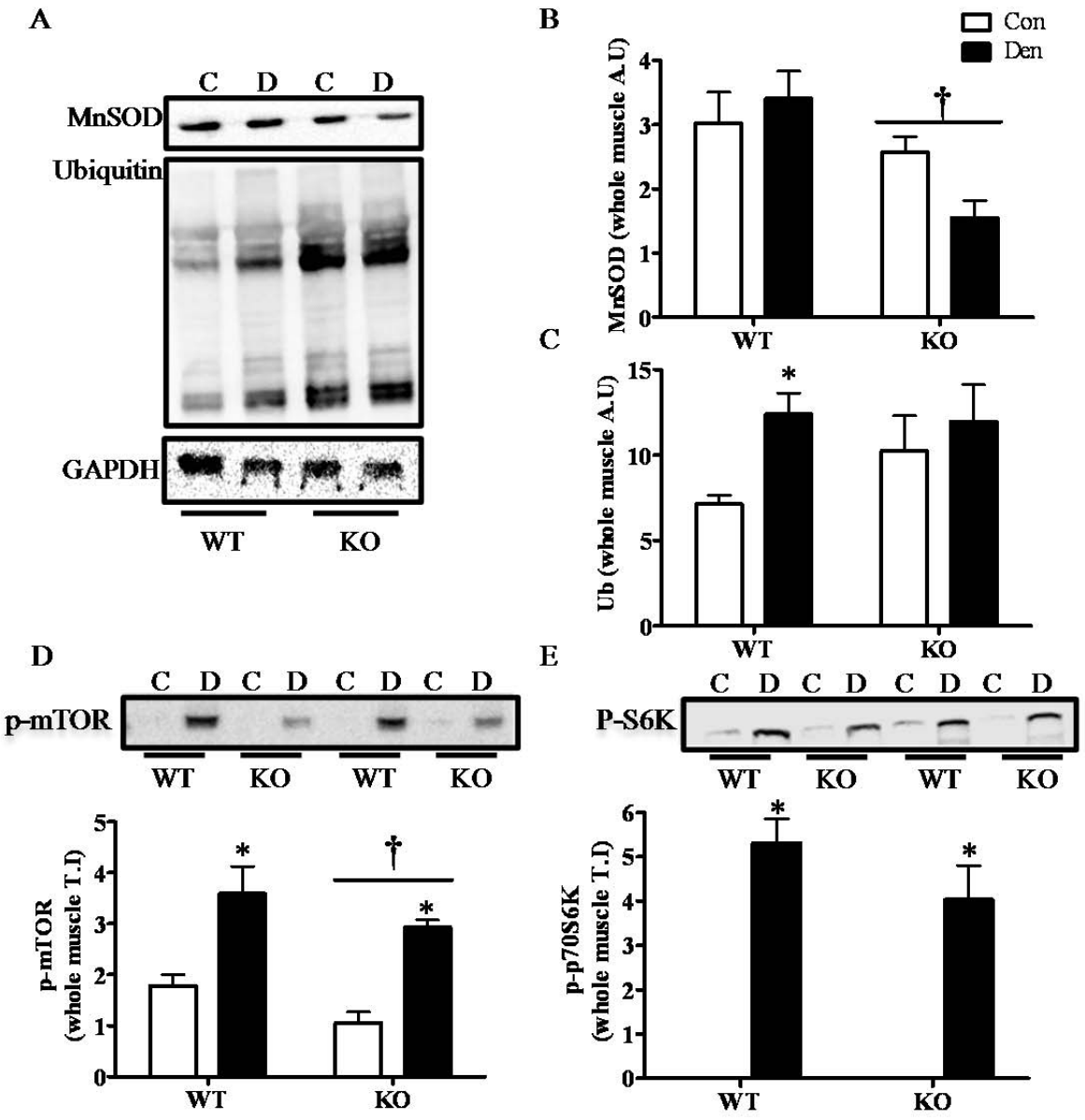


Fig. S7: Whole muscle protein measures. A-E. Blots and quantification of whole muscle protein expression in TA muscle in Con, Den, WT and KO animals. A. Representative blots. Quantification of B. MnSOD; C. Ubiquitin; D-E. Representative blot and quantification of D. p-mTOR; E.p-S6K. *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH was used as a loading control (n=4-8 for all groups).

Fig.S8

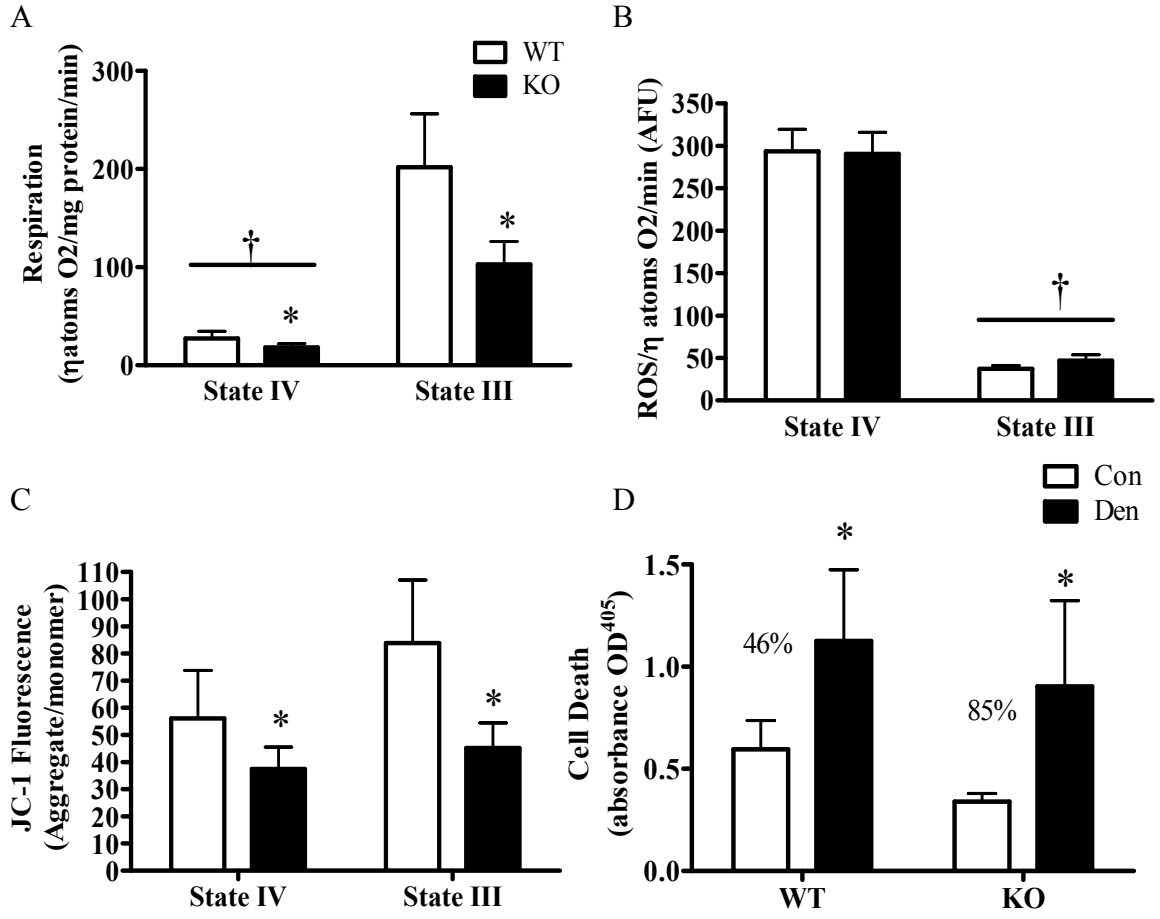


Fig. S8: Mitochondrial Function and cell death. A. State III and state IV respiration measured in mitochondria isolated from WT and KO animals. B. State III and state IV ROS productions in mitochondria isolated from WT and KO animals. C Mitochondrial membrane potential measured by flow cytometry, State IV and state III in WT and KO animals. D. Apoptotic cell death as measured by DNA fragmentation using a cell death ELISA in Con and Den WT and KO animals. *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. (n=4-8 for all groups).

Fig.S9

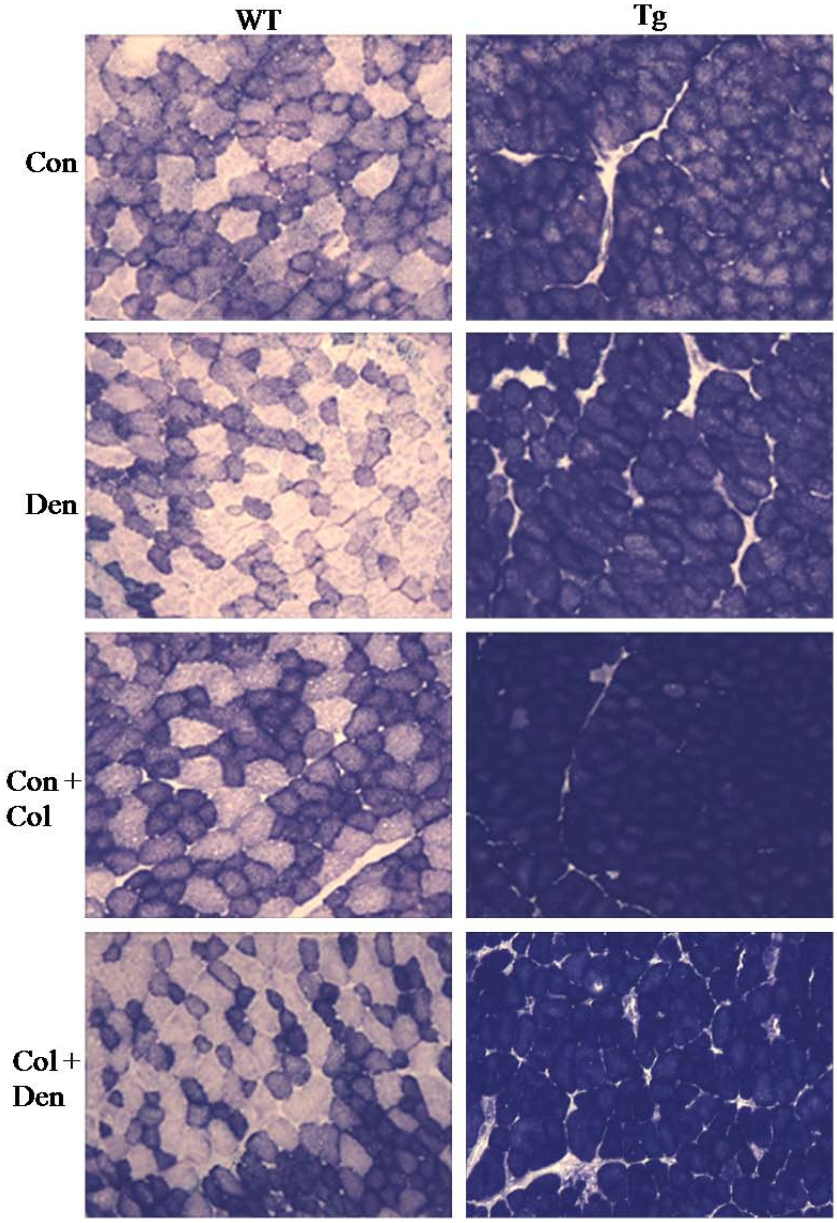


Fig. S9: Animal model characteristics. SDH staining of TA muscle cross sections from WT, PGC-1α Tg Control (Con) and Denervated (Den) both treated vehicle with Colchicine (Col).

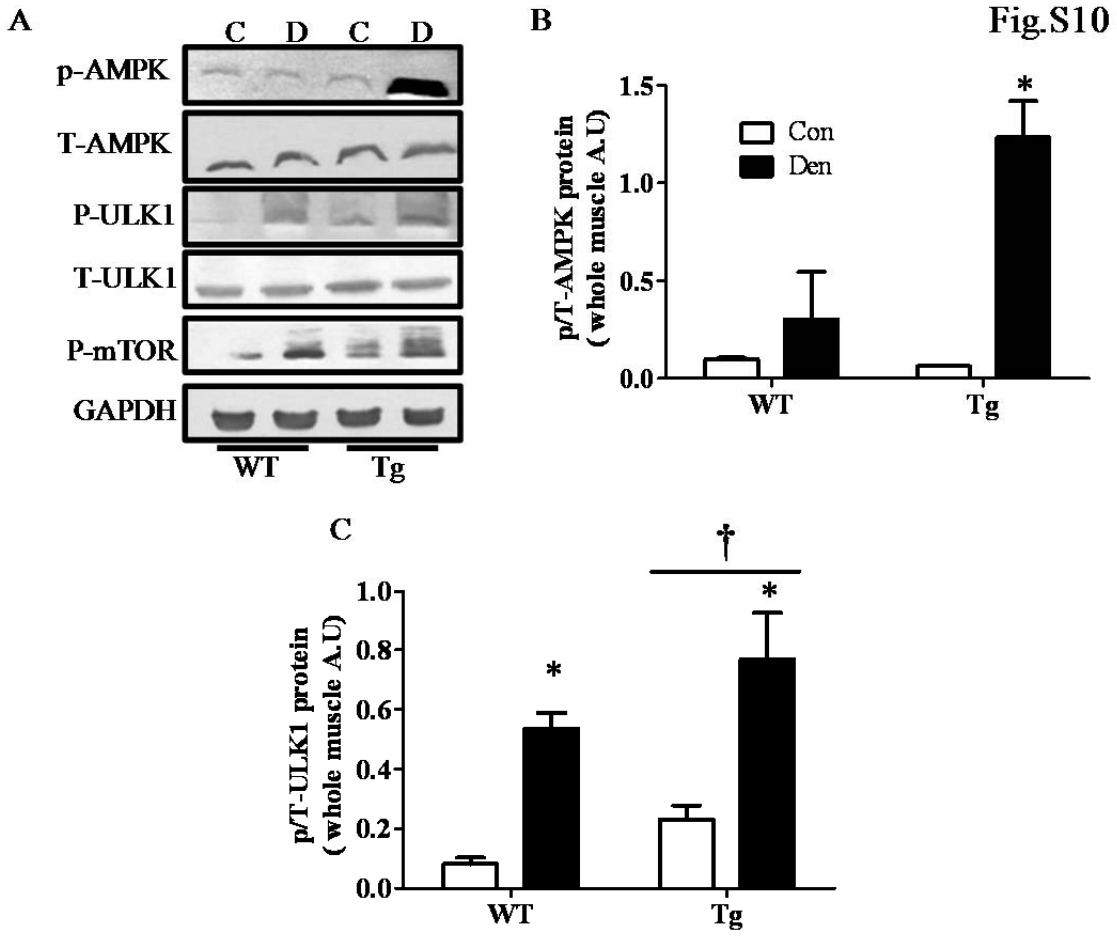


Fig. S10: Whole muscle protein measures. A-C Blots and quantification of whole muscle protein expression in TA muscle in Con, Den, WT and Tg animals. A. Representative blots. Quantification of B. p/T-AMPK; C. p/T-ULK1. *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH, and total protein were used as a loading controls where appropriate (n=3-4 for all groups).

Fig.S11

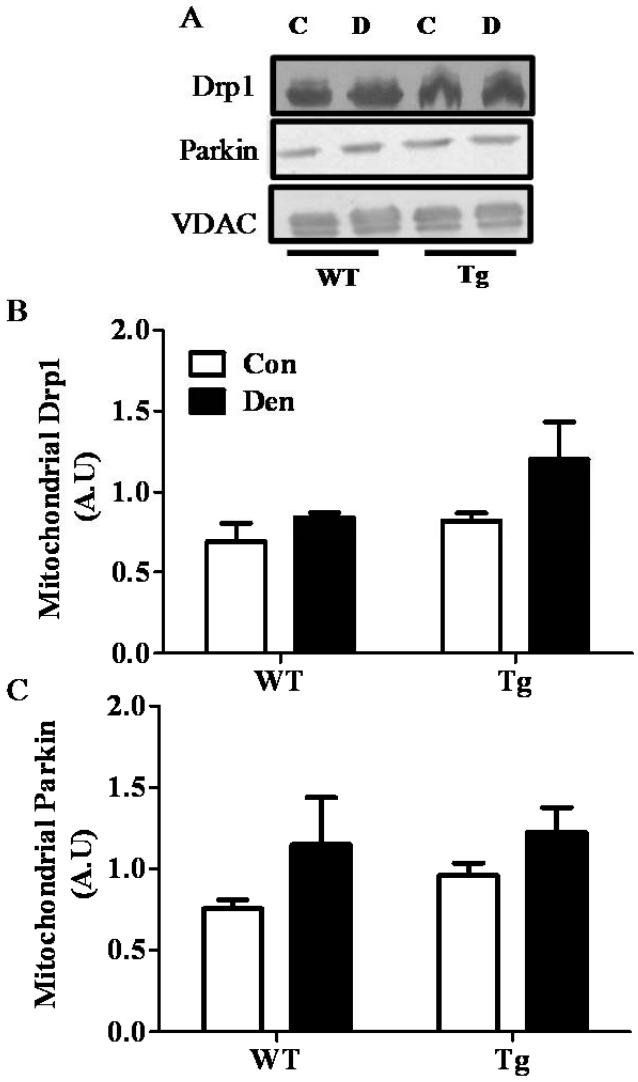


Fig. S11: Proteins on isolated mitochondria. A-C Blots and quantification of proteins on isolated mitochondria. A. Representative blot and quantification of B. Drp1; C. Parkin .VDAC was used as a loading control (n=3-4 for all groups).

Fig.S12

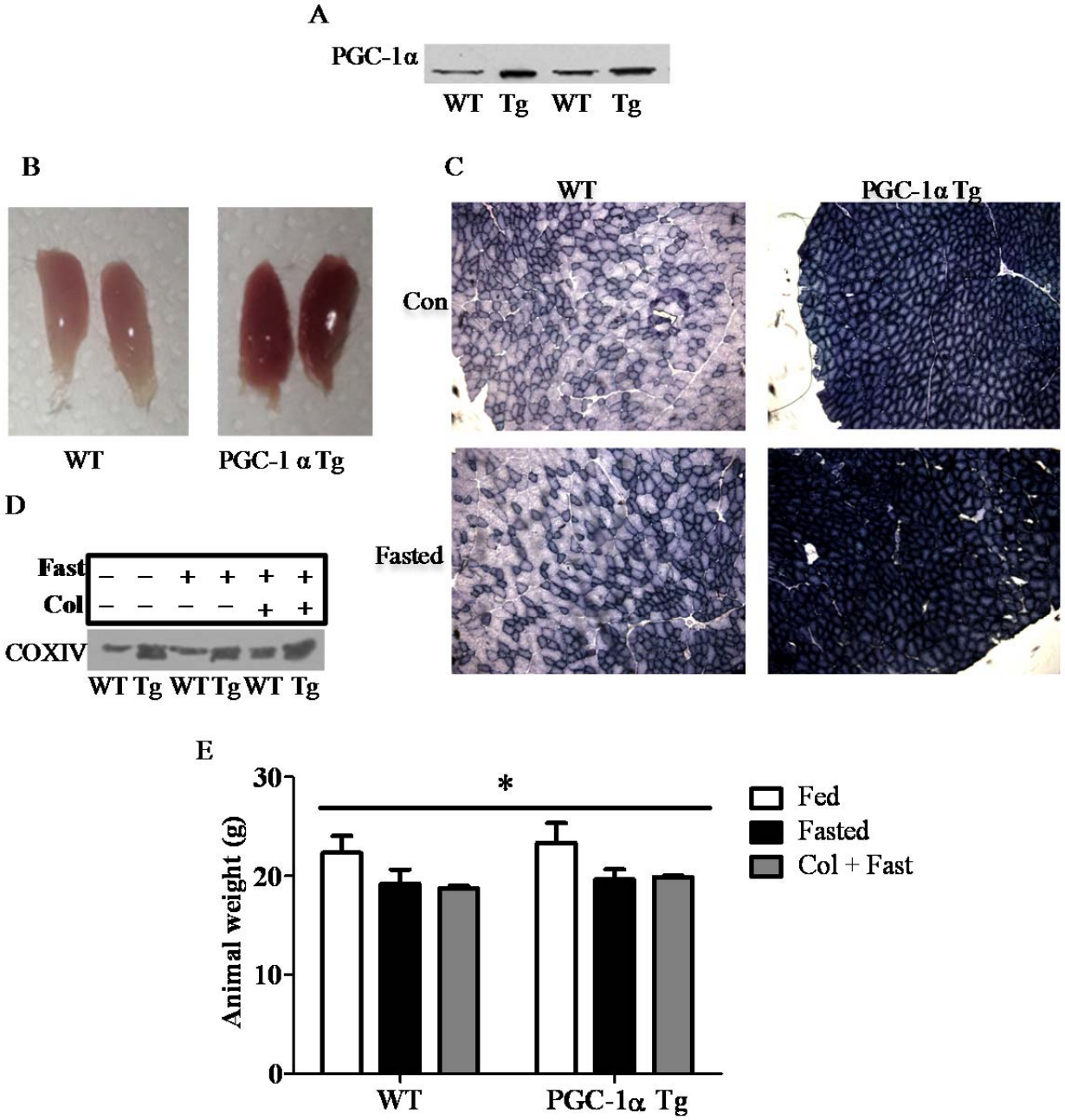


Fig S12: Animal model characteristics. A) Representative blot of PGC-1α protein confirming its over expression in PGC-1α Tg animals. B) Representative pictures of TA muscle from WT and PGC-1α Tg animals. C) SDH staining of TA muscle cross sections. D) COXIV protein content as a marker of mitochondrial abundance. E) Animal weight in grams. *P<0.05 main effect of fasting

Fig.S13

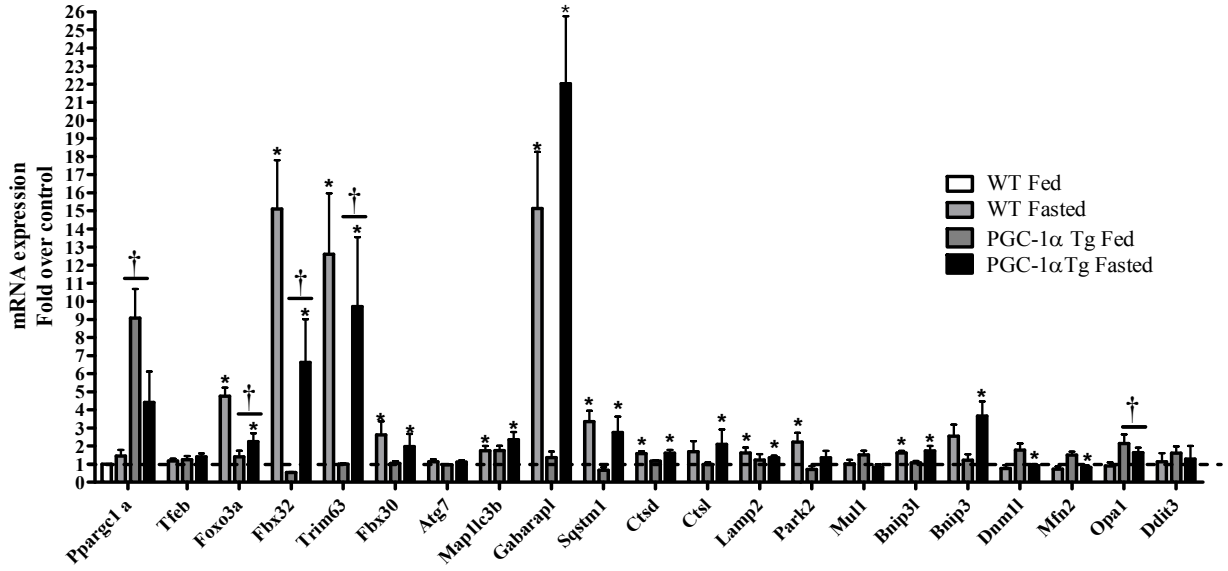


Fig. S13: Gene expression measured by real time PCR. mRNA fold change between wild type (WT) and PGC-1α Tg fed and fasted (24h). All groups were compared to WT Con, and Gapdh and Actb were used as housekeeping genes *P<0.05 significant effect of fasting (24h). †P<0.05 significant effect of genotype(n=3 for all groups).

Fig.S14

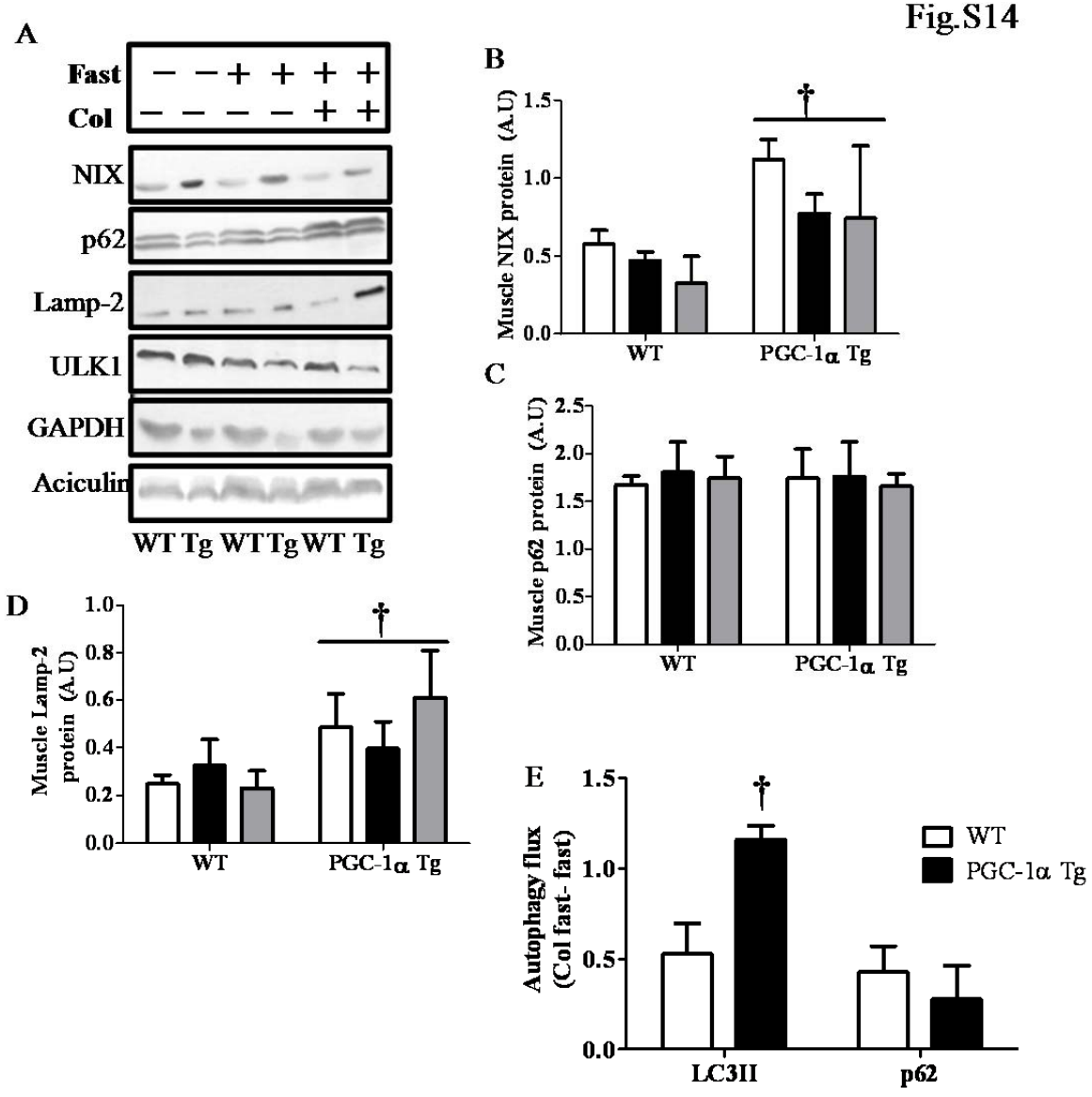


Fig S14: Autophagy protein expression and flux. A) Representative blots of autophagic proteins and B-F) quantification of protein expression and autophagy flux in WT and PGC-1αTg animals that were fed (Control) or fasted, with or without colchicine (Col) treatment; B) NIX; C) p62; D) Lamp-2; E) autophagy flux. GAPDH was used as loading control. *P<0.05 significant effect of treatment. †P<0.05 significant effect of genotype. GAPDH was used as a loading control (n=3 for all groups).

Fig.S15

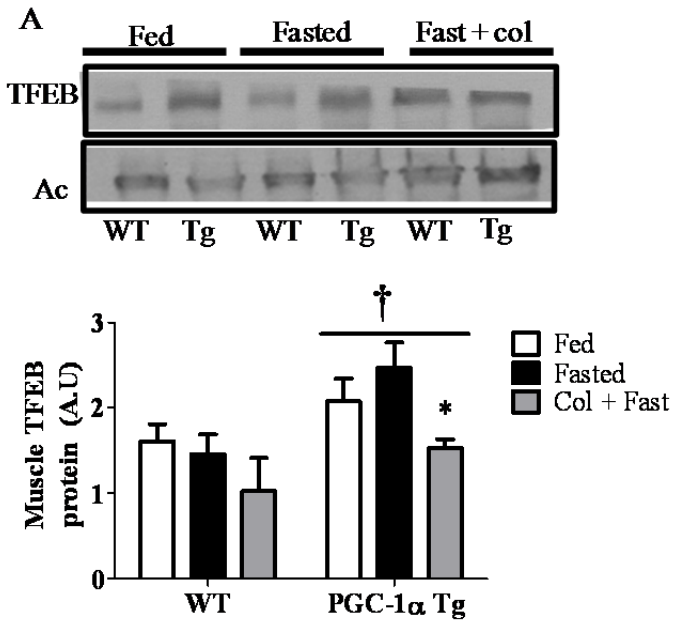


Fig. S15: Whole muscle TFEB. Representative blot and quantification of whole muscle TFEB protein levels in WT and PGC-1αTg animals that were fed or fasted, with or without colchicine (Col) treatment. Aciculin was used as loading control. *P<0.05 significant effect of treatment. †P<0.05 significant effect of genotype (n=3 for all groups).

CHAPTER 5:

The role of PGC-1α during acute exercise-induced autophagy and mitophagy in skeletal muscle

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Running title: PGC-1α, exercise and autophagy in muscle

Abbreviations:
AMPK, AMP-activated knase; Atg7, autophagy related 7; Con, control; COX, cytochrome oxidase; COXIV, cytochrome oxidase subunit IV; EDL, extensor digitorum longus; Ex, exercise; Ex+R, exercise and recovery; FOXO3, Forkhead box O3; KO, Knockout; Lamp-2, lysosome associated membrane protein 2; LC3/Maplc3, microtubule-associated protein 1 light chain 3; Nix/Bnip3l, Bcl-2/adenovirusE1B 19kDa interacting protein 3-like; NPC1, Niemann-Pick C1; p38 MAPK, p38 mitogen activated protein kianse; p62/Sqstm1, sequestosome 1; Parkin/park2, RBR E3 ubiquitin protein ligase; PGC-1α, Peroxisome Proliferator co-activator 1 alpha; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TA, tibialis anterior; Tfam, mitochondrial transcription factor A; TFEB, Transcription factor EB; Tg, transgenic; WT, wild type.

Abstract

Regular exercise leads to systemic metabolic benefits which require the remodeling of energy resources in skeletal muscle. During acute exercise the increase in energy demands initiate mitochondrial biogenesis, orchestrated by the transcriptional co-activator PGC-1α. Much less is known about the degradation of mitochondria following exercise, although new evidence implicates a cellular recycling mechanism, autophagy/mitophagy, in exercise-induced adaptations. How mitophagy is activated, and what role PGC-1α plays in this process during exercise, has yet to be evaluated. Thus, we investigated autophagy/mitophagy in muscle immediately following an acute bout of exercise (Ex), or 90 min following exercise (Ex+R) in wild type (WT) and in PGC-1α knockout animals (KO). Deletion of PGC-1α resulted in a 40% decrease in mitochondrial content, as well as a 25% decline in running performance which was accompanied by severe acidosis in KO animals, indicating metabolic distress. Ex induced significant increases in gene transcripts of various mitochondrial (e.g COXIV, Tfam) and autophagy-related genes (e.g. p62, LC3) only in WT, but not in KO animals. Exercise also resulted in enhanced targeting of mitochondria for mitophagy, as well as increased autophagy and mitophagy flux in WT animals. This effect was attenuated in the absence of PGC-1α. We also identified NPC1, a transmembrane protein involved in lysosomal lipid trafficking, as a target of PGC-1α that is induced with exercise. These results suggest that mitochondrial turnover is increased following exercise, and that this effect is at least in part co-ordinated by PGC-1α.

Introduction

The merits of physical activity are numerous and well documented. Regular exercise leads to improved cardiovascular health, cognition, metabolism, oxidative capacity, and fatigue resistance, among many other beneficial consequences. Moreover, increased contractile activity is also protective of muscle mass and function under a myriad of pathologies. Although alterations in skeletal muscle metabolism, as well as its secretome, have been documented to contribute to the pleiotropic benefits of regular exercise, the molecular mechanism underpinning these adaptations remain unclear.

Skeletal muscle possesses a remarkable capacity to adapt to alterations in contractile activity, a property referred to as muscle plasticity. This type of cellular remodeling often requires a shift in metabolic profile, with amendments to the structure of the mitochondrial network, as well as changes in mitochondrial content. Organelle density is determined by the balance between its synthesis and degradation. Mitochondrial biogenesis is regulated transcriptionally through the coordinated expression of nuclear and mitochondrial genes, orchestrated largely by the transcriptional co-activator peroxisome proliferator gamma coactivator-1α (PGC-1α) (18, 41). Conversely, organelle degradation is mediated by a selective form of macroautophagy known as mitophagy (13, 15). During mitophagy, dysfunctional mitochondria are first segregated from the healthy network by fission and are then tagged for elimination (14, 37). Mitochondrial targeting can occur either via mitophagy-specific receptors such as Nix and Bnip3 (14, 26, 30), or by the ubiquitination of mitochondrial outer membrane proteins by E3 ligases such as Parkin and Mul1 (4, 21, 25). Tagged mitochondria are then recognized by, and engulfed into, double membrane vesicles termed autophagasomes. Autophagosomes are subsequently delivered to the lysosome for proteolytic degradation.

Several recent studies have indicated that autophagy is activated following an acute bout of endurance exercise (5, 6, 8–10, 20, 22) and this may contribute to chronic exercise-induced improvements in muscle health and oxidative capacity. Indeed, deficient autophagy results in lack of exercise-mediated metabolic benefits, as well as progressive degeneration in mitochondrial function and performance (6, 20, 22). Coincidentally, PGC-1α levels have also been demonstrated to increase following an acute bout of endurance exercise (2), and PGC-1α is localized to the nucleus during the post exercise recovery period. The significance of this is that absence of PGC-1α also results in diminished exercise-induced metabolic benefits (2, 16). However, it is currently unknown whether PGC-1α plays a role in acute exercise-induced autophagy, or in the regulation of mitophagy flux. Thus, we set out to investigate the function of PGC-1α in mediating autophagy and mitophagy induction in skeletal muscle following an acute bout of exercise. Our results should shed light on how the mitochondrial biogenesis and degradation pathways may interact to ensure proper mitochondrial remodeling, and thus contribute to muscle plasticity as a result of exercise.

Materials and Methods

Animal generation, treatment and exercise

PGC-1α whole body KO animals and C57BL/6 wild-type (WT) were housed in a 12:12-h light-to-dark cycle, and given food and water *ad libitum*. The generation and characterization of PGC-1α KO mice have been previously described (1, 19). To evaluate autophagy flux, animals were treated with either colchicine or an equal volume of vehicle (water) through an intraperitoneal injection every 24 hours at a dose of 0.4mg/kg/day (12) for 2 days prior to the day of exercise/ sacrifice, with the final injection taking place 24h prior to sacrifice. After two days of habituation to the treadmill and colchicine or vehicle injections (water; Veh), the EX and Ex+R groups were run to failure on a treadmill (Fig. 1C). The exercise bout was terminated when the animals could

not continue running after 3 consecutive shocks. Animals were sacrificed either immediately (Ex) or 90 min post exercise (Ex+R). Muscle was then excised, and either immediately frozen in liquid nitrogen for later use in protein or gene expression analysis, or used fresh for cellular fractionation. All procedures involving animals were approved and conducted in accordance with the regulations of the York University Animal Care Committee in compliance with the guidelines set forth by the Canadian Council on Animal Care.

Blood Lactate

Blood lactate was measured prior to, immediately post-exercise, and following 90 min of recovery. A 0.2μL blood sample was obtained by tail nick and immediately analyzed using the Lactate Scout+ analyser (EKF-diagnostic GmbH, Magdeburg, Germany).

Histology

Cytochrome oxidase (COX) and succinate dehydrogenase (SDH) staining was performed on 10-μm cross sections of digitorum longus (EDL) muscles as previously described (24). Briefly, frozen muscle sections adhered to glass slides were dried and subsequently incubated with either COX or SDH reaction solutions for 30 min in the dark at 30 °C. Each slide contained sections from all conditions, in order to ensure equal staining across the groups. After washing with PBS, glass cover slips were mounted onto the slides with DPX Mountant for histology (Fluka, 44581) and sealed. Images of stained muscle sections were captured with a Nikon 90i eclipse upright microscope using a ×20 objective.

COX activity

Cytochrome c oxidase (COX) enzyme activity was measured as previously detailed (38) by determining the rate of oxidation of fully reduced cytochrome *c* by isolated enzymatic extract, evaluated as a change in absorbance at 550 nm using a microplate reader (Bio-Tek Synergy HT).

Gene expression analysis

Quantitative real-time PCR was performed to determine mRNA expression levels. Total RNA was isolated using TRIzol reagent (Invitrogen, 15596-026). RNA was reverse transcribed into cDNA using a Superscript III first strand synthesis kit (Invitrogen, 18080-044) according to manufacturer instructions. The primers used for gene expression analysis are listed in Table 1 and were designed based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi). Analyses were performed with SYBR® Green chemistry (PerfeC$_T$a SYBR® Green Supermix, ROX, Quanta BioSciences, 95055-500) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). *Gapdh* and *Actb* were used in combination as housekeeping genes.

mRNA array

The gene expression of 84 key autophagic genes were profiled by RT^2 Profiler Autophagy PCR Arrays (SABioscience, PAMM-084) as recommended by the manufacturer. RT-PCRs were performed in 96-well plate format using StepOnePlus™ Real-Time PCR System. Fold changes in autophagic gene expression from KO samples relative to WT control samples were calculated using the $\Delta\Delta C_t$ method with the integrated software package for PCR Array Systems provided by the manufacturer (RT^2 Profiler PCR Array Data Analysis Template v3.3). $\Delta\Delta C_t$ values from each sample were normalized by three housekeeping genes that did not change across the conditions.

Immunoblotting

Protein extracts from frozen TA cryosections (22), isolated mitochondria, or nuclear extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were blocked with 5% skim milk or 5% BSA solution. Membranes were incubated overnight at 4°C with the

appropriate concentration of primary antibody. The following antibodies were used: P-AMPK (Thr 172), T-AMPK, P-p38 (Thr 180/Tyr 182), T-p38, LC3, and Parkin (Cells Signaling Technology), GAPDH, VDAC and NPC1 (Abcam), DRP-1 (BD Transduction Laboratories), p62 (Sigma-Aldrich), PGC-1α (Millipore Corporation), and Ubiquitin (Enzo Life Sciences). Membranes were subsequently washed and incubated with the suitable HRP-conjugated secondary antibody for 1h at room temperature, and visualized with enhanced chemiluminescence. Quantification was performed with Image J Software (NIH, Bethesda, MD, USA) and values were normalized to the appropriate loading control.

Autophagy and Mitophagy flux calculation

Autophagy flux was calculated by the Δ change in LC3II and p62 protein levels in whole muscle extracts, between colchicine treated and untreated animals of the same condition and genotype. Where Δ change = (colchicine treated-Vehicle treated)/Vehicle treated.

Mitophagy flux was calculated by the Δ change in LC3II and p62 protein levels in isolated mitochondria, between colchicine treated and untreated animals of the same condition and genotype.

Cellular Fractionation

Enriched mitochondrial and nuclear cellular subfractions were isolated by differential centrifugation, as previously described (38). Briefly, muscles were minced on ice and homogenized using a Teflon pestle and mortar, and suspended in mitochondrial isolation buffer (MIB; 250 mM Sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) supplemented with protease (Complete, Roche, 1169749801) and phosphatase inhibitor cocktails (Cocktail 2 and 3, Sigma, P5726 and P0044). The homogenates were centrifuged at 1,000 g for 10 min at 4 °C to pellet the nuclei while mitochondrial and cytosolic fractions were

contained within the supernate. The supernate fraction was re-centrifuged at 16,000 g for 20 min at 4 °C to pellet the mitochondria. The mitochondrial pellet was washed twice and resuspended in a one-fold dilution of MIB. Mitochondria were subsequently sonicated 3x3sec to yield the enriched mitochondrial fraction. Pellets containing nuclei were re-suspended in nuclear lysis buffer (1.5 mM $MgCl_2$, 0.2 mM EDTA, 20mM HEPES, 0.5M NaCl, 20% glycerol, 1% Triton-X-100), incubated on ice for 30min, and then sonicated 3x10 sec followed by a final centrifugation step of 15min at 16,000g. The supernate was collected to obtain the enriched nuclear fraction. Protein concentrations within the samples were determined using the Bradford method. Fraction purity was determined by Western blot analysis of each fraction for the loading controls of all fractions (Fig.S3).

Statistics

Comparisons between WT and KO, Con, Ex and Ex+R animals were evaluated using two-way analyses of variance (ANOVA) on each of the treatment conditions. Bonferroni post-tests were performed when applicable. All values represent the mean ± S.E. Data were considered statistically different if $P < 0.05$.

Results

PGC-1α KO animals exhibit diminished mitochondrial content, reduced endurance capacity and metabolic stress with exercise. Deletion of PGC-1α resulted in significantly lower mitochondrial content in skeletal muscle, as demonstrated by reduced succinate dehydrogenase (SDH) as well as cytochrome oxidase (COX) staining, and a ~40% decrease in COX activity (Fig.1 A-B). To examine the involvement of PGC-1α in exercise-induced autophagy, 3 month old PGC-1α knockout (KO) and wild type (WT) animals were subjected to an acute bout of incremental treadmill running (Fig.1C-E). The lack of PGC-1α resulted in

diminished endurance performance, as the KO animals ran significantly less when compared to their WT counterparts (Fig. 1D). Moreover, KO animals exhibited a ~40% higher blood lactate basally, which increased by 3.8-fold with exercise, compared to a 2.8-fold increase in WT animals (Fig.1E). We also noted that the blood lactate of KO animals did not return back to baseline as effectively as that of WT animals during the 90 min recovery period. This indicates increased metabolic stress and a greater reliance on glycolysis in the KO animals. Confirmation of metabolic stress was evident from the changes in the phosphorylation of the metabolically sensitive p38 and AMPK kinases. Exercise resulted in a ~2.5-fold increase in the phosphorylation of AMPK in WT animals, while KO animals exhibited a ~4-fold induction (Fig 2A-B). Phosphorylation of p38 MAPK was elevated by ~50%, with exercise, in both WT and KO animals. However, KO animals expressed higher levels of phosphorylated p38 overall, which did not return to baseline during recovery (Fig. 2A, C).

PGC-1α localization to the nucleus and gene expression are elevated in response to exercise resulting in the induction of mitochondrial biogenesis in WT animals. Exercise induced the localization of PGC-1α to the nucleus (Fig. 3A) which was accompanied by an 8.5-fold increase in PGC-1α mRNA (*Ppargc1a*) expression following recovery (Fig. 3B). The increase in PGC-1α with exercise resulted in ~60% and ~50% inductions in the expression of the downstream targets cytochrome oxidase subunit IV (*COXIV*) and mitochondrial transcription factor A (*Tfam*), which were not evident in the KO animals (Fig. 3C-D). Thus, an acute bout of exercise was sufficient to induce the onset of mitochondrial biogenesis, in a PGC-1α-dependent manner, an effect that was not observed in KO animals.

Acute exercise results in the induction of autophagy, and this process is differentially regulated in PGC-1α KO animals. During autophagy, p62 and LC3 are consumed within the

lysosome while accompanying their respective organelle targets. A continuous transcriptional contribution is, therefore, required in order to avoid exhaustion of these important autophagy factors. We observed ~80% and ~94% inductions of *Maplc3b* (LC3) and *sqstm1* (p62) transcript levels with exercise, respectively. However, this effect was abolished in KO animals (Fig 4A and B). In contrast, the transcript levels of additional autophagy (*Atg7, Becn1*) and lysosomal (*CtsD, Lamp2*) markers were not different between WT and KO animals, and did not change with exercise or recovery (data not shown). Our acute exercise protocol was sufficient to induce LC3 lipidation in the muscle of WT animals, however we did not observe this increase in the KO mice until after the recovery period, indicating a delayed response to exercise in KO animals (Fig. 4C-D). The levels of p62 did not change immediately following exercise in WT animals, but increased with recovery (Fig. 4C, E). Conversely, p62 levels were elevated both immediately after exercise and following recovery in KO animals (Fig.4 C, E). This is indicative of impaired autophagosome degradation of p62 in KO animals.

One major difficulty in evaluating autophagic flux is the extremely dynamic and transient nature of autophagosomes, since the average half-life of this organelle is about 10 min (20). Therefore, we evaluated autophagy flux by measuring the percent change in LC3II and p62 protein *in vivo* with interperitoneal administration of the microtubule destabilizing drug colchicine, as previously described (12). Colchicine blocks autophagosome degradation by destabilizing the microtubule tracks on which they travel to the lysosome for degradation, resulting in the accumulation of autophagosomes, and thus in a back-log of LC3II and p62. Interestingly, LC3II flux tended to increase immediately following exercise and after recovery in WT animals, but was lower overall and did not follow a similar trend in KO animals (Fig. 4F). We also observed a trend for an increase in p62 flux with exercise in both WT and KO animals

(Fig. 4G), however this tendency was attenuated in the KO animals. Taken together, these results indicate an induction of autophagy with exercise in WT animals that is impaired in mice lacking PGC-1α.

Mitochondrial targeting for degradation is enhanced following an acute bout of exercise, but this signaling is attenuated in mice lacking PGC-1α. In order to investigate mitophagy induction and the role of PGC-1α in this process, we measured the presence of autophagy markers in isolated mitochondrial subfractions. Exercise induced a ~5.2-fold increase in the localization of LC3II to the mitochondria in WT animals, which decreased with recovery (Fig. 5A-B). Although mitochondrial localization of LC3II tended to increase with recovery in KO animals, this did not reach statistical significance. Moreover, LC3II flux was significantly elevated with exercise (~4.4-fold, Fig. 5C) in WT animals only, and this increase was completely abolished by the lack of PGC-1α. The localization of p62 to the mitochondria did not change with exercise or recovery, in either WT or KO animals (Fig. 5A, D). However, p62 flux was increased following recovery by 65% and 56% in WT and KO animals, respectively (Fig. 5E).

In order to elucidate the mechanisms behind mitochondrial targeting with exercise we examined the mitochondrial E3 ubiquitin ligase Parkin, which has been well documented to be intimately involved in mitophagy (25). Interestingly, Parkin mRNA levels were elevated ~3-fold, while mitochondrial localization of the protein was induced ~3.5-fold following exercise, which remained elevated during the recovery period in WT animals (Fig. 6 A-C). Parkin mRNA tended to increase with exercise in KO animals, but this increase was not statistically significant (Fig. 6A). Importantly, parkin localization to the mitochondria was both delayed, and attenuated in the KO animals, increasing by 2.6-fold only after recovery (Fig. 6B-C). Elevated Parkin in WT animals translated to a ~60% increase in the ubiquitination of mitochondrial proteins following

exercise, and this effect was absent in KO animals (Fig. 6B, D). Since an important prerequisite for mitochondrial degradation by autophagy is organelle fragmentation (37), we also examined mitochondrially-localized Drp-1, a fission protein that translocates to mitochondria and facilitates their fragmentation. We found a ~2.2-fold increase in the localization of Drp-1 to the mitochondrial subfraction following exercise, which returned to baseline during recovery in WT animals (Fig. 6B, E). No change in mitochondrial Drp-1 levels was observed in KO animals. Thus, these results indicate an increased targeting of mitochondria for mitophagy post-exercise which is, at least in part, dependent on PGC-1α.

Exercise-mediated alterations in transcriptional regulators of autophagy are not different between WT and KO animals. The transcriptional regulation of autophagy with exercise has not been thoroughly examined. Therefore, we investigated FoxO3 and TFEB, two well known transcriptional regulators of the autophagy-lysosome system. *Foxo3* expression was induced 2.8-fold with exercise in WT animals, and was consistently higher in KO animals across all conditions (Fig. 7A). T*feb* mRNA expression was not altered during exercise in either WT or KO animals, but tended to increase after recovery in WT animals only (Fig. 7B). Thus, both TFEB and FoxO3 may play a role in the transcriptional regulation of autophagy, but neither appears to be regulated by PGC-1α in this context.

NPC1 is a novel autophagy factor regulated by PGC-1α.

In order to identify additional autophagy factors that may be under the control of PGC-1α, we performed an unbiased PCR-based mRNA array and compared the expression of 84 autophagy related genes (for complete list of genes see table S1) in WT and PGC-1α KO animals. Several genes were dramatically down-regulated in KO as compared to WT animals (Fig. 8A), including *mTOR*, *Nfkb1*, *Pik3cg*, and *NPC1*. We chose to further investigate NPC1, a novel transmembrane protein responsible for cholesterol trafficking in late endosomes and lysosomes. Mutations in

NPC1 result in Niemann-Pick disease type C, an autosomal recessive neurovisceral lipid storage disorder, which is accompanied by impaired autophagy (17, 33). We first confirmed our array findings with real time PCR and found that *NPC1* expression was indeed diminished by the lack of PGC-1α (Fig. 8B). *NPC1* expression was induced by ~80% with exercise, and returned to basal levels during recovery in WT animals. The exercise-mediated increase in NPC1 was abolished in KO animals. We also found that NPC1 protein levels were significantly lower in KO animals as compared to their WT counterparts (Fig. 8C). We, therefore, have identified NPC1 as a novel autophagy factor that is induced with exercise, and this induction appears to be PGC-1α-dependent.

Discussion

Skeletal muscle is a malleable tissue that rapidly adapts to its metabolic environment. Much research has focused on establishing the mechanism responsible for this remarkable plasticity, as it holds within great therapeutic potential for a vast array of muscle and metabolic pathologies. The energy demands stemming from muscle contraction are known to initiate signaling cascades which lead to increased mitochondrial biogenesis in order to enhance the energetic potential of muscle. This exercise signaling largely converges on PGC-1α, a transcriptional co-activator responsible for orchestrating the mitochondrial biosynthesis program. However, it is not known how mitochondrial degradation is regulated during exercise, and the role of PGC-1α in this process has not been conclusively determined. The purpose of this study was to examine the activation of autophagy and mitophagy during an acute bout of exercise, and to evaluate the involvement of PGC-1α in this process.

PGC-1α has been documented time and again to drive mitochondrial biogenesis and expression of oxidative genes (2, 18, 28, 29). It is no surprise then that KO animals are

profoundly deficient in mitochondria, as observed biochemically by more pale SDH and COX staining, as well as diminished COX activity. This reduction in mitochondrial content translated to a functional deterioration in endurance performance. PGC-1α KO mice exhibited metabolic distress during exercise, evident by elevated lactic acid levels that did not effectively resolve with a 90 min recovery period. This was accompanied by reduced endurance performance compared to WT animals. KO animals also exhibited greater increases in the activation of the metabolic sensor AMPK and cellular stress sensor p38, further supporting metabolic distress in these animals. WT animals displayed normal elevations in the activation of these kinases with exercise, which returned back to basal levels during the recovery period. Our results confirm that the process of mitochondrial biogenesis is initiated with the first bout of exercise, and that PGC-1α is instrumental in this process (2, 28). PGC-1α has been documented to localize to the nucleus shortly after the commencement of exercise (7, 31). We noted an increase in nuclear PGC-1α in WT animals immediately following exercise which was followed by a strong induction in the transcript levels of the co-activator, particularly following the recovery period. These increases in PGC-1α transcript and nuclear localization were accompanied by the induction of mitochondrial genes encoded by the nuclear genome. This increase was abolished in animals lacking PGC-1α. These findings add further substantive support for the importance of PGC-1α in mediating adaptations in oxidative capacity within muscle as a result of exercise.

Several studies have documented the induction of autophagy following an acute bout of exercise (5, 6, 8, 9). Here we demonstrate for the first time that the transcript levels of autophagy factors LC3B and p62 are induced immediately following an acute bout of exercise, and that this increase is mediated by PGC-1α.We did not detect any alterations in the transcript levels of these factors in KO animals 90 min post exercise. We also noted an increase in LC3 lipidation with

exercise in WT animals. This response was delayed in KO animals and did not occur until after the recovery period, when LC3II levels returned to baseline in WT mice. Interestingly, we did not note a decrease in p62 levels in WT animals with exercise, as has been previously described (6, 22). This could be a result of differences in the exercise protocol employed between the studies, and perhaps due to the increases in p62 mRNA that we noted with exercise. In KO animals we observed the opposite effect, where p62 levels were elevated following exercise. Because of the lack of increase in p62 mRNA levels in KO animals, we conclude that the increase in p62 protein with exercise is likely due to impaired degradation by autophagy in these animals. We did not note any basal differences in LC3 or p62 levels between the genotypes, indicating that this is an exercise-mediated effect. Moreover, we also observed a trend for increases in LC3II and p62 flux in WT animals as determined with colchicine treatment experiments, but this trend was not found in KO mice. Indeed, LC3II flux was lower in KO animals. These results indicate that autophagy induction, gene expression and flux are all induced by exercise, and that this induction is compromised in mice lacking PGC-1α. Thus, PGC-1α appears to play a role in the regulation of exercise-induced autophagy.

Activation of mitophagy with exercise has only recently been documented, and was deemed to be required for the removal of dysfunctional mitochondria following damaging downhill running exercise (22). However, the regulation of mitophagy in exercising muscle has not been thoroughly examined. Our findings demonstrate that mitophagy signaling, as well as flux, are induced with an acute bout of exercise, and that this effect is diminished in animals deficient in PGC-1α. In our hands, exercise-induced mitochondrial localization and flux of LC3II was abolished in KO animals. Interestingly, no alterations in p62 localization to the mitochondria were observed with exercise in either genotype, and p62 flux was similarly elevated in WT and

KO animals following recovery only. We also noted that parkin plays a role in exercise-induced mitophagy, as there was an increase in the localization of this E3 ligase to the mitochondria. Enhanced abundance of ubiquitinated proteins within the mitochondrial subfraction with exercise was also observed in the WT animals. In contrast, parkin localization to mitochondria was delayed in PGC-1α KO animals, occurring following recovery, and no increase in mitochondrial protein ubiquitination was observed. It is interesting to note that, the parkin-PGC-1α axis presents a potential point of communication between mitochondrial biogenesis and degradation following exercise. Parkin has been documented to target the transcriptional repressor Paris (ZNF746) for degradation, thus releasing the Paris-mediated repression of PGC-1α transcription (36). This would act to enhance mitochondrial biogenesis to compensate for increased mitochondrial degradation.

The impairment in mitophagy that we observed in KO animals could also be due, in part, to altered organelle dynamics. Fission is a prerequisite for mitophagy (37), and in our model the localization of the fission protein Drp-1 to the mitochondria was increased after exercise in WT, but not in KO animals. This further supports our findings that mitophagy is activated with exercise, and that PGC-1α is involved in this process.

Although several factors have been identified to participate in the transcriptional regulation of autophagy (3, 23, 35, 42), little is known about this process in skeletal muscle, and the regulation of this process with exercise has not been investigated. Therefore, we examined the expression of FoxO3 and TFEB, two transcriptional regulators that have been well documented to induce autophagic gene expression in different cells and tissues. FoxO3 transcript levels were induced with exercise suggesting a role for this protein in mediating the increased expression of autophagy genes with exercise. However, we also noted an overall higher expression of FoxO3

in KO animals, indicating that FoxO3 is not likely to mediate PGC-1α-induced autophagy. This is in line with previous evidence implicating PGC-1α in the suppression of FoxO3 under atrophic conditions (32). We also investigated TFEB, a master transcriptional regulator of the autophagy-lysosome system (27, 35), previously documented to play a role in the induction of PGC-1α expression in the liver during nutrient deprivation (34). We noted a trend for increased TFEB transcript levels during recovery only in WT animals. The lack of this trend in KO animals suggests a potential role for TFEB in PGC-1α-mediated autophagic induction. Indeed, we have previously observed reduced TFEB protein levels in KO animals when compared to their WT controls (unpublished observations) under basal conditions. It is possible that PGC-1α may bind and co-activate TFEB on the promoter of autophagy-related genes, however, this warrants further investigation.

In an attempt to further characterise the endogenous role of PGC-1α in autophagy regulation we performed an unbiased mRNA array to examine 84 genes involved in various aspects of autophagy. Our array data reveal that multiple genes that were down-regulated in KO animals. We further focused on Niemann-Pick C1 (NPC1), a novel transmembrane protein involved in the regulation of cholesterol trafficking from late endosomes and lysosomes. NPC1 protein and mRNA were both strongly down regulated in PGC-1α KO animals, but were induced with exercise in WT animals, suggesting a role for this protein in exercise-induced adaptations. NPC1 has not been previously studied in this context, but mutations in NPC1 result in Neimann-Pick disease, a devastating lysosomal storage disease characterized by defective autophagy and increased cholesterol load (39). Moreover, polymorphisms or haploinsufficiency in the NPC1 gene have been correlated with obesity and type 2 diabetes (11, 40). Very little is currently known about NPC1 and its role in skeletal muscle.

Taken together, our findings indicate that exercise-induced metabolic adaptations involve augmented mitochondrial turnover that engages concomitant increases in both degradation and biogenesis. Our results also demonstrate that the transcriptional co-activator PGC-1α coordinates mitochondrial biogenesis and mitophagy immediately following exercise, and that both of these processes are compromised in the absence of the co-activator. This study sheds light on the mechanisms underpinning mitochondrial turnover induced by exercise, implicating PGC-1α in orchestrating this process.

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Authors' contributions

AV participated in the design of the study, collected the data, analyzed the data, performed statistical analysis, helped write and edit the manuscript. LDT and MP collected data and provided technical assistance. DAH conceived of the study, participated in its design and coordination, provided guidance and assistance with data interpretation, and helped write and edit the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Fig.1 Lack of PGC-1α results in diminished mitochondrial content and reduced exercise performance. A. Representative images of COX and SDH staining of EDL muscle from Con WT and KO animals. B. COX activity as a surrogate measure of mitochondrial content in WT and KO animals (scale bar: 100μm). C. After 2 days of habituation to the treadmill animals were run to until failure utilizing an incremental exercise protocol on a 0% incline. Animals began with a warm up period of 5min at 5m/min and 10min at 10m/min followed by a period of endurance running for 45min at 15m/min. Finally, running speed was increased by 1m/min every 2 min until animals refused to continue. D. Running performance in WT and KO animals treated with vehicle (water, veh) or 0.4mg/kg colchicine (col), measured by total distance ran. E. Blood lactate levels measured in WT and KO animals prior to exercise (Con), immediately following exercise (Ex) and following 90 min of recovery (Ex+R). *P<0.05, significant effect of exercise. †P<0.05, significant effect of genotype (n=4-12 for all groups).

Fig.2 Signaling kinases are activated with exercise. A-C. Blots and quantification of signaling kinases in control (Con), exercise (Ex),and exercise with recovery (Ex+R) in WT and KO animals. A. Representative blots. Quantification of B. P-AMPK; C. P-p38. *P<0.05 significant effect of exercise. †P<0.05, significant effect of genotype. Total protein and GAPDH were used as a loading controls (n=5-9)

Fig.3 Exercise induces increased nuclear localization and expression of PGC-1α, and mitochondrial biogenesis. A. Blot and quantification of PGC-1α levels in the nuclear subfraction of WT animals under basal conditions (con), after exercise (Ex) and following recovery (Ex+R). B. PGC-1α gene expression, mRNA fold change immediately following

exercise and with recovery in WT animals, all groups are compared to Con, *Gapdh* and *Actb* were used as housekeeping genes. C-D. Gene expression analysis of mitochondrial factors, mRNA fold change with exercise with and without recovery in WT and KO animals, all groups were compared to WT Con, *Gapdh* and *Actb* were used as housekeeping genes; C. COXIV; D. Tfam. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype (n=4-9).

Fig.4 Expression of autophagy genes and induction of autophagy with exercise are differentially regulated in PGC-1α KO animals. A-B. Gene expression of autophagy factors, mRNA fold change in WT and KO animals either in Con, Ex or Ex+R, all groups were compared to WT Con, *Gapdh* and *Actb* were used as housekeeping genes; A. LC3; B. p62. C-E. Blots and quantification of autophagic protein in whole muscle extracts in control (Con), exercise (Ex) and exercise with recovery (Ex+R) in WT and KO animals treated with vehicle (water) or colchicine (col) 0.4mg/kg/day for 2 days. C. Representative blots. Quantification of D. LC3BII; E. p62; F-G. Autophagy flux as determined by percent change in protein content from Col treated and Veh treated WT and KO animals in Con, Ex and Ex+R groups; F. LC3II; G.p62. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype. GAPDH was used as a loading control (n=5-9).

Fig.5 Exercise-induced mitophagy signaling and flux are attenuated in PGC-1α KO animals. A-E. Blots and quantification of autophagic proteins and flux in isolated mitochondrial fractions in control (Con), exercise (Ex), and exercise and recovery (Ex+R) in WT and PGC-1α KO (KO) animals, treated with vehicle (water) or colchicine (col) 0.4mg/kg/day for 2 days. A. Representative blots. Quantification of B. LC3BII protein; C. LC3II flux; D. p62 protein; C. p62 flux. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype. VDAC was used as loading control (n=7-9).

Fig.6 Lack of PGC-1α results in attenuated exercise-mediated mitophagy signaling. A. Parkin gene expression, mRNA fold change in WT and KO, Con, Ex, and Ex+R animals, all groups were compared to WT Con, *Gapdh* and *Actb* were used as housekeeping genes. B-E. Blots and quantification of proteins in isolated mitochondrial subfractions. B. Representative blots. Quantification of C. Parkin; D. ubiquitin; E. Drp-1. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype. VDAC was used as loading control. (n=4-9).

Fig.7 Transcriptional regulators of autophagy with exercise. A-B. Gene expression of transcriptional regulators of autophagy, mRNA fold change in Con, Ex and Ex+R in WT and KO animals all groups were compared to WT Con, *Gapdh* and *Actb* were used as housekeeping genes; A. FoxO3; B. TFEB. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype (n=4-9).

Fig.8 Identification of NPC1 as a PGC-1α-regulated autophagy factor, through PCR-array analysis. A. Heat map comparing the expression of 84 autophagy related genes between WT and KO animals using mRNA-array. Green indicates reduction while red indicates increase in gene expression, the brighter the colour the greater the change in gene expression. For the full list of fold changes and statistical significance see table S1. B. Gene expression of NPC1, expressed as mRNA fold change in Con, Ex, and Ex+R groups in WT and KO animals, all groups were compared to WT Con, *Gapdh* and *Actb* were used as housekeeping genes. C. Representative blot and quantification of NPC1 in TA muscle extracts. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype. GAPDH was used as loading control. (n=3-4).

Table 1 Primer sequences based on gene transcripts available in GenBank.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
Coxiv	CTCCAACGAATGGAAGACAG	TGACAACCTTCTTAGGGAAC
Tfam	GAAGGGAATGGGAAAGGTAGA	AACAGGACATGGAAAGCAGAT
Bnip3l	GGAAAGCGGCACAGAGAA	GAATGACGCCAGTGCTGAT
Park2	GTCTGCAATTTGGTTTGGAGTA	GCATCATGGGATTGTCTCTTAAA
Sqstm1	TGTGGTGGGAACTCGCTATAA	CAGCGGCTATGAGAGAAGCTAT
Maplc3b	GCTTGCAGCTCAATGCTAAC	CCTGCGAGGCATAAACCATGTA
Atg7	TTTCTGTCACGGTTCGATAATG	TGAATCCTTCTCGCTCGTACT
Lamp2	GCTGAACAACAGCCAAATTA	CTGAGCCATTAGCCAAATACAT
Catsd	TTTGCCAATGCTGTCGTACT	AGCGAGTGTGACTATGTGTGAG
TFEB	AGCTCCAACCCGAGAAAGAGTTTG	CGTTCAGGTGGCTGCTAGAC
Foxo3	ATGGACGACCTGCTGGATAAC	GGAGCTCTTGGCGGTATATG
Beclin1	AGGCTGAGGCGGAGAGATT	TCCACACTCTTGAGTTCGTCAT
NPC1	AGCATCACCGCATCTTACAA	GCATGGCTGTTCTGGAAGTAA
Actb	TGTGACGTTGACATCCGTAA	GCTAGGAGCCAGAGCAGTAA
Gapdh	AACACTGAGCATCTCCCTCA	GTGGGTGCAGCGAACTTTAT

Fig.1

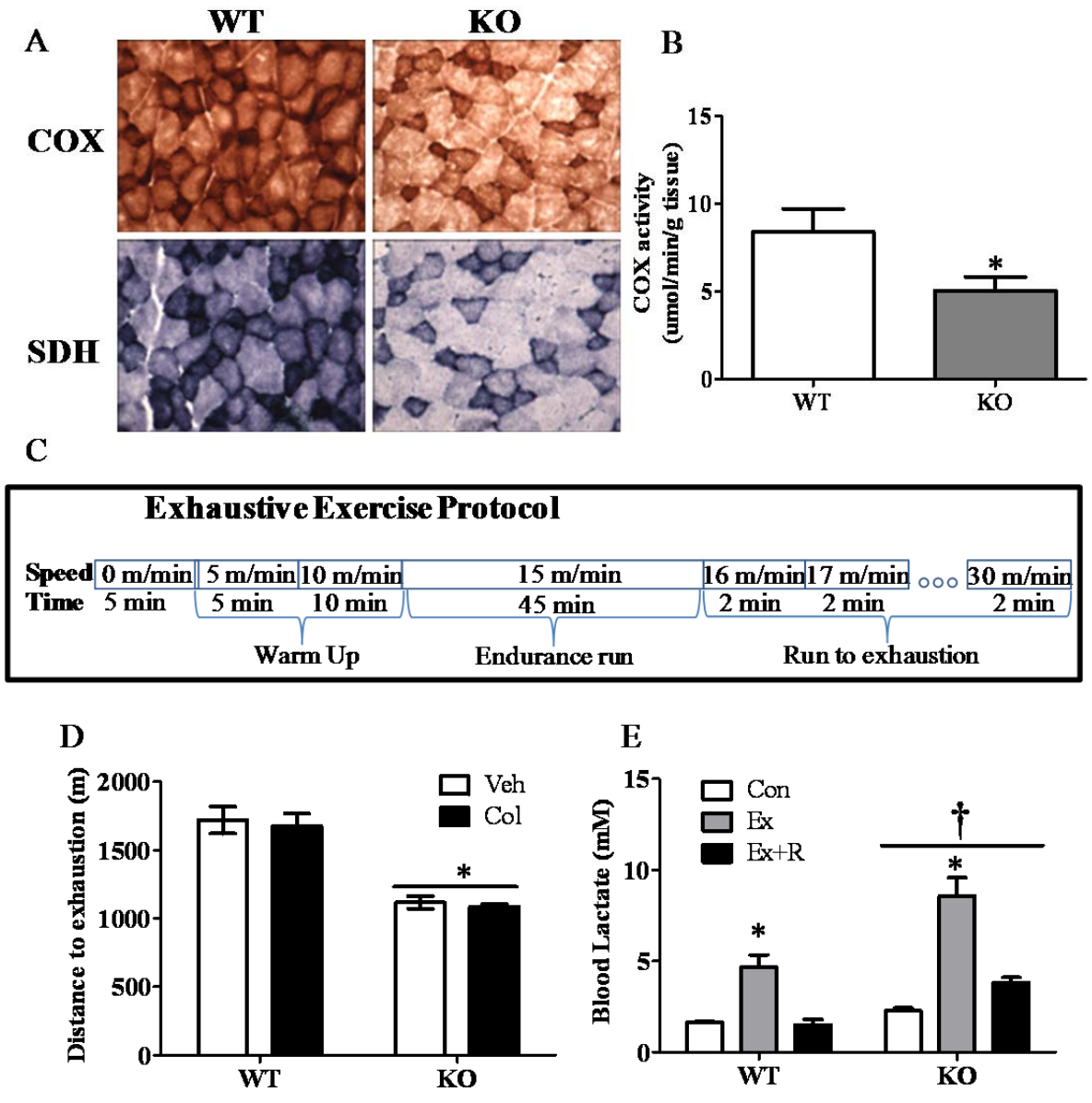

A
WT
KO
COX
SDH
B
COX activity (umol/min/g tissue)
15
10
5
0
*
WT
KO
C
Exhaustive Exercise Protocol
Speed
Time
0 m/min
5 min
5 m/min
5 min
10 m/min
10 min
15 m/min
45 min
16 m/min
2 min
17 m/min
2 min
ooo
30 m/min
2 min
Warm Up
Endurance run
Run to exhaustion
D
Distance to exhaustion (m)
2000
1500
1000
500
0
Veh
Col
*
WT
KO
E
Blood Lactate (mM)
15
10
5
0
Con
Ex
Ex+R
*
*
†
WT
KO

Fig.2

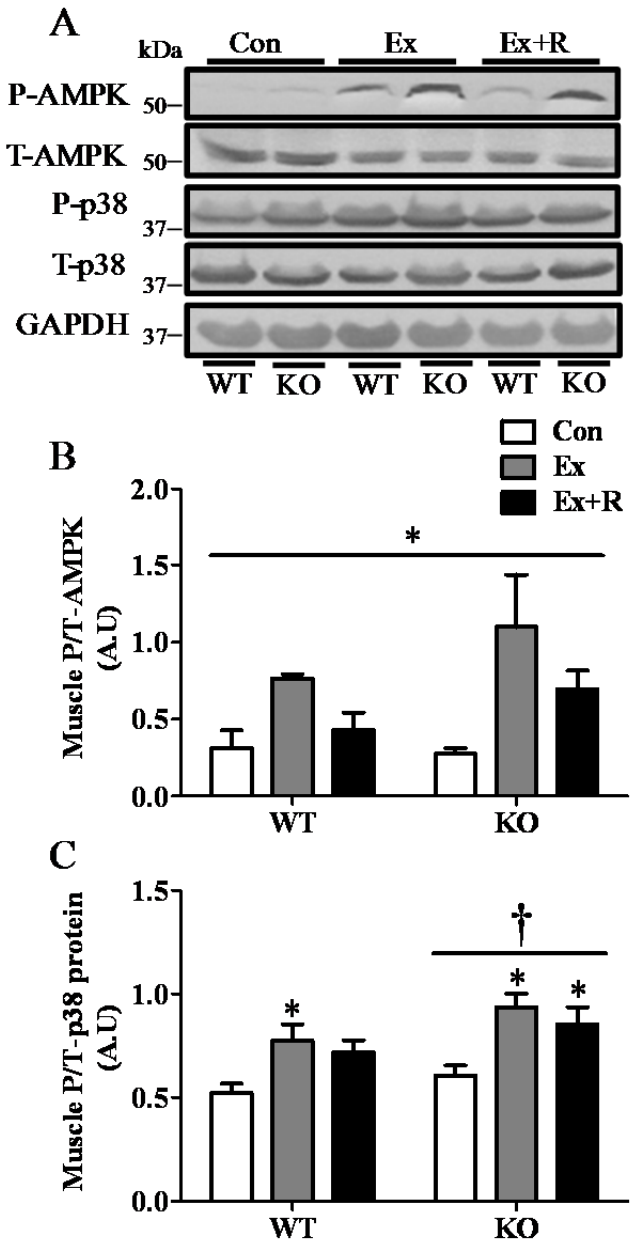

A
kDa
Con
Ex
Ex+R
P-AMPK
50–
T-AMPK
50–
P-p38
37–
T-p38
37–
GAPDH
37–
WT
KO
WT
KO
WT
KO
B
Con
Ex
Ex+R
Muscle P/T-AMPK (A.U)
2.0
1.5
1.0
0.5
0.0
*
WT
KO
C
Muscle P/T-p38 protein (A.U)
1.5
1.0
0.5
0.0
†
*
*
*
WT
KO

Fig.3

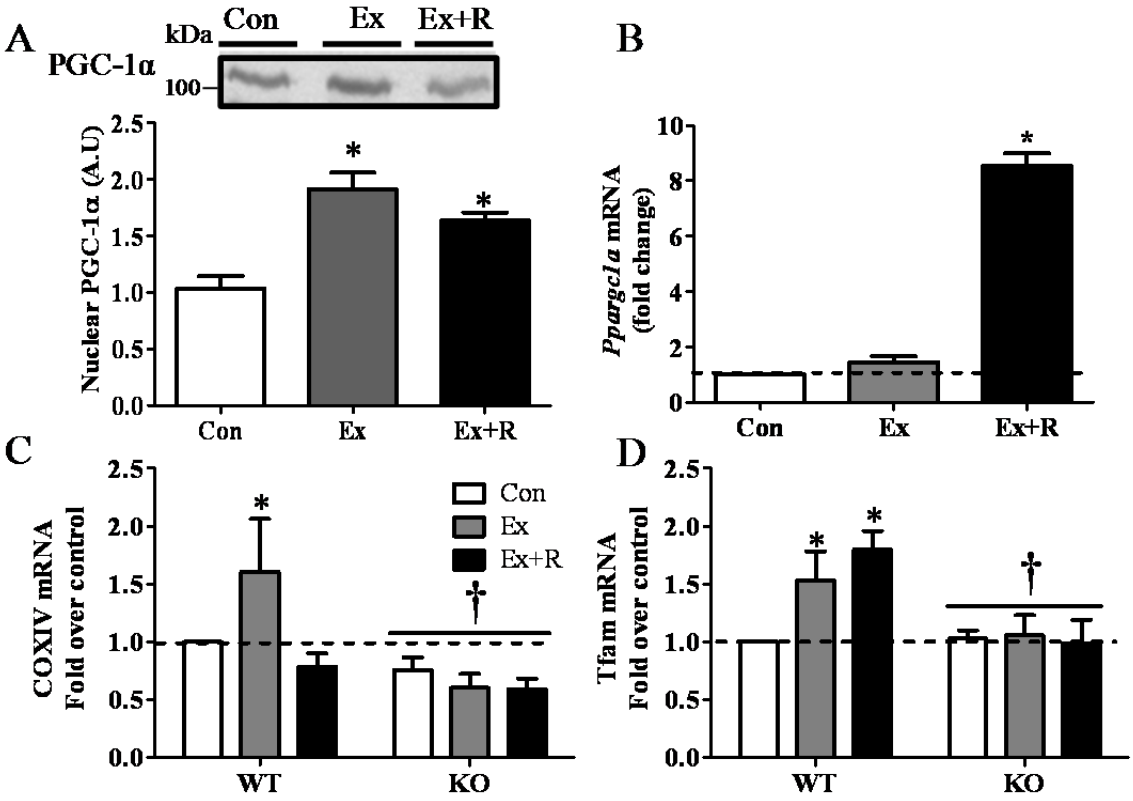

A
kDa
Con
Ex
Ex+R
PGC-1α
100
Nuclear PGC-1α (A.U)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Con
Ex
Ex+R
B
Ppargc1a mRNA
(fold change)
10
8
6
4
2
0
*
Con
Ex
Ex+R
C
COXIV mRNA
Fold over control
2.5
2.0
1.5
1.0
0.5
0.0
*
Con
Ex
Ex+R
†
WT
KO
D
Tfam mRNA
Fold over control
2.5
2.0
1.5
1.0
0.5
0.0
*
*
†
WT
KO

Fig.4

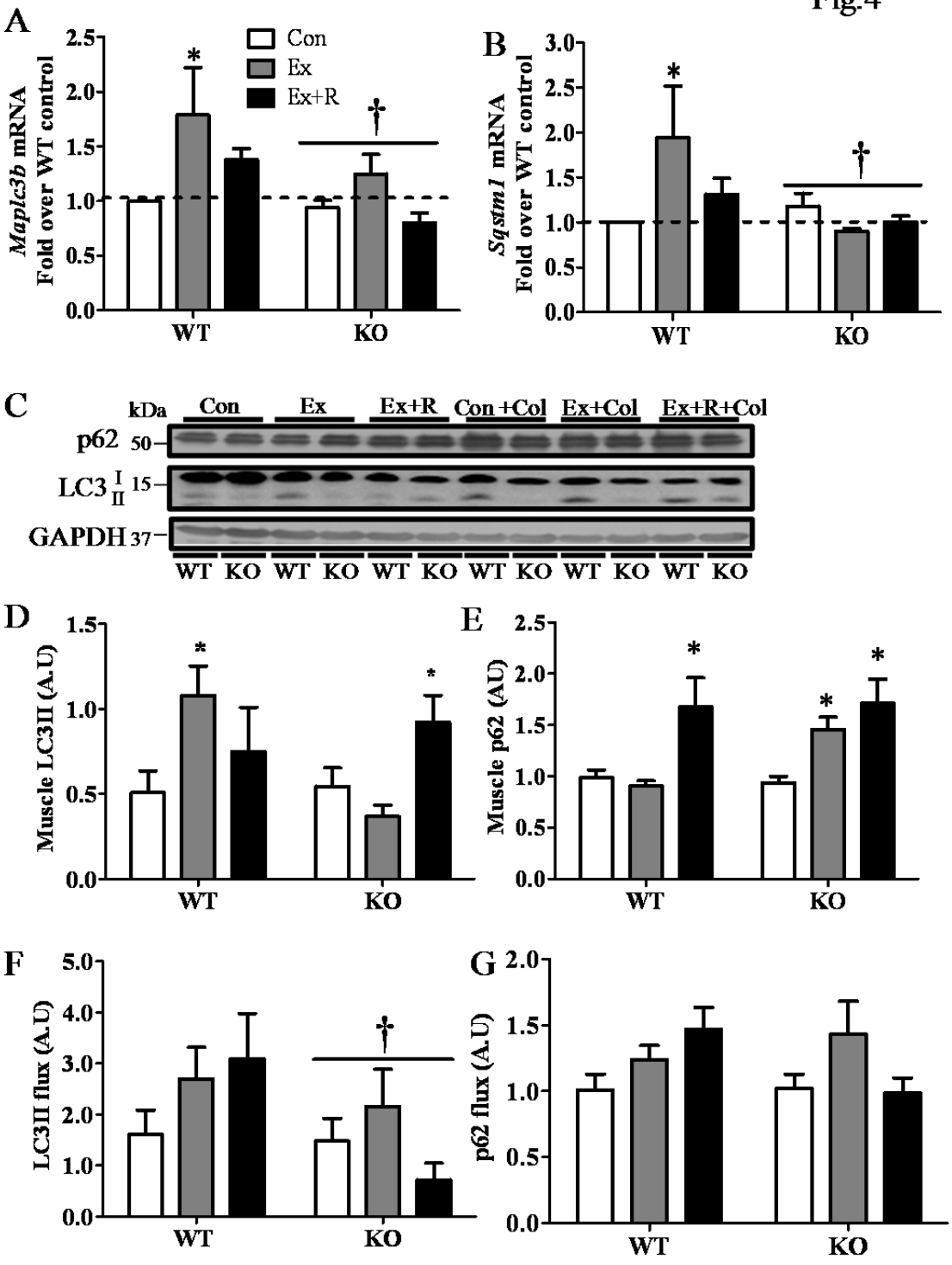

A
Maplc3b mRNA
Fold over WT control
Con
Ex
Ex+R
WT
KO
B
Sqstm1 mRNA
Fold over WT control
C
kDa
Con
Ex
Ex+R
Con +Col
Ex+Col
Ex+R+Col
p62
LC3 I II
GAPDH
WT KO WT KO WT KO WT KO WT KO WT KO
D
Muscle LC3II (A.U)
E
Muscle p62 (AU)
F
LC3II flux (A.U)
G
p62 flux (A.U)

Fig.5

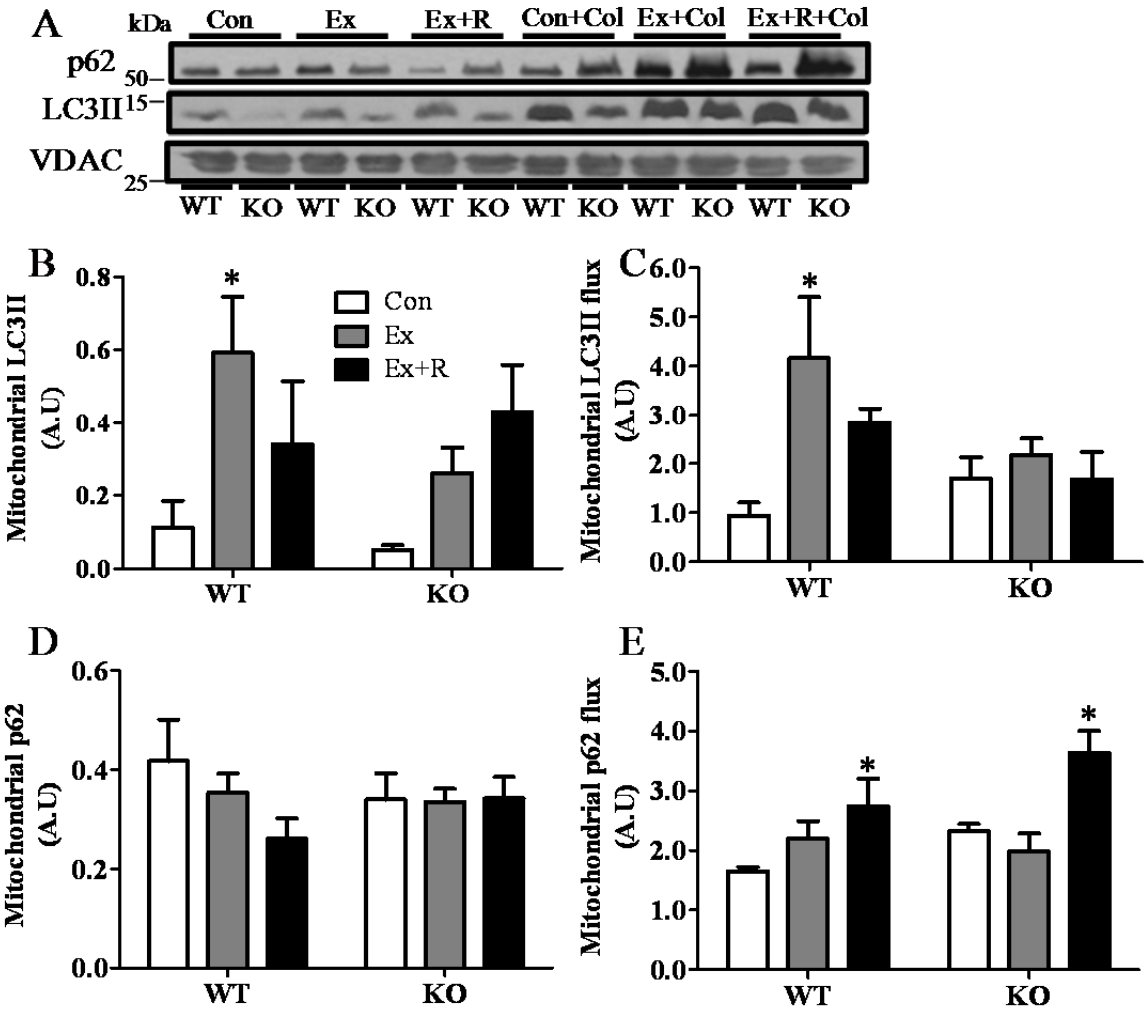

A
kDa
Con
Ex
Ex+R
Con+Col
Ex+Col
Ex+R+Col
p62
50
LC3II
15
VDAC
25
WT KO WT KO WT KO WT KO WT KO WT KO
B
Mitochondrial LC3II (A.U)
Con
Ex
Ex+R
WT
KO
C
Mitochondrial LC3II flux (A.U)
D
Mitochondrial p62 (A.U)
E
Mitochondrial p62 flux (A.U)

Fig.6

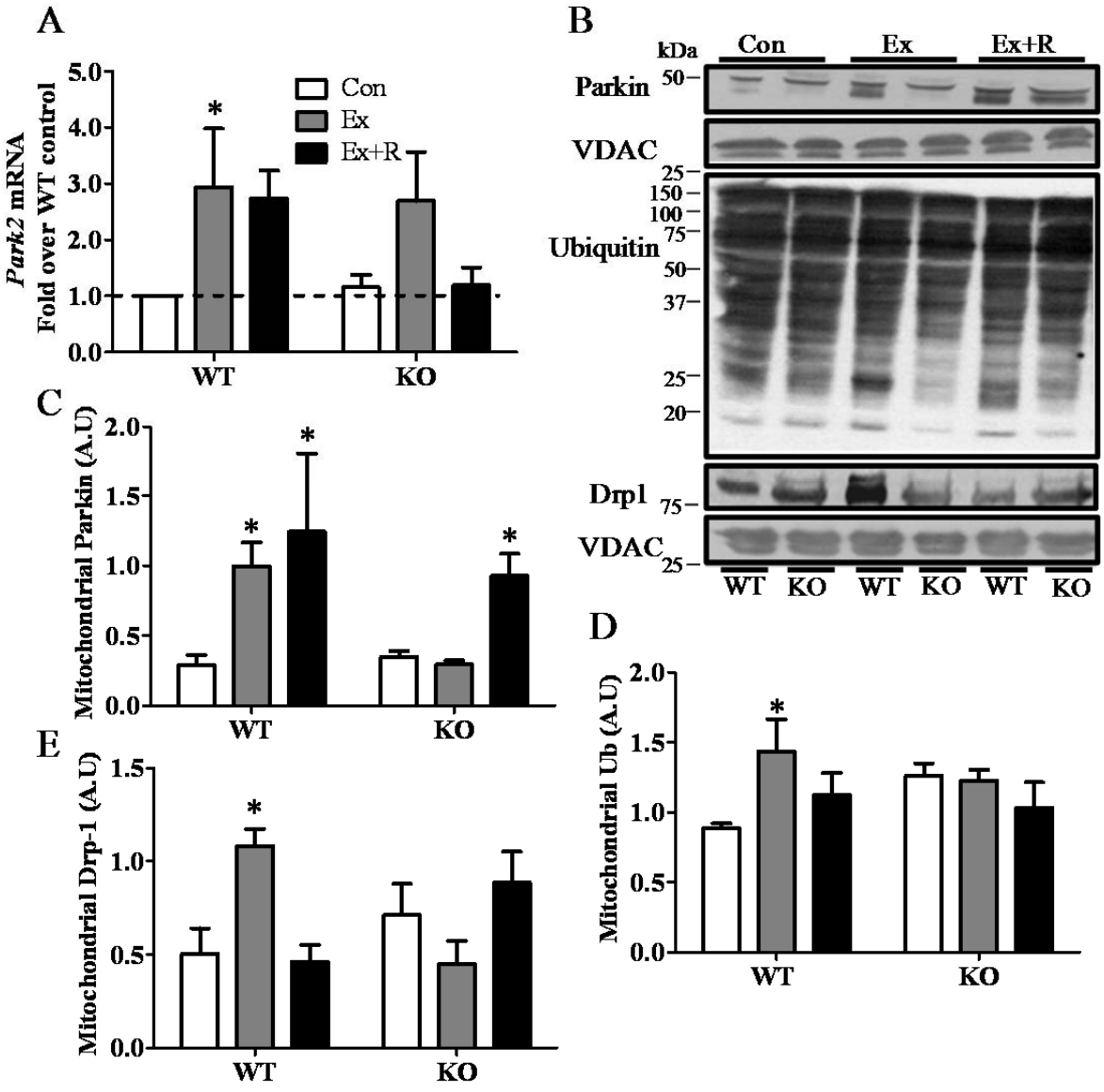

A
Park2 mRNA
Fold over WT control
5.0
4.0
3.0
2.0
1.0
0.0
Con
Ex
Ex+R
*
WT
KO
B
kDa
Con
Ex
Ex+R
Parkin
50
VDAC
25
150
100
75
Ubiquitin
50
37
25
20
Drp1
75
VDAC
25
WT
KO
WT
KO
WT
KO
C
Mitochondrial Parkin (A.U)
2.0
1.5
1.0
0.5
0.0
*
*
*
WT
KO
D
Mitochondrial Ub (A.U)
2.0
1.5
1.0
0.5
0.0
*
WT
KO
E
Mitochondrial Drp-1 (A.U)
1.5
1.0
0.5
0.0
*
WT
KO

Fig.7

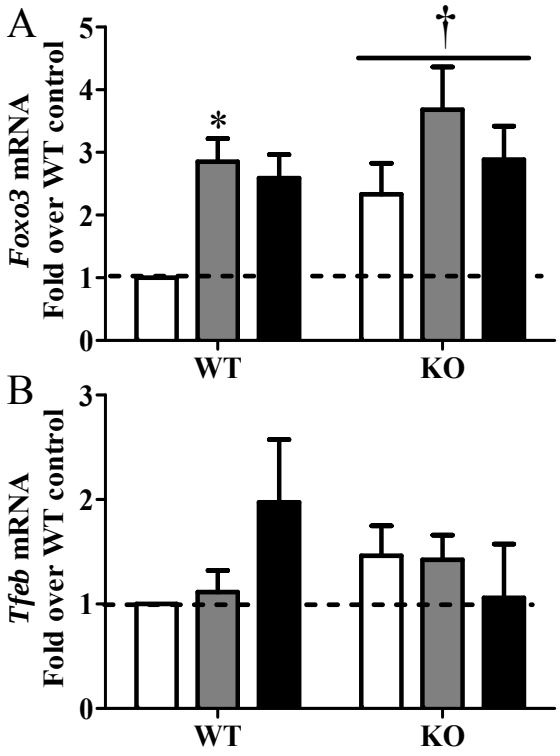
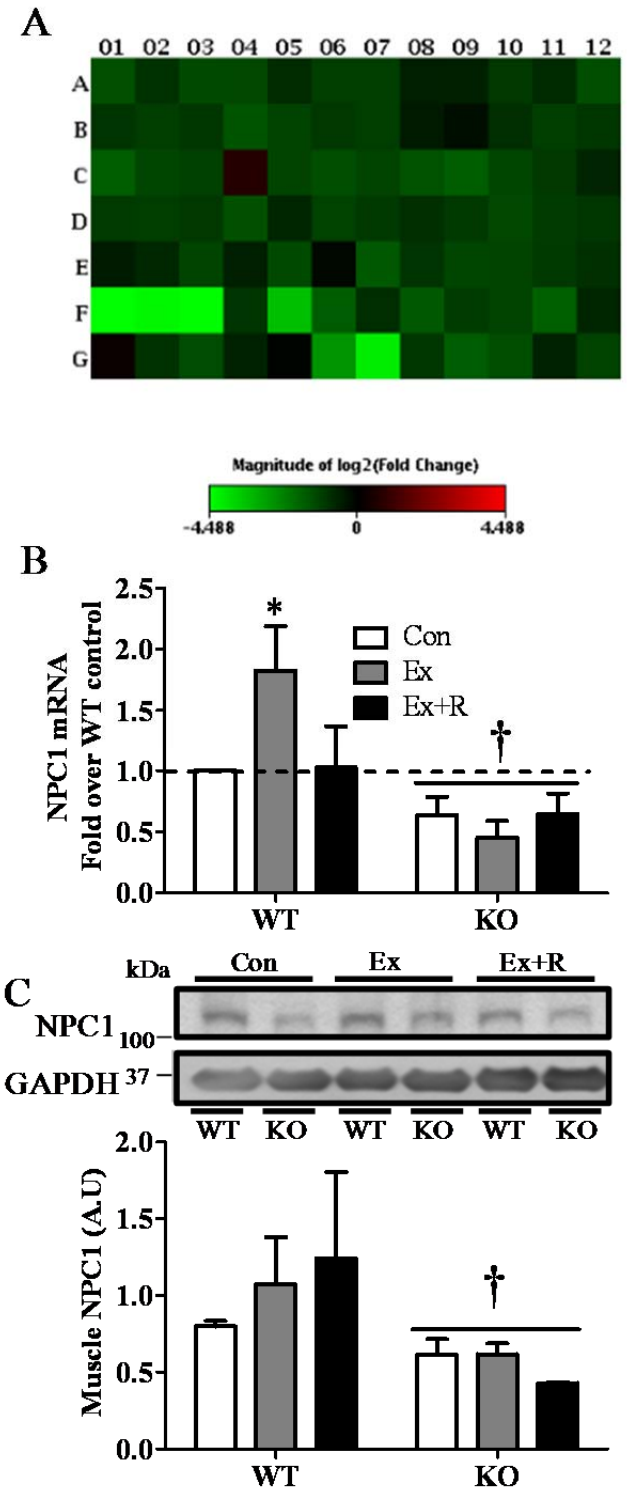

A
Foxo3 mRNA
Fold over WT control
5
4
3
2
1
0
*
†
WT
KO
B
Tfeb mRNA
Fold over WT control
3
2
1
0
WT
KO

Fig.8


A
01 02 03 04 05 06 07 08 09 10 11 12
A
B
C
D
E
F
G
Magnitude of log2(Fold Change)
-4.488
0
4.488
B
NPC1 mRNA
Fold over WT control
2.5
2.0
1.5
1.0
0.5
0.0
*
†
Con
Ex
Ex+R
WT
KO
C
kDa
Con
Ex
Ex+R
NPC1
100
GAPDH
37
WT
KO
WT
KO
WT
KO
Muscle NPC1 (A.U)
2.0
1.5
1.0
0.5
0.0
†
WT
KO

Supplementary Figures For

The role of PGC-1α during acute exercise-induced autophagy and mitophagy in skeletal muscle.

Anna Vainshtein[1], Liam D. Tryon[1], Marion Pauly[1], and David A. Hood[1]

Table S1 mRNA array data

Gene Symbol	p value	Fold change	Gene Symbol	p value	Fold change
Akt1	0.76	-2.67	Eif2ak3	0.75	-2.08
Ambra1	0.61	-1.83	Eif4g1	0.67	-2.11
App	0.73	-2.49	Esr1	0.58	-1.96
Atg10	0.70	-2.39	Fadd	0.75	-2.67
Atg12	0.76	-1.71	Fas	0.58	-1.61
Atg16l1	0.63	-2.16	Gaa	0.65	-2.26
Atg16l2	0.64	-2.12	Gabarap	0.67	-2.02
Atg3	0.59	-1.43	Gabarapl1	0.61	-1.78
Atg4a	0.60	-1.47	Gabarapl2	0.74	-2.01
Atg4b	0.65	-1.99	Hdac1	0.70	-2.4
Atg4c	0.60	-1.67	Hdac6	0.62	-2.1
Atg4d	0.68	-2.61	Hgs	0.61	-1.95
Atg5	0.62	-1.87	Hsp90aa1	0.58	-1.39
Atg7	0.67	-2.13	Hspa8	0.59	-1.64
Atg9a	0.65	-1.96	Htt	0.70	-2.29
Atg9b	0.54	-2.82	Ifng	0.29	-1.43
Bad	0.76	-2.25	Igf1	0.77	-2.45
Bak1	0.62	-2	Ins2	0.52	-1.1
Bax	0.62	-2.15	Irgm1	0.96	-2.9
Bcl2	0.51	-1.39	Lamp1	0.62	-1.87
Bcl2l1	0.48	-1.2	Map1lc3a	0.67	-2.36
Becn1	0.60	-1.73	Map1lc3b	0.76	-2.27
Bid	0.68	-2.16	Mapk14	0.64	-2.02
Bnip3	0.68	-1.93	Mapk8	0.63	-1.79
Casp3	0.81	-3.15	Mtor	0.03	-22.44
Casp8	0.75	-2.35	Nfkb1	0.05	-20.36
Cdkn1b	0.66	-2.19	Npc1	0.05	-22.12
Cdkn2a	0.44	1.55	Pik3c3	0.73	-1.89
Cln3	0.65	-2.27	Pik3cg	0.05	-10.45
Ctsb	0.74	-2.6	Pik3r4	0.59	-3.07
Ctsd	0.68	-2.31	Prkaa1	0.60	-1.76
Ctss	0.83	-2.73	Pten	0.20	-2.94
Cxcr4	0.97	-3.15	Rab24	0.66	-2.06
Dapk1	0.70	-2.32	Rb1	0.97	-2.23
Dram1	0.68	-2	Rgs19	0.51	-3.22
Dram2	0.65	-1.56	Rps6kb1	0.61	-1.59

Table S1
Continued

Snca	0.72	1.13
Sqstm1	0.67	-1.84
Tgfb1	0.68	-2.55
Tgm2	0.57	-1.5
Tmem74	0.29	-1.04
Tnf	0.00	-6.3
Tnfsf10	0.04	-18.34
Trp53	0.74	-1.94
Ulk1	0.47	-3.06
Ulk2	0.28	-2.59
Uvrag	0.54	-1.52
Wipi1	0.74	-2.25

Table. S2

Habituation

Day 1	Day 2	Day 3
5 min 0m/min	5 min 0m/min	5 min 0 m/min
5 min 5 m/min	5 min 5 m/min	5 min 5 m/min
	10 min 10 m/min	10 min 10 m/min
		45 min 15 m/min
		1 m/min every 2 min until exhaustion

Table S2: Habituation Protocol. Animals were acclimatized to the treadmill for two days prior to exhaustive exercise on day 3.

Fig.S1

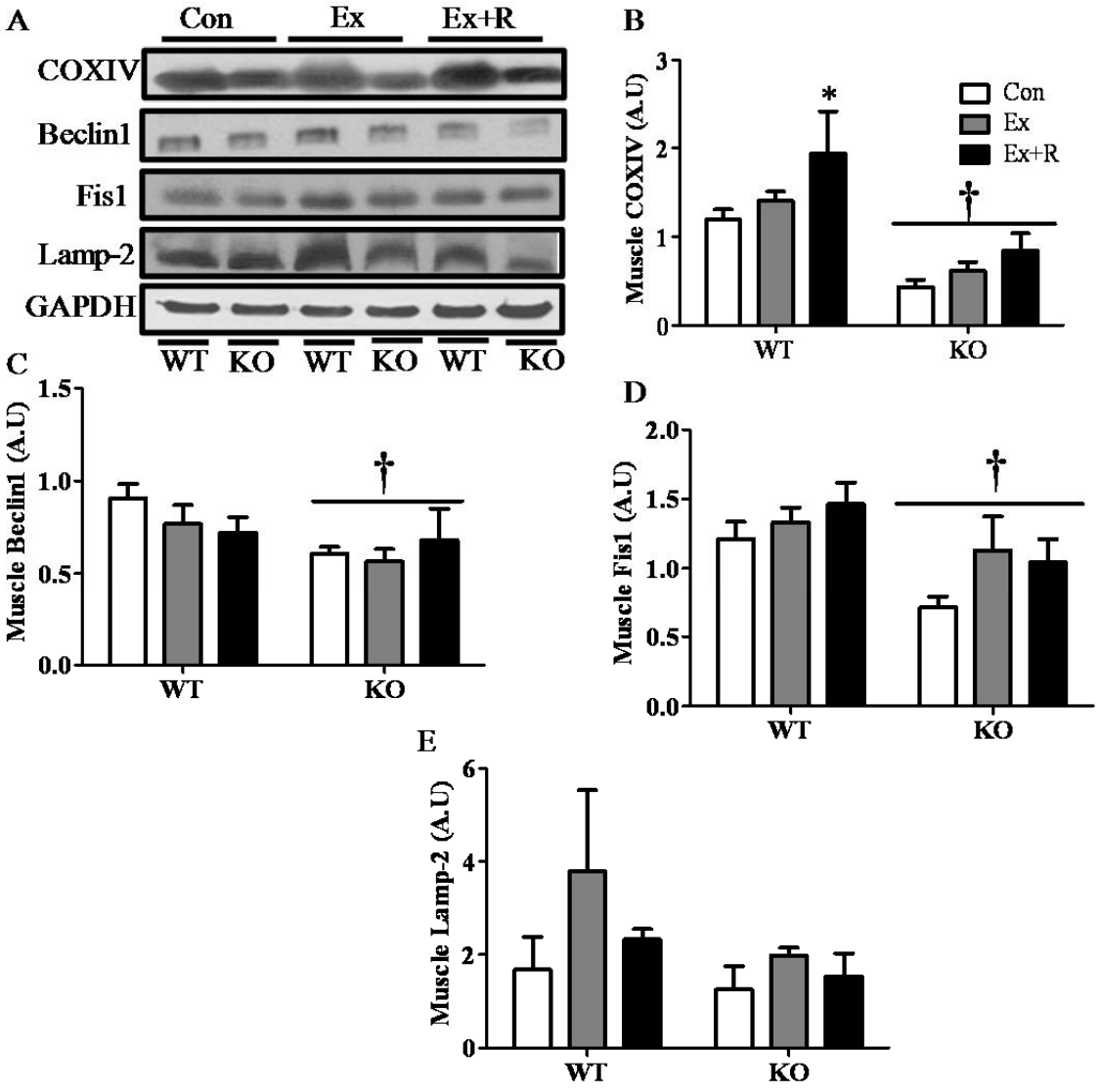


Fig. S1: Whole muscle protein measures. A-E Blots and quantification of whole muscle protein expression in TA muscle in Con, Ex, and Ex+R, WT and KO animals. A. Representative blots. Quantification of B. COXIV; C. Beclin1 ; D. Fis1 ; C. Lamp-2. * $P<0.05$ significant effect of exercise.. †$P<0.05$ significant effect of genotype. GAPDH was used as a loading control (n=4-8).

Fig.S2

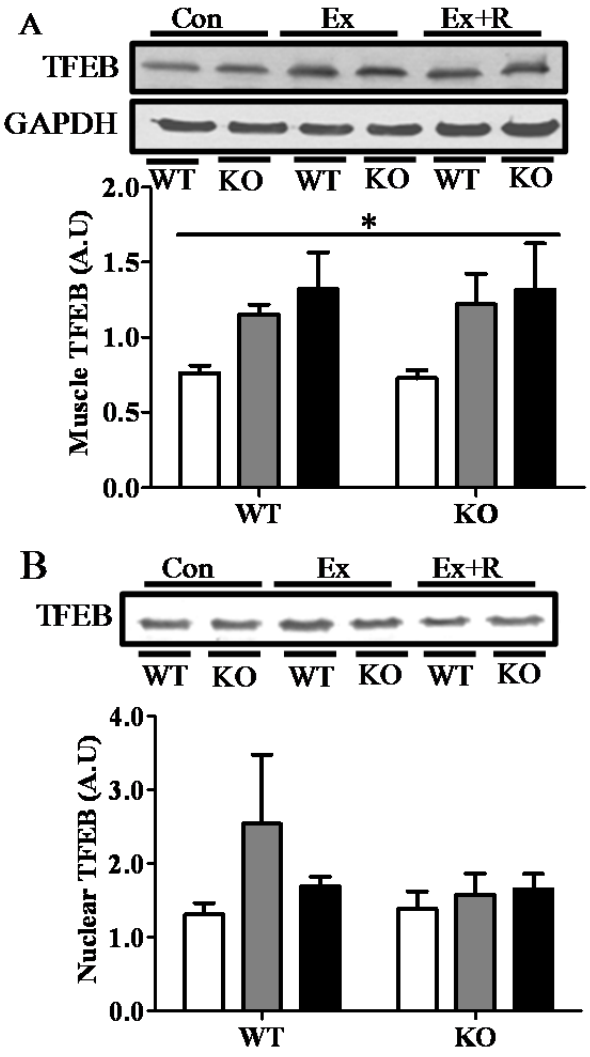


Fig S2: TFEB protein expression and localization. A. Representative blot and quantification of whole muscle TFEB protein expression in TA muscle in Con, Ex, Ex+R, WT and KO animals. B. Representative blot and quantification of nuclear TFEB *P<0.05 significant effect of exercise GAPDH, was used as a loading control where appropriate (n=7-9 for all groups).

Fig.S3

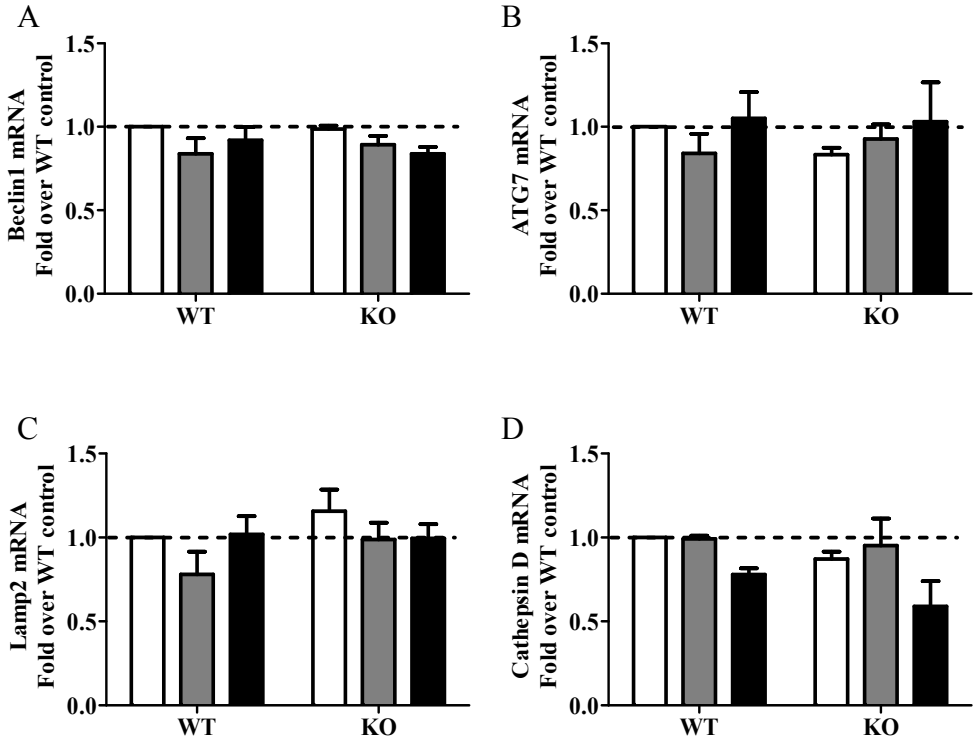


Fig. S3: mRNA expression of autophagy and lysosomal markers. A-D Gene expression as measured by real time PCR. mRNA fold change between wild type (WT) and PGC-1α KO, Con, Ex and Ex+R. All groups were compared to WT Con, and Gapdh and Actb were used as housekeeping genes (n=4-9)

Fig.S4

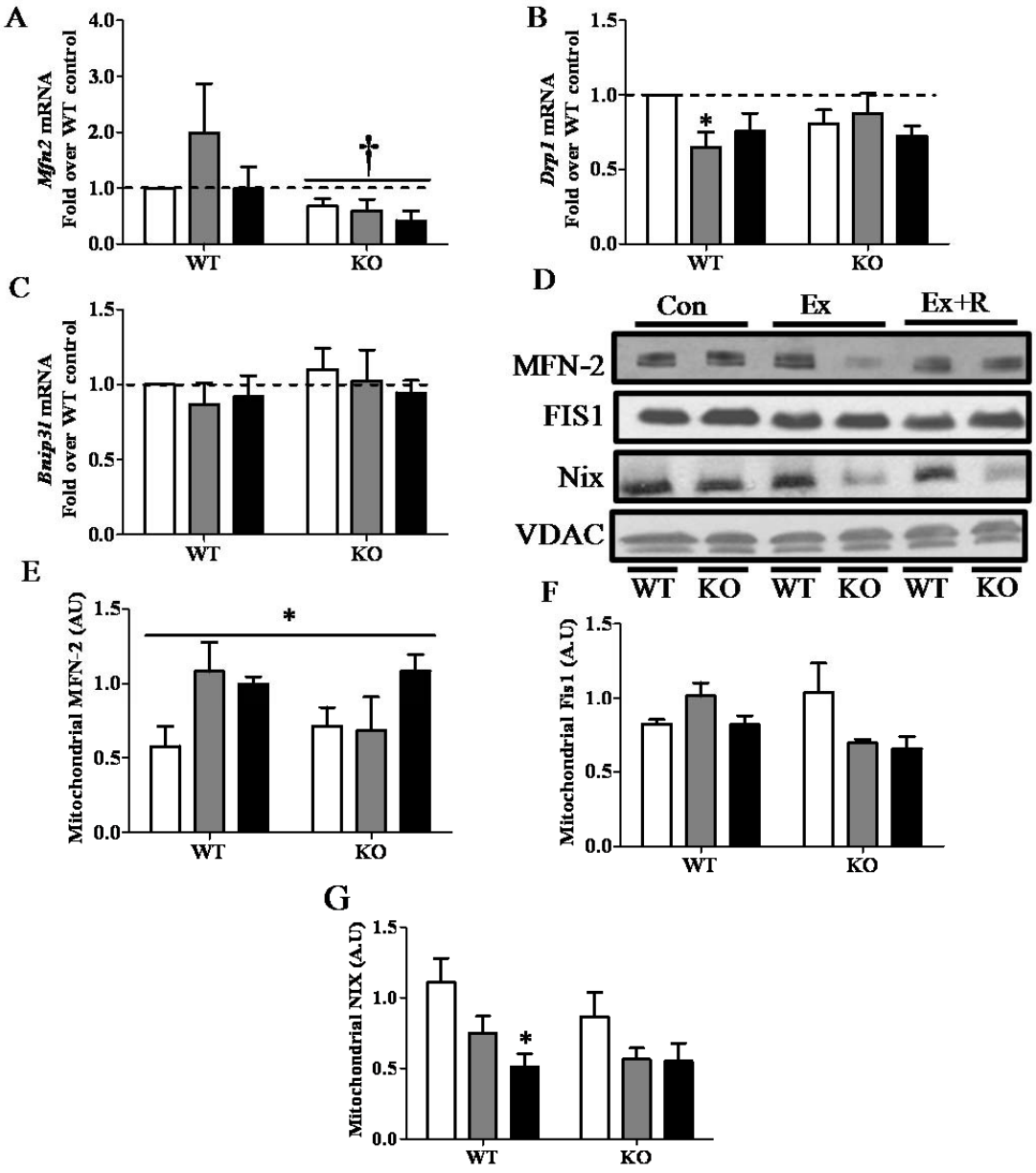


Fig. S4. **Mitochondrial dynamics and mitophagy.** A-C Gene expression measured by real time PCR. mRNA fold change between wild type (WT) and PGC-1α KO (KO) control (Con) and Exercise (Ex) and exercise with recovery (Ex+R). All groups were compared to WT Con, and Gapdh and Actb were used as housekeeping genes. A. Mfn2; B. Drp1; C. Bnip31 (Nix). D-G. Blots and quantification of proteins on isolated mitochondria D. Representative blots. Quantification of E. MFN-2; F. Fis1; F. Nix. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype. VDAC was used as a loading control (n=7-9 for all groups).

Fig. S5

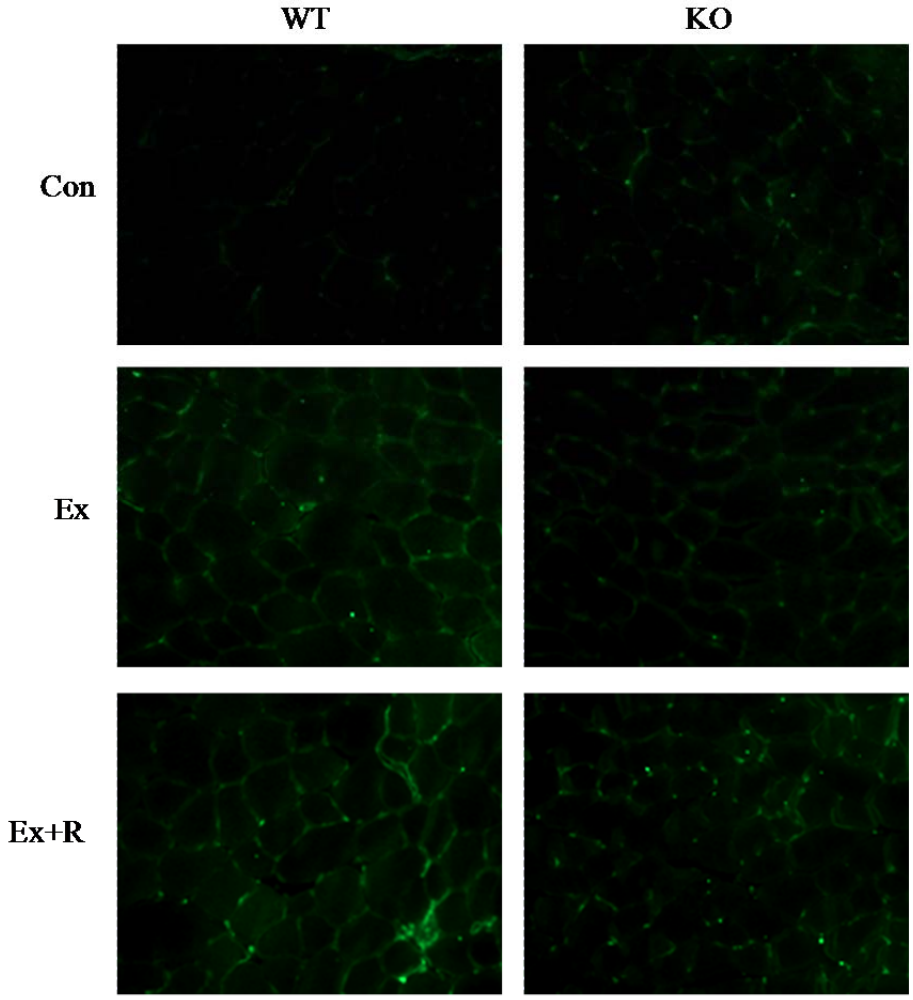


Fig. S5. p62 immunofluorescence. TA muscle sections from WT and KO animals, Con, Ex and Ex+R, immunostained for p62 (Green), visualized with a flourescence microscope using a 20x lens.

Fig. S6

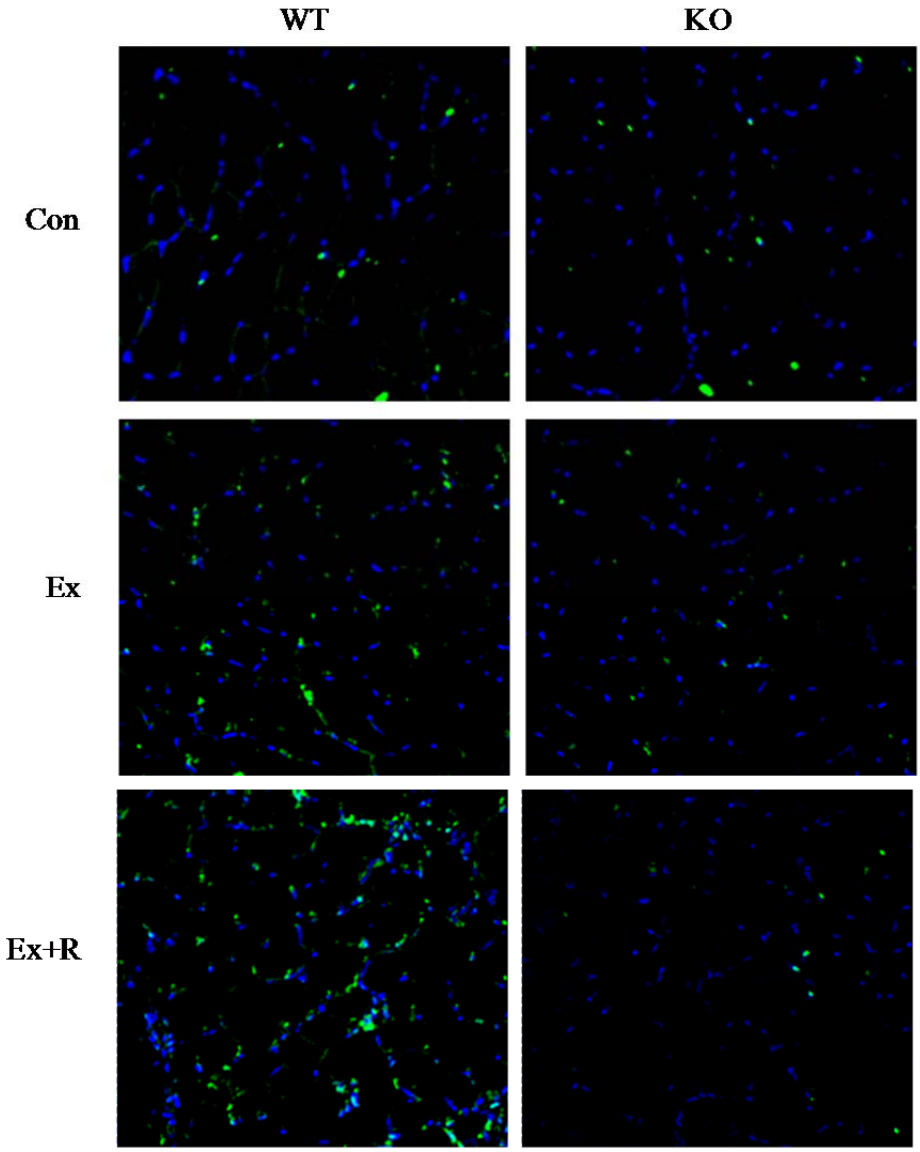


Fig. S6. TFEB immunofluorescence. TA muscle sections from WT and KO animals, Con, Ex and Ex+R, immunostained for TFEB (Green), and nuclei are stained with Dapi (Blue), visualized with a flourescence microscope using a 20x lens. Aqua coulor represents TFEB localized to Nuclei.

Fig. S7

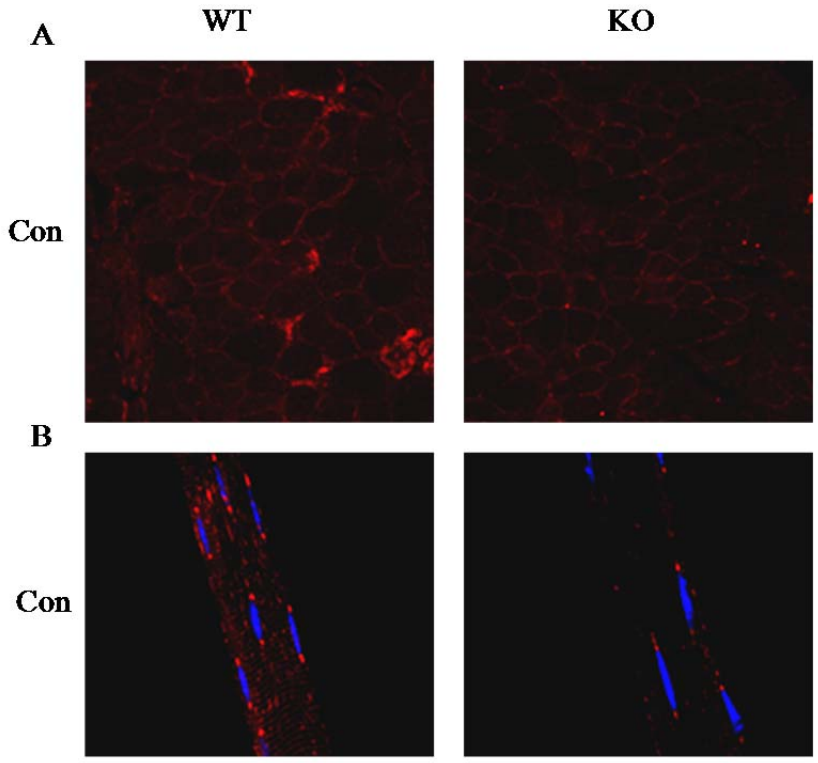


Fig. S7. Lamp-2 immunofluorescence. A. TA muscle sections from WT and KO animals immunostained for Lamp-2 (Red), and nuclei are stained with Dapi (Blue), visualized with a flourescence microscope using a 20x lens. B. Confocal images of fixed single fibers immuno-stained lysosomal Lamp-2 (Red). nuclei are in blue (Dapi). visualized with a flourescence microscope using a 60x lens

CHAPTER 6:

Summary and Conclusions

PGC-1α is a transcriptional co-activator that is a *bona fide* regulator of oxidative metabolism in many tissues and organs. This co-activator and its family members co-ordinate mitochondrial biogenesis, and have also been documented to contribute to overall organelle and tissue health. Indeed, PGC-1α is cytoprotective under a plethora of perturbations. In skeletal muscle, enhanced expression of the co-activator is sufficient to protect against loss of muscle mass and function with aging, heart failure, denervation and starvation. To date, the focus of PGC-1α research has been centered around mitochondrial biogenesis, and its role in mitochondrial degradation has been largely ignored.

Autophagy is a highly conserved proteolytic pathway responsible for the degradation of dysfunctional organelles as well as protein aggregates through the lysosomal machinery. The selective removal of mitochondria through autophagy has been dubbed mitophagy. Mitophagy, like autophagy, occurs basally in all tissues, and aids in cellular housekeeping by mediating the turnover of long-lived molecules and organelles. Under conditions of metabolic stress, autophagy/mitophagy can be upregulated and act as an adaptive response system, essential for cell survival and the maintenance of energetic balance. Aberrant autophagy contributes to cellular dysfunction, and can exacerbate muscle wasting during atrophic condtions. Autophagy is also activated with exercise, and it mediates some of the metabolic benefits incurred by physical activity. The transcriptional regulation of autophagy is not well understood, and governors of this process have only recently begun to emerge. Moreover, how mitochondrial turnover is coordinated during alterations in muscle contractile activity has not been thoroughly examined as of yet. The purpose of my dissertation was to investigate the role of PGC-1α in the regulation of

autophagy/mitophagy under the spectrum of muscle contractility. Using denervation and exercise as models of muscle use and disuse, we hoped to uncover the cross-talk between mitochondrial biogenesis and degradation machinery during these divergent metabolic stresses.

Objective 1: We first investigated the role of PGC-1α in denervation-induced autophagy and mitophagy using a loss of function model. Wildtype (WT) and PGC-1α knockout (KO) animals were unilaterally denervated for 7 days. KO animals were mitochondria-deficient, and presented signs of myopagthy. Denervation enhanced the expression of autophagy genes as well as proteins, and the lack of PGC-1α resulted in diminished autophagy flux both basally and in response to denervation. This was assessed by the percent change in LC3 and p62 in animals treated with the microtubule destabilizing drug colchicine. Moreover, deletion of the co-activator abolished denervation-induced increases in the lysosomal marker, Lamp-2. We also determined that enhanced mitophagy participates in the rapid loss of mitochondrial content observed with disuse, and that PGC-1α is required for this activation, as KO animals exhibited diminished basal and denervation-induced mitophagy flux. Moreover, mice lacking PGC-1α expressed significantly lower levels of the mitophagy receptor Nix, both under steady state conditions and in response to denervation. In search of a mechanism for PGC-1α action, we measured protein expression and nuclear localization of TFEB, a transcriptional regulator of autophagy and lysosomal genes. We found that KO animals had reduced levels of TFEB, thus suggesting an interaction between the two transcriptional regulators. Thus, PGC-1α plays a role in autophagy and mitophagy flux both basally and in response to denervation, and this effect may involve an interaction with TFEB.

Objective 2: In order to further establish the effect of PGC-1α on autophagy during denervation, we used a gain-of-function model, where wildtype (WT) and PGC-1α overexpressing (Tg)

animals were denervated for 7 days. Overexpression of PGC-1α resulted in a more oxidative muscle phenotype which was protected against the denervation-induced loss of muscle mass, and mitochondrial content. Interestingly, we observed enhanced expression of the essential autophagy protein LC3, lysosomal representatives Lamp-2 and Cathepsin D, as well as the mitophagy receptor Nix in Tg animals compared to WT. We also noted a significant increase in TFEB protein levels with PGC-1α overexpression. Despite these findings, mitochondrial targeting for degradation in Tg animals was reduced both basally and with denervation. These results are in contrast with our hypothesis, but are consistent with the protective role of PGC-1α on mitochondrial content and function, as well as muscle mass with denervation. Thus, PGC-1α plays a role in the regulation of some autophagy and lysosomal proteins during denervation, but enhanced expression of the co-activator appears to protect mitochondria from degradation during denervation.

Objective 3: Autophagy induction has been documented to occur with exercise. Therefore, we wanted to confirm these findings and further investigate the activation of mitophagy following an acute bout of exercise, as well as to determine the role of PGC-1α in this process. To this end, we subjected WT and KO animal to an exhaustive bout of acute exercise (Ex) and a subset of animals was allowed to recover for 90 min (Ex+R). First we confirmed the importance of the co-activator in the induction of mitochondrial biogenesis following exercise, and noted a lack of increase in COXIV and Tfam post exercise in animals deficient in PGC-1α. We also illustrated that an acute bout of exhaustive exercise was sufficient to induce both autophagy and mitophagy, and that this activation was dependent on PGC-1α, as lack of the co-activator abolished exercise-mediated induction of these processes. Moreover, we observed that mitophagy induced with exercise appears to involve the E3 ubiquitin ligase parkin as well as ubiquitination of

mitochondrial substrates, and this too was mediated by PGC-1α. Thus, PGC-1α orchestrates mitochondrial turnover after exercise by enhancing both mitochondrial biogenesis and degradation.

Taken together, these studies confirm the fundamental role of PGC-1α in the regulation of mitochondrial content and health in skeletal muscle. We also indicate that PGC-1α plays a regulatory role in stress-induced autophagy/mitophagy in skeletal muscle in a stimulus-dependent manner, with the ultimate goal of maintaining metabolic homeostasis, thus contributing to muscle plasticity.

CHAPTER 7:

Future Directions

Based on the findings from the three studies outlined in this dissertation it is evident that PGC-1α is intimately involved in the regulation of autophagy and mitophagy during alterations in muscle contractile activity. It is also clear that autophagy/mitophagy and PGC-1α play a major role in muscle plasticity. However, several questions remain unanswered and could form the basis for future studies. A few of these are outlined below.

1) How does PGC-1α regulate autophagy in skeletal muscle?

Although our studies demonstrate a role for PGC-1α in the regulation of autophagy/mitophagy especially following exercise, the mechanisms by which PGC-1α operates remain unclear. Several viable culprits of PGC-1α may be responsible for its action, including transcription factors known to be co-activated by PGC-1α, such as PPARα and HIF-1α or other transcription factors not yet validated, such as TFEB and FoxOs, or others that remain to be identified. In our current studies we utilized animal models where the co-activator was chronically deleted in the whole body, or overexpressed in skeletal muscle. These can lead to complex cellular adaptations to the chronic alterations in PGC-1α. It would, therefore, be of interest to study the effects of acute deletion and overexpresion of PGC-1α in an isolated system using a cell culture model. Acute deletion of PGC-1α using siRNA or shRNA, would constitute a useful model to study the effects of lack of PGC-1α on the expression and flux of autophagy/mitophagy. Moreover, tandem-tagged probes that can be targeted to LC3 or the mitochondria exist, and could be useful for the dissection of autophagy and mitophagy progression in cellular models where PGC-1α levels are manipulated basally, and in response to

metabolic stress such as serum starvation. To try and tease out mechanism, and to confirm that the effects we observed on autophagy in our animal models were indeed directly mediated by PGC-1α, it would be useful to examine whether re-introduction of PGC-1α to cells lacking the co-activator would rescue autophagy/mitophagy. Moreover, to confirm interactors of PGC-1α, we could immuno-precipitate the co-activator and examine by immunoblot whether any of our predicted factors (i.e TFEB) co-precipitate.

2) What role does NPC1 play in skeletal muscle?

Using our array analysis we uncovered NPC1 to be under the control of PGC-1α. The role of this protein in skeletal muscle is completely unknown. Neimann-Pick C1 is a transmembrane protein responsible for the trafficking of lipids and cholesterol. Mutations in this protein results in Neimann-Pick type C1 disease, a devastating neurovisceral disease characterized by increased lipid and cholesterol load. However, a majority of studies examining the etiology of this disease have focused on neuronal cells. Since patients with the disease were found to be hypotonic, it would be of interest to investigate if this is a by-product of the neurodegeneration occurring in these patients, or if this effect is mediated by altered skeletal muscle metabolism and signaling. Since this protein has never been examined in the context of muscle health, we could start by investigating the expression of NPC1 in various atrophic conditions such as denervation or aging. However, to really delve into the role of this protein in skeletal muscle we could obtain NPC1 knockout animals that are commercially available (Jasckson labs). To determine the functional role of NPC1 in skeletal muscle, we would first have to characterise the muscle of these animal by measuring muscle mass and cross sectional area, as well as make functional measurements such as force generating capacity and fatigability. Then, to assess muscle morphology, we could use electron microscopy and histochemical stains such as hematoxylin

and eosin, as well as succinate dehydrogenase in order to get a crude estimate of mitochondrial mass. To see any abnormalities in lipid load we could use oil red O staining. Based on our findings, we could then further look at mitochondrial function and content in these animals as well as assess autophagy protein expression and flux. Since NPC1 has never been examined in the context of skeletal muscle, the options here are truly endless.

3) Does exercise induce autophagy and mitophagy in the heart?

Autophagy and mitophagy play an important role not only in skeletal muscle but also in cardiac tissue. Indeed, deficient autophagy/mitophagy in cardiomyocytes is detrimental and results in apoptotic cell death. Moreover, mitophagy is required for cardioprotection induced by pre-conditioning. During cardiac infarction autophagy/mitophagy are protective of cardiac tissue, and rescue cardiomyocytes from imminent cell death. Indeed, we have some preliminary data that autophagy is a highly active process in the heart, and that it is further activated during exercise. But the role of PGC-1α in cardiac autophagy/mitophagy has never been documented. It would therefore be of interest to investigate autophagy and mitophagy activation in the heart following acute exercise, and to determine the role of PGC-1α in these processes. Since cardiovascular disease is currently the second leading cause of death in Canada (Statistics Canada) this could have vast implications for public health.

APPENDIX A- Additional Data

The role of PGC-1α in the unfolded protein response

Rationale

In chapters 4 and 5 we established a regulatory role for PGC-1α in autophagy and mitophagy during varying muscle contractile states. However, the mechanism by which PGC-1α regulates autophagy has not been elucidated. The unfolded protein response (UPR) is a cellular stress response system that is activated when unfolded or misfolded proteins accumulate in the lumen of the endoplasmic reticulum (ER). The ultimate goal of this mechanism is to restore cellular homeostasis by reducing protein translation, increasing protein degradation and boosting ER folding capacity. However, if these efforts fail to restore steady state, the UPR can trigger apoptosis. Indeed, various UPR factors have been documented to enhance autophagy in response to cellular distress in order to avoid cellular demise (1–3, 5). Moreover, PGC-1α has been documented to modulate UPR in response to exercise via co-activation of the transcription factor ATF6α (4). The PGC-1α-UPR axis has also been deemed important for mediating exercise-induced adaptations. However, it is not clear what role PGC-1α plays in the UPR under altering muscle contractile states and if this interaction contributes to autophagy induction. Therefore, in this study we investigate the role of PGC-1α in the regulation of UPR protein expression in skeletal muscle utilising both loss and gain of function models under basal conditions, and in response to denervation, and acute exercise.

Results

Unfolded protein response is enhanced in PGC-1α Tg animals. In order to investigate the role of PGC-1α on the basal expression of proteins involved in the UPR we utilised wild type (WT), PGC-1α KO (KO) as well as PGC-1α Tg (Tg) animals. Western blot analysis revealed that despite a trend there was no significant difference in the expression of the ER chaperone Bip (Fig. 1B) or the transcription factors CHOP (Fig. 1C) and ATF6 (Fig. 1F) between WT animals and KO or Tg animals. However, we did find a significant ~5.5-fold difference in Bip expression between KO and Tg animals. We also observed 2.4- and 2.5-fold higher CHOP and ATF6 expression in Tg compared to KO animals. Protein levels of p-eif2α were similar between WT and KO animals, but were significantly induced in Tg animals (Fig. 1D).

Unfolded protein response is induced by denervation. To assess the involvement of PGC-1α in UPR during denervation WT, KO and Tg animals were unilaterally denervated by severing the sciatic nerve, while the contralateral limb served as an internal control. Denervation resulted in a 2-fold decrease in Bip protein levels in WT animals, while lack of PGC-1α resulted in a 10-fold lower overall Bip protein expression with no further changes following denervation (Fig 2B). CHOP protein levels were induced 3.5- and 2.5-fold with denervation in WT and KO animals, respectively (Fig 2C). Phosphorylation of eif2α was similarly induced by ~3-fold in both WT and KO animals (Fig 2D).

PGC-1α overexpression resulted in greater basal levels of Bip protein, which were not further altered by denervation in the Tg animals (Fig. 3B). CHOP and p-eif2α protein levels were basally higher in Tg animals when compared to their WT counterparts and Tg animals were also better able to up-regulate these proteins in response to denervation (Fig. 2C-D). This resulted in additive, 17- and 2-fold inductions in CHOP and p-eif2α, respectively, in denevated Tg animals when compared to WT controls.

Unfolded protein response in WT and PGC-1α KO animals in response to an acute bout of exhaustive exercise. PGC-1α has been previously documented to upregulate the UPR in response to an acute bout of exercise. We, therefore, investigated the expression of UPR genes and proteins in WT and PGC-1α KO animals immediately following an acute bout of exhaustive exercise and after a 90min recovery period. Bip mRNA was induced by 2.8-fold immediately following exercise in WT animals only, and this exercise-induced increase was abolished by the lack of PGC-1α (Fig. 4A). Moreover, Bip protein levels progressively declined following exercise in WT animals only (Fig. 4C). Bip protein levels were overall lower in KO animals and were not further altered by exercise. CHOP levels were significantly induced immediately following exercise in WT animals only, while KO animals expressed overall lower CHOP levels which were not further altered with exercise (Fig. 4D). We did not find any variations in the levels of p-eif2α following exercise in either WT or KO animals (Fig. 4F).

Conclusions

The unfolded protein response is a major cellular quality control mechanism that is activated when the ER is overwhelmed by damaged proteins. Our results indicate that the UPR is activated in skeletal muscle during alterations in metabolic demands and PGC-1α appears to be involved in this process. Interestingly, two seemingly opposing stimuli: muscle disuse, such as denervation and increased contractility such as exercise, both resulted in enhanced activation of the UPR. Moreover, although it appears that overall activation of the UPR is perturbed when PGC-1α is lacking and enhanced when the co-activator is overexpressed, the relationship between PGC-1α and the UPR appears complex and stimulus as well as factor specific. Under basal conditions differences in UPR protein expression could only be detected when PGC-1α levels were vastly different, i.e between KO and Tg animals and not WT animals. Moreover, PGC-1α overexpression, enhanced the UPR during denervation. However, the lack of PGC-1α appeared to attenuate UPR only following exercise with little effect on UPR induction during denervation, with the exception of Bip. Indeed, PGC-1α has been previously documented to regulate Bip expression though ATF6α (4). Therefore, PGC-1α appears to play a complex role in the regulation of the UPR, which provides further insight into the many ways by which this co-activator contributes to skeletal muscle metabolic plasticity.

Fig.1

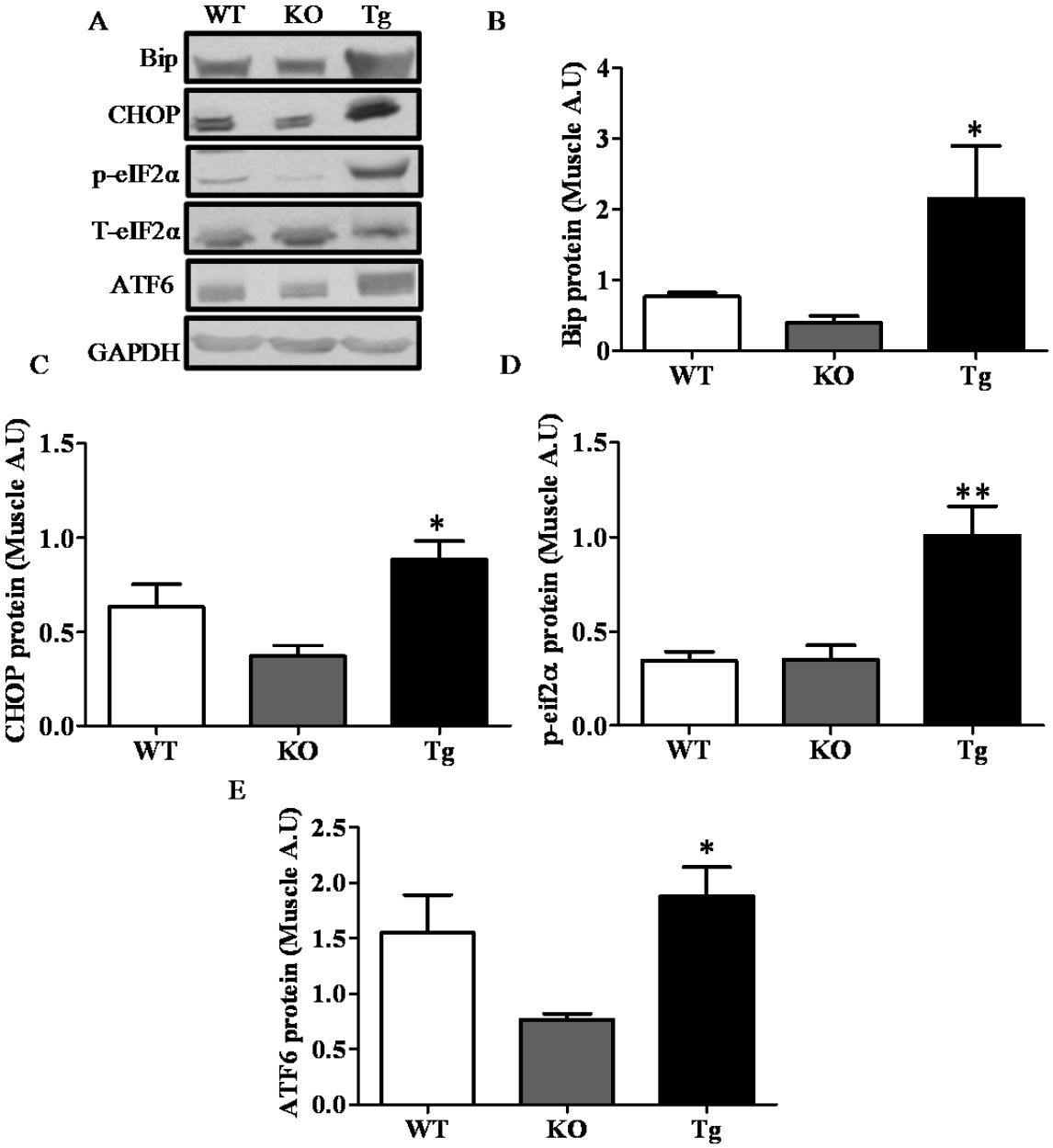


Figure 1. Unfolded protein response in WT, PGC-1α KO and Tg animals A-E. Blots and quantification of UPR-related protein expression in TA muscle in WT, KO, and Tg animals. A. Representative blots. Quantification of B. Bip; C. CHOP; D. p-eif2α. E. ATF6.*P<0.05 significant difference between KO and Tg. **P<0.05 significant difference between Tg and both WT and KO effect of genotype. GAPDH was used as a loading control (n=4 for all groups).

Fig.2

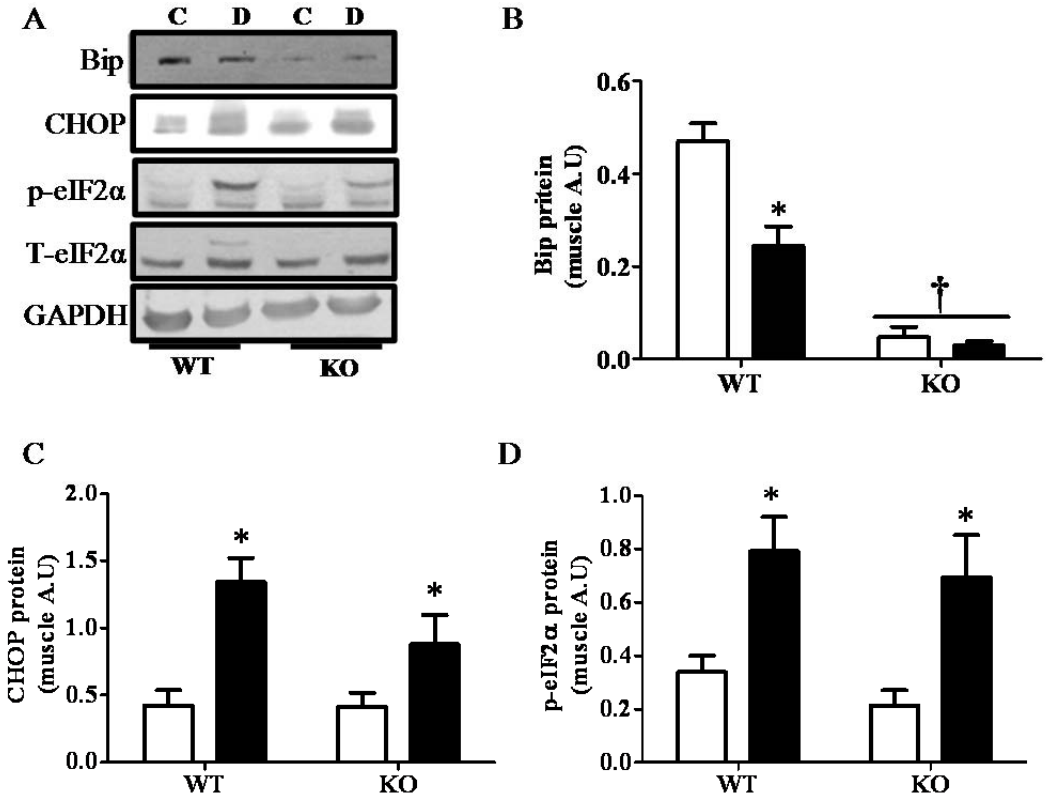


Figure 2. Unfolded protein response in WT and PGC-1α KO animals in response to denervation. A-D. Blots and quantification of UPR-related protein expression in TA muscle in Con, Den, WT and KO animals. A. Representative blots. Quantification of B. Bip; C. CHOP; D. p-eif2α. *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH was used as a loading control (n=4 for all groups).

Fig.3

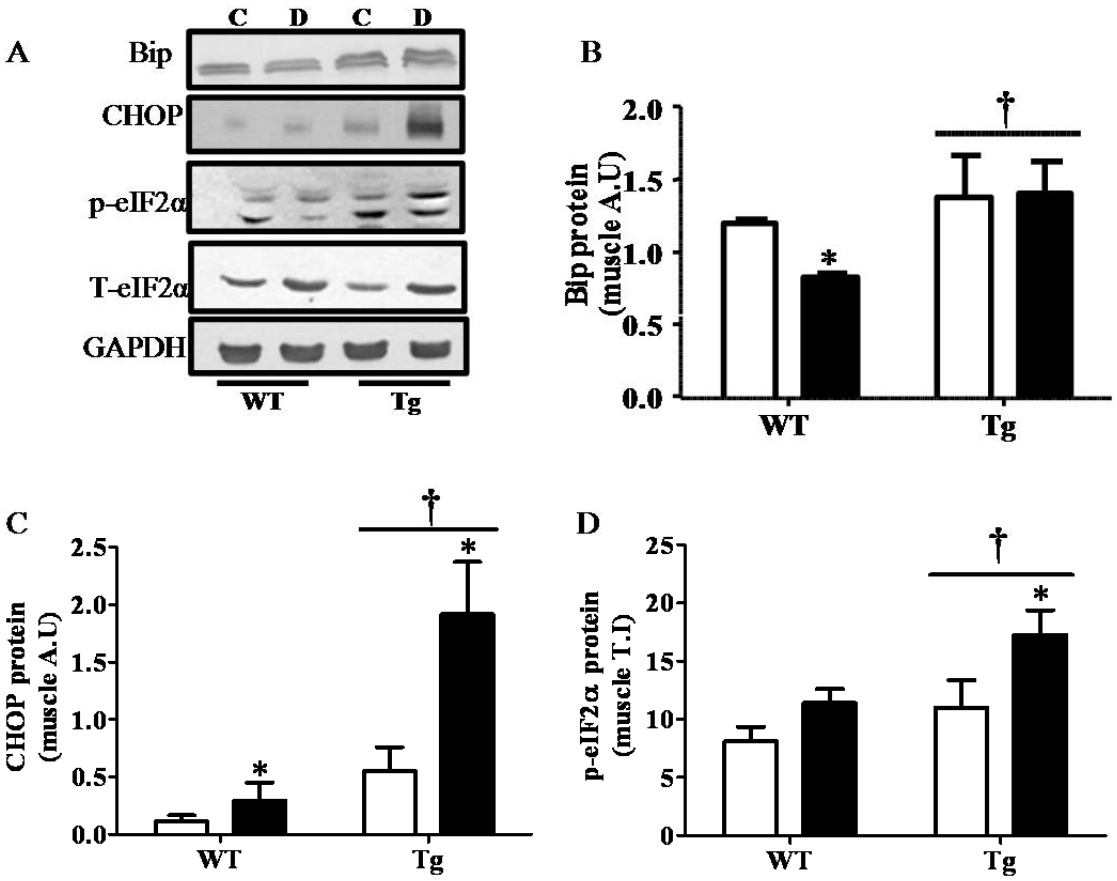


Figure 3. Unfolded protein response in WT and PGC-1α Tg animals in response to denervation. A-D. Blots and quantification of UPR-related protein expression in TA muscle in Con, Den, WT and Tg animals. A. Representative blots. Quantification of B. Bip; C. CHOP; D. p-eif2α. *P<0.05 significant difference between Con and Den. †P<0.05 significant effect of genotype. GAPDH was used as a loading control (n=4 for all groups).

Fig.4

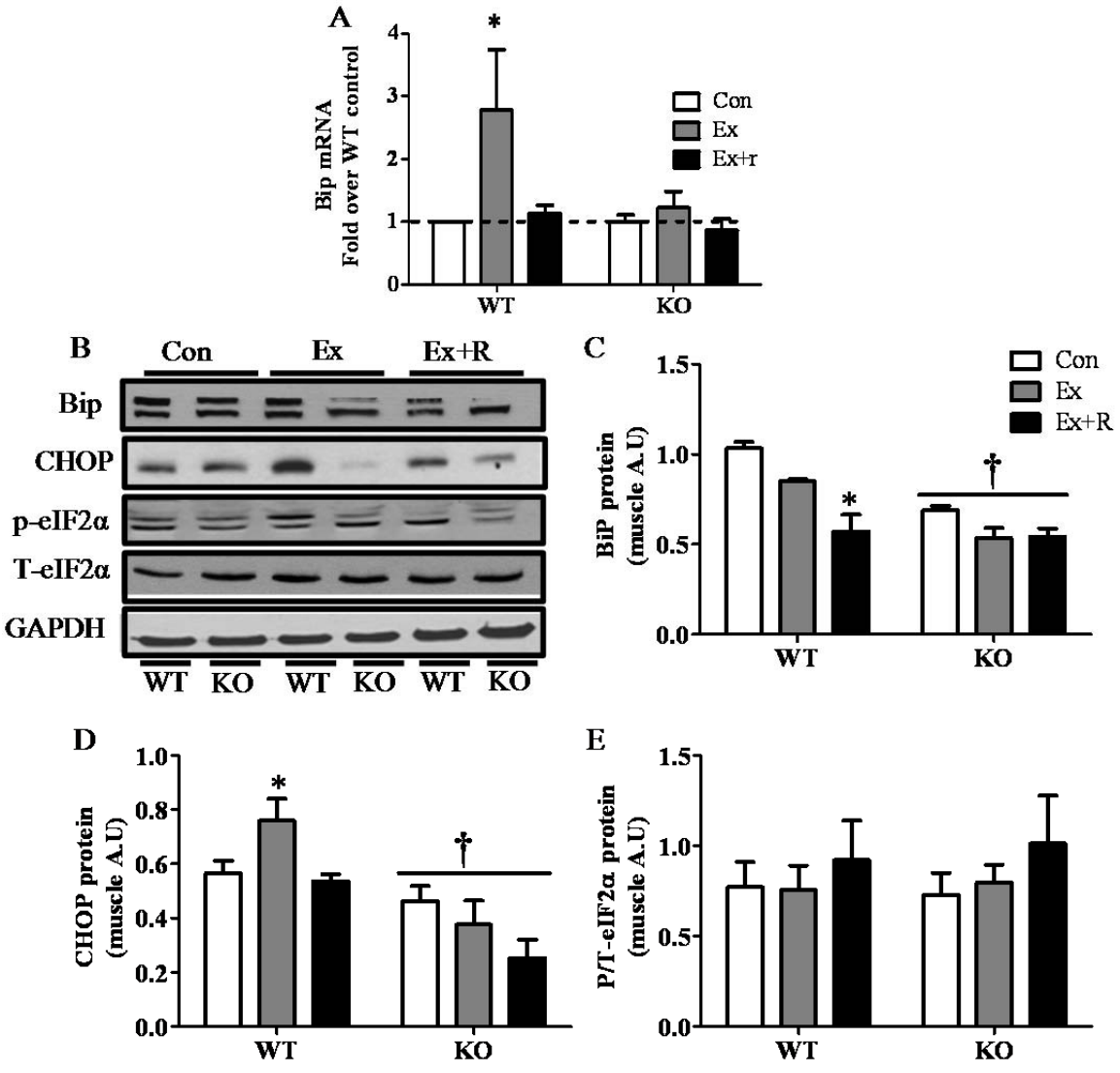


Figure 4. Unfolded protein response in WT and PGC-1α KO animals in response to an acute bout of exhaustive exercise and 90 min of recovery. A Bip gene expression measured by real time PCR. mRNA fold change between wild type (WT) and PGC-1α KO (KO) control (Con) and Exercise (Ex) and exercise with recovery (Ex+R). All groups were compared to WT Con, and Gapdh and Actb were used as housekeeping genes. B-E. Blots and quantification of UPR-related protein expression in TA muscle in Con, Ex, and Ex+R in WT and KO animals. B. Representative blots. Quantification of C. Bip; D. CHOP; E. p-eif2α. *P<0.05 significant effect of exercise. †P<0.05 significant effect of genotype. GAPDH and total protein were used as loading controls where appropriate (n=7-9 for all groups).

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APPENDIX B- Extended Procedures

Protein Extracts from muscle sections

Reagents:

1. Lysis buffer: (Make stock and aliquot, store at -20)
   50mM Tris pH 7.5
   150mM NaCl
   10mM $MgCl_2$
   0.5mM DTT
   1mM EDTA
   10% Glycerol
2. 20% SDS stock
3. 10% Triton x-100 stock
4. Protease and Phosphatase inhibitors: Complete 50x stock (from tablet Roche) , phosphatase inhibitor cocktail 2 (sigma #P5726) and phosphatase cocktail 3 (sigma #P0044) (both cocktails come as 100x stock)

Procedure:

1. Cut muscle frozen in liquid N_2

- 20 sections 20um for Gastrocnemius (can also domore i.e 40 sections)
- 40 sections 20um for Tibialis A nterior
- EDL: *pulverize with mortar
  *re suspend in 200ul of lysis buffer
  * Homogenize with pestle

Place eppendorf with cut sections into Liq. N_2 immediately when finished cutting to avoid thawing.

2. Make working stock of lysis buffer with final concentrations of 2% SDS, 1% Triton x-100 detergents and 1x protease/phosphatase inhibitors. (100ul of stock per sample) .

i.e: For 1ml of lysis buffer add
100ul of 20% SDS
100ul of 10% Triton x-100
20ul of complete
10 ul phosphatase inhibitor cocktail 2
10ul phosphatase inhibitor cocktail 3

3. Take eppendofs with cut sections out of Liq. N_2 and place on ice with open lids (to avoid explosion), be careful not to spill any sections that are on the lid or the sides. Add 100ul of working lysis buffer stock to each sample. If some sections are stuck on the side or in the lid, briefly centrifuge.
4. Thermomix for 10 min at 70ºC at 700rpm in thermomixer
5. Centrifuge for 10 min at 4ºC at 13,000rpm
6. Collect supernate into a newly labelled eppendorfs
7. Quantify protein concentration with BCA/ Bradford, freeze leftover lysis buffer to use as blank for [protein].

Single fiber fixation isolation and Immunofluorescence

Immunofluorescence staining of isolated fixed EDL fibers for imaging using a confocal microscope. (Adapted from **Raben N, Shea L, Hill V, Plotz P**. Monitoring autophagy in lysosomal storage disorders. *Methods Enzymol* 453:417-49, 2009.)

Materials and Reagents:

1. 2% Paraformaldehyde in 0.1 M sodium phosphate buffer:
    a) Make by mixing equal volumes of :
    b) 4% paraformaldehyde and
    c) 0.2M phosphate buffer, pH 7.2. For 100ml: Add 1.93g Na_2HPO_4 and 0.75g NaH_2PO_4 volume up to 100 with H_2O.
5. PBS
6. 6 well plate with silicone in the wells and pins
7. Glass slides and cover slips
8. 50% glycerol in PBS
9. 0.04% saponin
10. Blocking solution 10% goat serum in PBS
11. 0.2% Triton X-100 in a 10% goat serum blocking solution in PBS.
12. Primary Ab in 10% goat serum in PBS
13. 0.5ug/ml of Dapi in PBS
14. Fluorescent secondary Ab in 10% goat serum in PBS
15. Mounting medium

Muscle fixation, and fiber isolation procedure:

1. Carefully isolate and excise EDL muscle, anochor the muscle onto the silicon covered 6 well dish with pins and submerge in 2% paraformaldehyde in phosphate buffer for at least 1h at room temperature.
2. Wash muscle with PBS and either dissociate immediately or place in 50% glycerol at 4˚C overnight, then store in -20 until further use.
3. After long term storage gradually transition muscle through diminishing concentrations of glycerol and mechanically tease apart in a puddle of 0.04% saponin using fine forceps.
4. Mount single fibers onto glass slide

Fiber immunostaining Procedure:

1. Permeabilize isolated fibers with 0.2% Triton X-100 in a 10% goat serum blocking solution.
2. Incubate fibers with the appropriate primary antibody diluted in blocking solution overnight at 4 °C.
3. Carefully wash fibers with PBS 3x5min
4. Incubate for 2 h at room temperature with the suitable fluorescent secondary antibody. (From here on make sure to protect fibers from light)
5. Carefully wash fibers with PBS 3x5min, add Dapi at a 0.5 μg/ml concentration to the first wash (in order to visualize the myonuclei).
6. Mount glass cover slip and dry thoroughly. Keep refrigerated and protected from light until imaging.

APPENDIX C- Other Contributions

During my doctoral studies, I made the following contributions to literature that are not included in my dissertation:

Peer-Reviewed publications

6. Tryon L.T., **Vainshtein A**., Memme J., Crilly M.J., Hood D.A. Recent advances in mitochondrial turnover during chronic muscle disuse. Integrative Medicine Research, 3(4): 153-220, 2014.

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1. O'Leary M.F.N., **Vainshtein A.**, Carter H.N., Zhang Y., and Hood D.A. Denervation-induced mitochondrial dysfunction and Autophagy in skeletal muscle of apoptosis-deficient animals. Am J Physiol Cell Physiol. 303(4):C447-54, 2012.

Published abstracts and conference proceedings

15. **Vainshtein A.**, Desjardins E.M., Sandri M., Hood D.A. PGC-1α at the Crossroads of Mitophagy and Mitochondrial Biogenesis. *Gordon Research Seminar - Autophagy in Stress, Development & Disease.* Il Ciocco, Italy, 2014. Selected speaker

14. **Vainshtein A.**, Desjardins E.M., Sandri M., Hood D.A. PGC-1α, a novel regulator of muscle autophagy? *Gordon Conference series - Autophagy in Stress, Development & Disease*. Il Ciocco, Italy, 2014.

13. **Vainshtein A.**, Desjardins E.M., Sandri M., Hood D.A. Mitochondria meet their maker: how PGC-1α regulates mitochondrial degradation via autophagy. *Proceedings of the Ontario Exercise Physiology Meeting, 2014.*

12. Carter H.N., Crilly M.J., O'Leary M.F.N., **Vainshtein A.**, Ostojic O. and Hood D.A. Chronic contractile activity induces a transcriptional profile to restore muscle mitochondrial

homeostasis with age. *Appl Physiol Nutr and Metab* 38, 2013.

11. Carter H.N., O'Leary M.F.N., Iqbal S.*, **Vainshtein A.***, Ostojic O.*, Pastore S.F. and Hood D.A. Profiling Mitochondrial Turnover in Aged Skeletal Muscle: Mitophagy Arrested? *Proceedings of the Muscle Health Awareness.* 2013. * contributed equally

10. **Vainshtein A.**, Zhang Y., Saleem A., Menzies K.J., and Hood D.A. Chronic disuse-induced autophagy is partially dependent on PGC1-alpha. *Appl Physiol Nutr and Metab* 38: Supplement 3, S550, 2012.

9. Carter H.N., O'Leary M.F.N., Iqbal S.*, **Vainshtein A**.*, Ostojic O.*, Pastore S.F., and Hood D.A. Profiling Mitochondrial Turnover in Aged Skeletal Muscle: Mitophagy Arrested? *Proceedings of the Muscle Health Awareness.* p.28 ,2012. * Contributed equally.

8. **Vainshtein A.**, and Hood D.A. Skeletal Muscle autophagic signaling in PGC-1alpha Knockout animals. *Gordon Conference series - Autophagy in Stress, Development & Disease*. Ventura, CA, 2012.

7. **Vainshtein A.**, Uguccioni G., and Hood D.A. PGC-1alpha-mediated modulation of autophagy. *Appl Physiol Nutr and Metab* 36: Supplement 2, S355, 2011.

6. Pastore S., **Vainshtein A.**, Hood D.A. The effects of exercise on circadian clock protein expression in skeletal muscle. *Appl Physiol Nutr and Metab* 36: Supplement 2, S344, 2011.

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