MECHANISMS OF p53-INDUCED MITOCHONDRIAL BIOGENESIS IN SKELETAL MUSCLE

AYESHA SALEEM

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ABSTRACT

p53 is a pleiotropic protein that is mutated in many types of cancers. It is involved in regulating various distinct cellular pathways including apoptosis, cell cycle arrest, senescence, autophagy, angiogenesis, and most relevant to this dissertation, cellular metabolism. While extensive research has been conducted on p53 in cancer biology, the role of this protein in modulating mitochondrial function in skeletal muscle has only been recently investigated. Thus the aim of this dissertation was to assess the mechanisms by which p53 induces and controls aerobic metabolism in skeletal muscle, and the subsequent impact it carries on exercise-mediated pathways that elicit an increase in mitochondrial bioenergetic capacity.

First, we examined the effect of p53 on mitochondrial protein import and complex IV assembly. Using subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria isolated from p53 wildtype (WT) and knockout (KO) mice, we discerned no effects of p53 on the rate of protein import. However, the expression of several proteins involved in the import process was reduced in the KO mice. Assembly of complex IV was impaired in the IMF mitochondria, along with the assembly co-factor Surf1, which may facilitate the previously documented attenuation of mitochondrial function in the p53 KO mice.

Next we evaluated whether p53 is recruited in response to endurance exercise. To assess this, we subjected C57Bl/6 mice to an acute run at 15m/min for 90 minutes $\pm$ 3 hours recovery, and subsequently measured alterations in content, and sub-cellular localization of p53. The nuclear p53 content decreased steadily with acute exercise and post-recovery, in sharp contrast to the increase in p53 abundance in SS and IMF

mitochondria. Concomitantly, higher levels of mitochondrial p53 were complexed with Tfam, the mitochondrial DNA (mtDNA) transcription factor, and with mtDNA at the D-loop with exercise and recovery. We identified putative p53 response elements in the D-loop, and hypothesized that p53 could be mediating mtDNA transcription in collaboration with Tfam. Further support for this was derived from the observation that the exercise-induced increase in mtDNA-transcribed protein COX-I was completely abrogated in p53 KO mice.

Lastly, we sought to determine the necessity of p53 to the exercise-induced changes that transpire within the muscle upon an imposed metabolic and physiological challenge such as a bout of endurance exercise. p38 MAPK activation was abolished, whereas AMPK and CaMKII signalling was attenuated with exercise in p53 KO mice. This occurred in tandem with lower levels of PGC-1α translocation into the nucleus, and subsequently attenuated the increase in mRNA content of genes involved in mitochondrial biogenesis. While non-exercised p53 KO mice displayed an impaired ability to undergo autophagy during basal conditions, there was no difference in the activation of autophagic proteins post-exercise in p53 KO vs. WT mice.

Collectively, our data illustrate that p53 is integral to maintaining baseline levels of mitochondrial content and function, and is intimately involved in, and necessary for the signaling process initiated upon acute endurance exercise that mediates mitochondrial biogenesis.

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LIST OF ABBREVIATIONS

$\Delta\Psi m$	mitochondrial membrane potential
2D	two dimensional
ACC	acetyl-CoA carboxylase
ADP	adenine diphosphate
AICAR	5-aminoimidazole-4-carboxamide riboside
AE	acute exercise
AER	acute exercise plus recovery
AIF	apoptosis-inducing factor
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANT	adenine nucleotide translocase
ATF2	activating transcription factor-2
ATP	adenosine triphosphate
ATG7	autophagy-related protein 7
BDNF	brain-derived neurotrophic factor
BN-PAGE	Blue Native PAGE
Ca^{2+}	calcium
CaMK	calcium/calmodulin-dependent protein kinase II
Caspase	cysteine-aspartic acid proteases
Cpn10	chaperonin 10
COX	cytochrome *c* oxcidase
COX I	cytochrome *c* oxidase subunit I
COXIV	cytochome *c* oxidase subunit IV
CPT1	carnitine palmitoyl transferase
CREB	cAMP response element binding protein
CS	citrate synthase
CtsD	cathepsin D
DAPK-1	death-associated protein kinase 1
DBD	DNA binding domain
DRAM	damage-related autophagy modulator
Drp1	dynamin-related protein 1
FGF2	fibroblast growth factor 2
FSTL-1	follistatin-like 1
HAUSP	herpesvirus associated ubiquitin-specific protease
Hsp	heat shock protein
ECL	enhanced chemiluminescence
ERK	extracellular signal-regulated kinase
ERR	estrogen-related receptor
ETC	electron transport chain
IGF-1	insulin-like growth factor-1
GAPHD	glyceraldehyde phosphate dehydrogenase
GLUT4	glucose transporter 4

IL	interleukin
IM	inner mitochondrial membrane
IMF	intermyofibrillar mitochondria
JNK	c-Jun N-terminal kinase
KO	knockout
Lamp2	lysosomal-associated membrane protein 2
LC3	light chain 3
LIF	leukemia inhibitory factor
MAPK	mitogen-associated protein kinase
Mdm2	mouse double minute 2
MEF2	myocyte enhancing factor 2
MHC	myosin heavy chain
Miro	mitochondrial rho
mM	millimolar
MPP	mitochondrial processing peptidase
mRNA	messenger ribonucleic acid
MSF	mitochondrial import stimulating factor
mtDNA	mitochondrial DNA
mtPTP	mitochondrial permeability transition pore
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex I
NES	nuclear export signal
NLS	nuclear localization signal
NMJ	neuromuscular junction
NRF-1/2	nuclear respiratory factor-1/2
OCT	ornithine transcarbamylase
OM	outer mitochondrial membrane
PAM	presequence translocase-associated motor
PAGE	polyacrylmide gel electrophoresis
PCR	polymerase chain reaction
PFT α	pifithrin-alpha
PGC-1α	PPAR-γ coactivator-1α
PGM	phosphoglycerate mutase
PINK1	PTEN-induced putative kinase 1
pol γ	mtDNA polymerase gamma
PPAR	peroxisome proliferator associated receptor alpha
PRD	praline-rich domain
PRC	PGC-1 related co-activator
PTEN	phosphatase and tensin homolog
rRNA	ribosomal ribonucleic acid
ROS	reactive oxygen species
RXR	retinoic X receptor
SAM	sorting and assembly machinery
SCO2	synthesis of cytochrome c oxidase 2

SDH	succinate dehydrogenase
SIRT1	sirtuin 1
SED	sedentary
Ser	serine
SR	sarcoplasmic reticulum
SS	subsarcolemmal
Surf1	surfeit locus protein 1
TAD	transcriptional activation domain
TET	tetramerization motif
Tfam	mitochondrial transcription factor A
TFB1M	transcription factor beta 1 mitochondria
TFB2M	transcription factor beta 2 mitochondria
Thr	threonine
TIGAR	tp53-induced glycolysis and apoptosis regulator
TIM	translocase of the inner membrane
TOM	translocase of the outer membrane
TSC2	tuberous sclerosis complex 2
µM	micromolar
Ub	ubiquitin
UV	ultraviolet
VDAC	voltage dependent anion channel
WT	wildtype
YY1	initiator element binding factor 1

CHAPTER 1:

INTRODUCTION

Individuals over the age of 65 years represent one of the fastest growing segments of the North American population. In elderly population, cancer is fast becoming an epidemic as approximately 40% of men and women in Canada will develop some form of this disease, and and approximately 1 out of 4 Canadians will die from cancer. (Canadian *Cancer Statistics*, 2009). The increasing costs associated with the care and management of cancer and associated co-morbidities are financially unfeasible and overburden our healthcare system. It is thus imperative that the molecular biology and pathology of cancer is studied to re-define and fine-tune potential therapeutic strategies available for cancer treatment and management.

The importance of the tumor suppressor protein p53 protein can be gleaned from the fact that over 50% of all human cancers carry a mutation in the *p53* gene. Re-introduction of p53 or restoration of its activity is sufficient to suppress the growth of tumors, both *in vitro* and when transplanted into mice (95; 139; 155). Following genotoxic stress, p53 is recruited within minutes to either induce cell cycle arrest and coordinate DNA repair, or depending on the extent of the damage, initiate cell death (141). Recent research has shed light on the ability of p53 to respond to more physiological stressors as well (141). p53 regulates cellular anti-oxidant defence, angiogenesis, fertility, autophagy, and orchestrating a balance between the anabolic and catabolic pathways within the cell (141). Additionally, in the last few years, research has demonstrated that p53 also plays a vital role in controlling cell metabolism. Since it is

commonly accepted that metabolic perturbations are a hallmark of cancer progression (45; 145), metabolism is thus being studied as a possible therapeutic target in cancer treatment, making p53-mediated regulation of cellular energetics a hot topic of investigation.

Increasingly sedentary lifestyles coupled with excess caloric intake have resulted in the emergence of inactivity-related chronic diseases and co-morbidities. Utilizing endurance exercise as a viable therapeutic modality for the treatment and management of age-associated chronic diseases is gaining wide acceptance by the medical community (146). In addition to improving metabolic performance, a plethora of epidemiological studies have shown that regular physical activity extends life expectancy and reduces morbidity. Elucidating the underlying causes and molecular signaling events, such as the role of p53 in the adaptive response to endurance exercise, carries great significance for the treatment of physical inactivity- and impaired metabolism-related diseases such as obesity, insulin resistance, type 2 diabetes, cancer and cardiovascular diseases. Since the primary adaptation to a regimen of endurance exercise is an increase in mitochondrial oxidative capacity, it is imperative to gain a thorough understanding of the canonical pathway of initiation and regulation of this process.

CHAPTER 2:

LITERATURE REVIEW

2.1. Overview of exercise-mediated mitochondrial biogenesis

A highly malleable tissue, skeletal muscle exhibits a remarkable range of plasticity in response to a number of physiological and pathophysiological stimuli. With endurance exercise training, there is a change in substrate metabolism, an increase in mitochondrial content, and an improved exercise tolerance. Since this was first noted many years ago (58), many researchers have confirmed and extended these observations. Exercise-mediated enhancement of muscle mitochondrial content is now a well established phenomenon in exercise physiology (59), and this increase in mitochondrial content per gram of tissue with exercise is termed mitochondrial biogenesis. This is evident in both subsarcolemmal (SS) mitochondria, those located underneath the sarcolemma membrane, and in intermyofibrillar (IMF) mitochondria, those interspersed between the myofibrils (89).

Cellular mediators of metabolic adaptations that respond to endurance exercise stimuli are complex, and are subject to multiple facets of regulation at the transcriptional and translational level. The exercise-mediated increase in mitochondrial content is largely due to an extension of the existing reticulum rather than de novo organelle synthesis (132). Notwithstanding being a main focus of research in exercise physiology, it is of great importance to continue to explore and delineate the underlying mechanisms of

mitochondrial biogenesis in order to complement the new data that emerge continually and further our understanding of this process.

The process of mitochondrial biogenesis is complex, requiring the coordinated induction of over 1,500 proteins encoded by both the nuclear and mitochondrial genomes as recently reviewed (124). Control over this process is mediated by a number of transcriptional regulators, including the members of the peroxisomal proliferator-activated receptor (PPAR) γ coativator-1 (PGC-1) family, nuclear respiratory factor 1/2 (NRF-1/2), cAMP response element binding protein (CREB), initiator element binding factor (YY1), estrogen-related receptor (ERR) α/γ, thyroid hormone receptor family, and myocyte-enhancing factor 2 (MEF2), among several others. Of the numerous studies devoted to defining the transcriptional regulation of mitochondrial biogenesis, an exciting and emerging role for p53 has surfaced (see below). However, of this myriad of factors, the lion's share of the attention has been given to the transcriptional co-activator, PGC-1α. The process itself begins much before the activation of these transcription factors, with the recruitment of cellular signalling kinases.

2.1.1. Signalling pathways regulating exercise-mediated mitochondrial adaptations

It is generally accepted that the increase in contractile activity, and subsequent metabolic demands during acute exercise, trigger various signalling cascades that converge to augment mitochondrial and metabolic gene transcription (27; 71). The cumulative effects of these repeated changes in gene transcription result in the incorporation of new skeletal muscle proteins to constitute the adaptive response over the

course of training. The precise signal transduction pathways are not completely understood, but evidence suggests that adenosine monophosphate (AMP), calcium, and reactive oxygen species (ROS)-induced activation of AMP-activated protein kinase (AMPK), calcium/calmodulin-activated protein kinase (CaMK), and p38 mitogen-activated protein kinase (MAPK), respectively, are key molecular events linked with muscle adaptation (60-62; 89). Activation of each of these signalling pathways in skeletal muscle is associated with increased mitochondrial content, likely through direct and indirect activation of regulatory transcription factors (5; 112; 154). Furthermore, these kinases increase their activity in response to exercise in a dose-dependent (intensity and duration of contractile activity) manner, and also based on the fiber-type composition of the muscle (88). More recently, a growing body of work has provided evidence for myokines, muscle-derived factors secreted upon exercise that can subsequently act in an autocrine or paracrine fashion to induce systemic adaptations subsequent to exercise training (107).

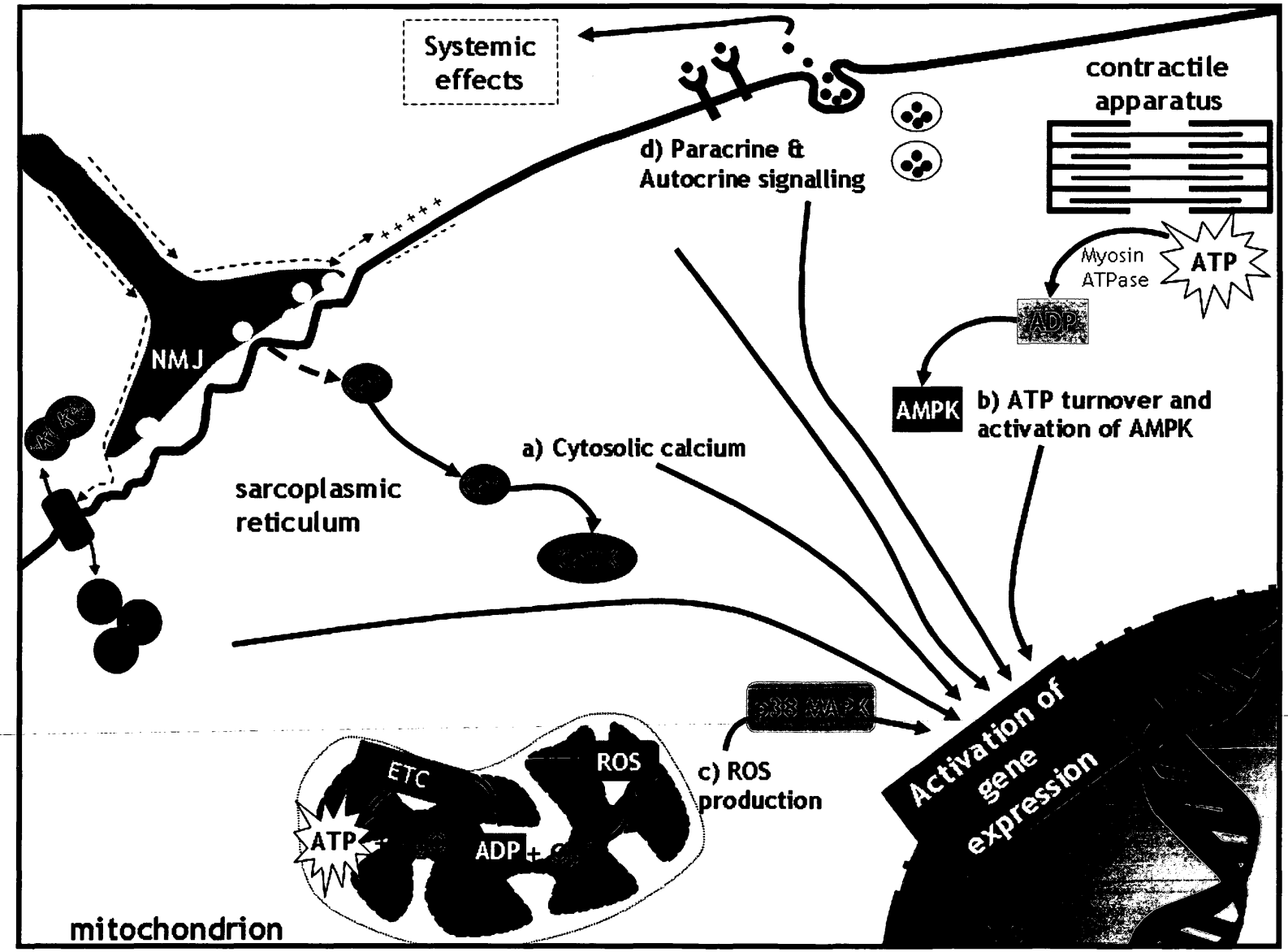
Systemic effects
contractile apparatus
d) Paracrine & Autocrine signalling
Myosin ATPase
ATP
ADP
AMPK
b) ATP turnover and activation of AMPK
NMJ
a) Cytosolic calcium
sarcoplasmic reticulum
p38 MAPK
ROS
ETC
ATP
ADP
c) ROS production
Activation of gene expression
mitochondrion

Figure 1. Overview of key intracellular signaling pathways hypothesized to mediate exercise-induced mitochondrial biogenesis in a skeletal muscle cell. (a) Upon motor unit recruitment, the action potential travels down a neuron and at the neuromuscular junction (NMJ), causes acetylcholine to be released into the synaptic cleft. Acetylcholine binds to its receptors on the post-synaptic muscle membrane, causing ligand-gated sodium (Na^+) channels to open and allow three Na^+ molecules in for every two potassium (K^+) molecules out of the cell thereby depolarizing the cell. As the action potential propagates across the sarcolemma membrane, it causes the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR). Calcium allows the cell to undergo contraction, while at the same time activating a series of kinases including Ca^{2+}/calmodulin-dependent protein kinase (CaMK). (b) Calcium binds to troponin and potentiates the interaction of actin and myosin, causing adenine triphosphate (ATP) to be broken down to produce adenine diphosphate (ADP) which is further processed to produce monophosphate (AMP). This results in the activation of AMP-dependant protein kinase (AMPK) via allosteric modulation by AMP. (c) ADP is also responsible for initiating the process of oxidative phosphorylation in the mitochondria, wherein oxygen is consumed along with ADP to produce ATP and water by the electron transport chain (ETC). Approximately 2-5% of the oxygen consumed is diverted to produce reactive oxygen species (ROS) which can either be quenched by the resident mitochondrial anti-oxidants, or exit the organelle to activate stress-responsive proteins or kinases such as p38 mitogen-activated protein kinase (p38 MAPK). (d) Lastly, exercise has been shown to result in the release of myokines, or muscle-derived humoral factors that are released into the blood to act in a paracrine or autocrine manner to induce systemic, or local mitochondrial biogenesis, respectively.

2.1.1.1. CaMK

Contractile activity or exercise results in an elevation in intracellular cytosolic Ca^{2+} concentration in skeletal muscle. Resting Ca^{2+} concentrations of 10-30 nM can be increased up to 100-300 nM in slow-twitch fibers, and 1-2 μM in fast-twitch fibers upon exercise (54). Augmented Ca^{2+} levels can mediate gene expression by activating key Ca^{2+}-depedent enzymes such as calcineurin, protein kinase C (PKC) and calcium/calmodulin-depedent protein kinase II, and IV (CaMKII, CaMKIV; (26). Support for Ca^{2+}-mediated increase in mitochondrial biogenesis is primarily derived from cell culture, where myocytes treated with calcium ionophores such A23187, ionomycin, or with caffeine, display a subsequent increase in mitochondrial enzymes and proteins, and regulatory transcriptional factors at the mRNA and protein level (26). Transgenic mice with constitutively active form of CaMKIV display increased muscle PGC-1α expression, and mitochondrial biogenesis (154), however CaMKIV knockout mice do not differ from wild-type littermates in the response to endurance training, including an up-regulation of PGC-1α (6). Furthermore, since CaMKIV is primarily found in neural tissue, its contribution to adaptations in mitochondrial biogenesis in skeletal muscle is thought to be limited. This indicates that other CaMK isoforms and Ca^{2+}-activated enzymes may play a vital role in regulating mitochondrial biogenesis in response to exercise. CaMKII has been shown to respond to both acute and chronic exercise, and its activation is positively correlated with increased oxidative capacity in skeletal muscle. While less is known about CaMKII in translating the metabolic adaptations, it has been illustrated to play a role in stimulating glucose uptake by regulating the expression of

GLUT4 (154). CaMKII is primarily regulated by autophosphorylation, and is thought to be activated by Ca^{2+} levels in a dose-dependent manner (29; 36).

2.1.1.2. AMPK

During contractile activity, there is a concurrent reduction in the ATP/ADP ratio and an elevation in AMP levels (46). AMPK functions as a metabolic stress sensor protein and can be allosterically activated by AMP up to 10-fold, and phosphorylation at Thr-172 in the kinase domain by upstream protein kinases causes >100-fold activation (22; 46; 50). Together with the allosteric effect of AMP, AMPK can be activated up to 1000-fold over baseline levels upon nutritional or oxidative stress. AMPK is phosphorylated at Thr-172 by the upstream tumor suppressor protein kinase LKB1, and also by CaMK-β, the latter activating AMPK even in the absence of an increase in AMP/ATP ratios (51). AMPK is a heterotrimeric complex, consisting of the catalytic α subunit (containing Thr-172) and two regulatory β and γ (containing the site for allosteric modulation by AMP) subunits (46). AMPK has been shown to be activated in response to exercise in humans, animals and in cell culture (62) and is responsible for acute, as well as chronic adaptations to endurance exercise. AMPK promotes GLUT4 translocation to the sarcolemma membrane and thus increases glucose uptake. It also initiates fatty acid oxidation by phosphorylating and inactivating Acetyl-CoA carboxylase (ACC)2, which produces malonyl-CoA, an inhibitor of carnitine-palmitoyl transferase-1 (CPT-1), the enzyme in the outer mitochondrial membrane responsible for fatty acid uptake (150; 151). With respect to long-term adaptations to exercise, AMPK increases GLUT4 expression (158), directly phosphorylates PGC-1α, a main regulatory protein involved in

mitochondrial biogenesis (68), and enhances Sirtuin 1 (SIRT1) activity which subsequently promotes the deacetylation of the PGC-1α leading to its activation (21). Furthermore, AMPK activation by 5-aminoimidazole-4-carboxamide riboside (AICAR), a well-known pharmacological drug that specifically triggers AMPK by mimicking AMP, elicits an increase in PGC-1α mRNA, protein and promoter activity, accompanied by concomitant amplification in mitochondrial enzyme activities such as citrate synthase, cytochrome *c* oxidase and malate dehydrogenase in skeletal muscle (60; 66; 149). These data illustrate the important role of AMPK in mediating exercise-induced adaptations.

2.1.1.3. ROS

Mitochondria are the main source of reactive oxygen species (ROS) production in the cell, and ROS are generated when an electron is inappropriately donated to an oxygen molecule in the electron transport chain at complex I and III. At baseline conditions, approximately 2-5% of oxygen is converted to ROS (17; 90). Accumulation of ROS damages cellular macromolecules such as proteins, lipids and DNA. The damage accrued by a lifetime of oxidative damage has been stipulated as the underlying cause for many degenerative diseases including aging, cancer and diabetes (67). However, evidence indicates a beneficial role for ROS in the cell as well. Exercise has been shown to increase formation of ROS in the muscle (28; 109), and ROS-activated downstream signals have been implicated in inducing mitochondrial biogenesis (61; 65; 125). ROS have been illustrated to induce mitochondrial reticulum formation, enhance mtDNA levels and mitochondrial mass. This increase was mediated specifically by the expression of NRF-1 and PGC-1α, which was enhanced following exogenous ROS treatment

indicating a positive role for ROS in mediating mitochondrial biogenesis (125; 129). Our lab has previously demonstrated that ROS enhance PGC-1α promoter activity in both an AMPK-dependent and independent manner (65).

Additionally, ROS are known to boost kinase activation (mainly mitogen-activated protein kinases or MAPKs) and reduce the activity of phosphatases such as calcineurin (23; 85; 135). MAPKs include c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAPK, of which p38 MAPK has a well-documented role in modulating the oxidative capacity of skeletal muscle (75). p38 MAPK is readily phosphorylated upon exercise (5; 40), and its downstream targets include p53, myocyte-enhancing factor 2 (MEF2) and activating transcription factor-2 (ATF-2) among others. p38 MAPK adaptively upregulates glucose transport in a MEF2 dependent manner (101), and is causally linked to elevations in PGC-1α promoter activity via engagement of ATF-2 (5).

2.1.1.4. Myokines

The last decade has witnessed an expanding appreciation of the role of skeletal muscle as an endocrine organ, replete with muscle-specific production of myokines that are secreted by the contracting skeletal muscle into the blood. The list of myokines produced by the muscle is growing steadily and includes myostatin, interleukin (IL)-6, IL-7, IL-8, IL-15, brain-derived neurotrophic factor (BDNF), leukemia inhibitory factor (LIF), fibroblast growth factor (FGF)-2, insulin-like growth factor (IGF)-1, follistatin-like 1 (FSTL-1), oncostatin M, and irisin to name a few (107). These myokines exact a diverse effect on muscle and other organs. Myostatin, LIF, IL-7 and IL-15 act on the

muscle in an autocrine manner and are involved in regulating muscle hypertrophy (19; 48; 56). BDNF, traditionally involved in ensuring neuronal cell growth, maturation, differentiation and survival, has been shown to increase fatty acid oxidation in an AMPK-dependent manner and enhance brown fat thermogenesis (98; 107; 108). IL-6 and IL-15 have been illustrated to play a role in lipid and glucose metabolism (103), insulin sensitivity (12) and increasing endurance capacity (33; 104; 116). IGF-1 and FSTL-1 promote osteogenesis (3; 74), and FGF-21 has been stipulated in improving glucose metabolism (96). Oncostatin M has been shown to inhibit mammary cell growth and induce apoptosis (57). The PGC-1α-dependent myokine irisin drives brown-fat development and improves glucose homeostasis (16). IL-6, IL-15, BDNF, oncostatin M and irisin increase at the mRNA, and protein level in muscle upon exercise, and are secreted into the blood by the contracting muscle (107). It is indeed exciting to speculate whether lack of exercise leading to an altered myokine expression profile is the underlying link between inactivity and inactivity-related chronic diseases.

2.1.2. Mitochondrial Protein Import

Mitochondrial synthesis is a highly regulated process that involves the coordination of both the nuclear and the mitochondrial genomes. mtDNA itself encodes for only 13 proteins, which implies that the rest of the protein content, about 1500 nuclear-transcribed proteins, must be imported into the mitochondria after being transcribed and translated in the cytoplasm (142). Proteins destined to become a part of the mitochondria must interface with the protein import machinery that spans the outer

and inner mitochondrial membranes, and increases in mitochondrial reticulum must be accompanied by an enhanced rate of protein import (89).

The protein import machinery consists of the translocases of the outer membrane (TOM) complex, and the transclocases of the inner membrane (TIM) complex (8). The TOM complex serves as the entry point for all mitochondrial destined protein and includes receptor proteins such as Tom20, Tom22 and Tom70 that interact with the precursor proteins that have been delivered to the mitochondria through the assistance of cytosolic chaperones such as heat shock protein (Hsp)90, Hsp70 and mitochondrial import stimulation factor (MSF). While Tom20 and Tom22 recognize the precursor proteins with an N-terminal presequence (1), Tom70 shows affinity for proteins with an internal targeting sequence (86; 87). The receptors transport the precursor proteins through the 400 kDa general import pore formed by the β-barrel protein Tom40. Once through the TOM complex, the preproteins can follow one of three major pathways: 1) preproteins with a presequence are transferred to the translocase of the inner membrane (TIM)23 complex for import into the matrix (14; 15); 2) proteins destined for the inner membrane rely on chaperone-like components of the intermembrane space and the protein insertion machinery of the inner membrane or TIM22 complex (127); 3) precursor proteins fated for outer membrane incorporation are integrated therein by the sorting and assembly machinery (SAM) complex (11). This review will focus on the import of matrix-destined mitochondrial proteins.

The TIM23 complex consists of Tim23, Tim50, and Tim17. The matrix-destined proteins translocate through the import channel formed by Tim23 (136). This

translocation requires mitochondrial membrane potential ($\Delta\psi m$) and ATP (15). $\Delta\psi m$ activates the channel itself, as well as providing an electrophoretic effect that drives the positively charged presequence of the preprotein into the matrix (10; 136). ATP drives the mitochondrial chaperone, mitochondrial Hsp70 (mtHsp70), which forms the core of the presequence translocase-associated motor (PAM), to liaise with the preprotein and pull it into the matrix (148). Mitochondrial processing peptidase (MPP) then removes the presequence from the preproteins, and mitochondrial chaperones such as mtHsp60 and mtHsp10 help fold the protein into its final conformation (47; 134). Studies have shown that the enhancement in mitochondrial function is closely related to improved rates of protein import. Several components of the import machinery such as Hsp70, MSF, mtHsp70, mtHsp10, Tom20, Tim17, Tim23, Tom22 and other members of the TOM and TIM complex display increased levels of expression in response to an exercise stimulus (42; 70; 131). Furthermore, import and assembly of tom40 increases upon chronic exercise (70), whereas denervation yielded a net reduction in the rate of protein import into the mitochondria (126). While the regulation of protein import into the mitochondria and its response during exercise remains enigmatic, it is clear that an improved ability to import more proteins would allow for a more streamlined process of mitochondrial assembly and therefore improve organelle function.

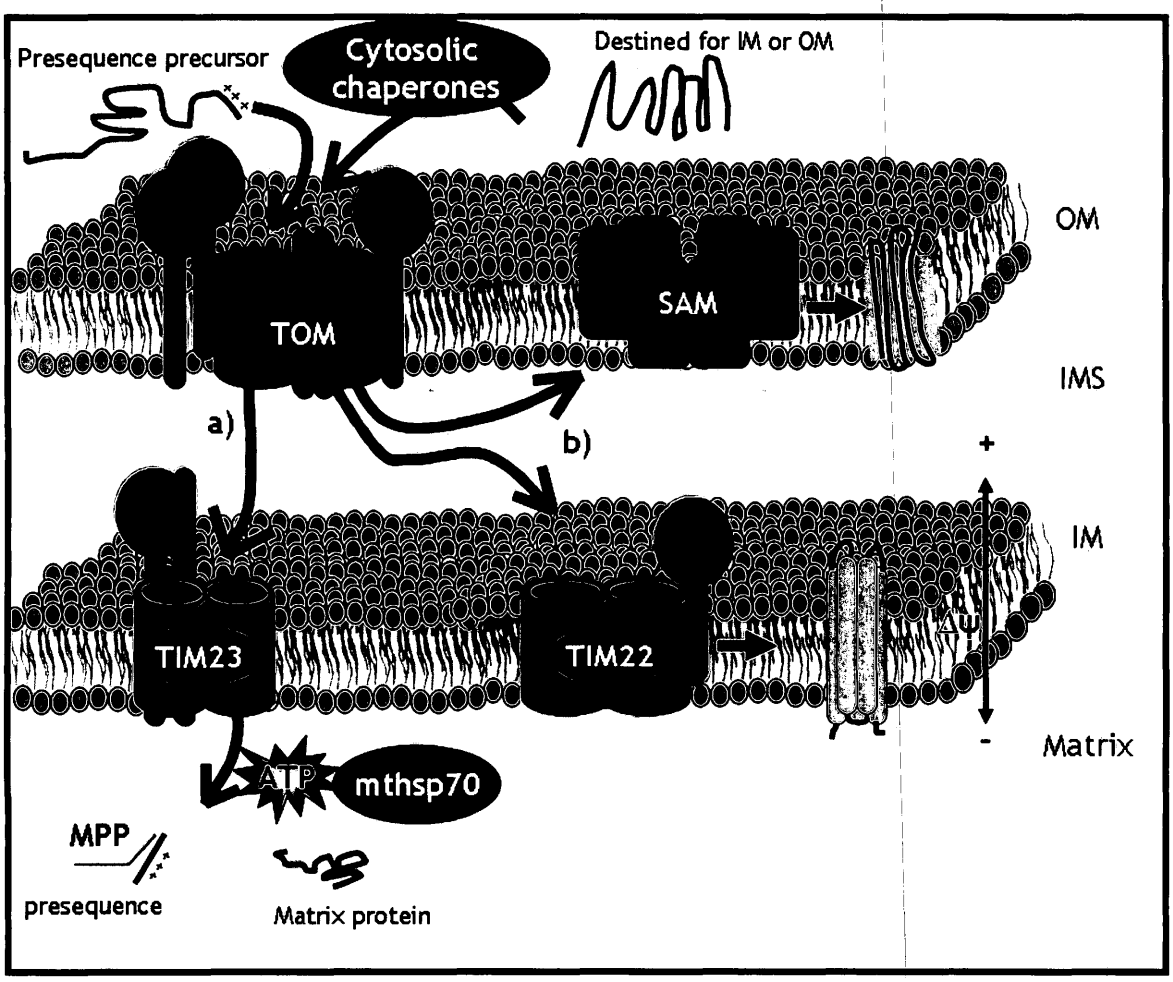

Figure 2. Mitochondrial Protein Import Pathway. Upon translation, many mitochondrial matrix-destined proteins are synthesized as precursor proteins with a cleavable N-terminal presequence. Other mitochondrial proteins may be bound for insertion into the outer or inner mitochondrial membranes. These proteins are translocated to the mitochondria with the help of cytosolic chaperones such as Hsp70, Hsp90 and MSF. At the outer mitochondrial membrane (OM), the precursor interacts with the translocases of the outer mitochondrial (TOM) receptor proteins such as Tom20. a) The translocases of the inner mitochondrial membrane (TIM23) complex assists in targeting the precursor protein to the matrix. The mitochondrial heat shock protein 70 (mtHsp70) drives the import of precursor proteins into the matrix in an ATP- and mitochondrial membrane potential ($\Delta\psi$)-dependent manner. The mitochondrial processing peptidase (MPP) cuts off the presequence and the protein is then folded and configured to its mature form. The mature form of the protein can then execute its function in the mitochondria (e.g. binding to mtDNA and regulating mtDNA-driven transcription). b) Proteins targeted to be incorporated into the IM of the mitochondria are directed to the TIM22 complex, and those that need to be inserted into the OM are sent to the sorting and assembly machinery (SAM) complex.

2.1.3. Autophagy/Mitophagy

Macroautophagy, hereafter referred to as autophagy, is the selective removal of damaged organelles and subcelluar structures, and includes the select elimination of mitochondria (mitophagy), endoplasmic reticulum (reticulophagy), peroxisomes (pexophagy), ribosomes (ribophagy), granules (crinophagy), and pathogens (xenophagy) through the lysosomal pathway (43; 73). A basal level of autophagy is maintained to perform general housekeeping of cell by turning over long-lived/damaged organelles and proteins. Decreased rates of autophagy are implicated in various neurological and metabolic disease. On the other hand, the process of autophagy can be adaptively increased upon metabolic stress, such as endurance exercise (123), to aid in cell survival. Autophagy is activated primarily through a surge in levels of ROS, and by amino acid restriction (43).

To maintain a healthy and bioenergetically competent pool of mitochondria in the cell, mitochondrial biogenesis must be complemented with mitophagy to remove dysfunctional mitochondrial units. It is presumed that mitochondria are turned over as an intact unit as the half-lives of mtDNA, cardiolipin, and inner membrane proteins are reportedly similar (41; 113). The process of mitophagy is initiated upon a loss of $\Delta\psi m$ or an elevation in ROS levels, which recruits PTEN-induced putative kinase 1 (PINK1) and E3 ubiquitin ligase Parkin to the outer mitochondrial membrane (39). Parkin ubiquitinates mitochondrial fusion protein 1/2 (Mfn1/2), and mitochondrial outer membrane GTPase called mitochondrial rho (Miro, involved in mitochondrial transport) effectively delinking the dysfunctional mitochondria from the healthier reticulum (39; 144). Autophagic

proteins p62 then bind to the ubiquitinated outer membrane mitochondrial proteins and engage the autophagosome (77). The autophagasome is inducted upon recruitment of Beclin-1, and is assembled by a series of conjugation reactions catalyzed by various autophagy related genes (Atgs) that result in the lipidation, and thus biochemical cleavage, of microtubule-associated light chain 3 (LC3) to LC3II (72; 99). Increase in LC3II expression is commonly used as a biomarker for activation of autophagy. Once the mitochondrion is enveloped by the autophagosome, it is delivered to the lysosome and subsequently broken down by the various resident lysosomal hydrolases. The macromolecules recycled are then released back into the cell to maintain energy balance.

In addition to triggering the synthesis of mitochondria, exercise has recently been recognized to play a part in the removal of damaged or dysfunctional mitochondria, thereby maintaining mitochondrial homeostasis (44; 44; 52; 52; 69; 69). Much work needs to be done to fully elucidate the intensity, frequency and duration of exercise required to initiate optimal autophagy/mitophagy in skeletal muscle, and to comprehensively document the exercise-mediated changes autophagic flux.

2.1.4. Role of PGC-1α in mitochondrial biogenesis

The PGC-1 family consists of PGC-1α, PGC-1β, and PGC-1 related co-activator (PRC). PGC-1α is a transcriptional co-activator and a critical regulator of the transcription of nuclear genes encoding mitochondrial proteins, and it has been implicated as a key mediator of the adaptive response to exercise in skeletal muscle (110).

Many of the putative signals triggering exercise-induced adaptations in skeletal muscle are activators of PGC-1α. For example, p38 MAPK can phosphorylate PGC-1α at three sites, releasing PGC-1α from a repressor protein (34; 111). AMPK activates PGC-1α by phosphorylating threonine-177 and serine-538 residues, leading to mitochondrial biogenesis (68). Our lab has shown that AMPK activation by AICAR, a well-known pharmacological drug that specifically triggers AMPK, elicits an increase in PGC-1α promoter activity, as well as mRNA expression (66). PGC-1α function is inhibited by acetylation and the NAD^+-dependent type III deacetylase, SIRT1, deacetylates and activates PGC-1α (102). Cellular reactive oxygen species (ROS) have also been identified as a stimulus regulating PGC-1α in promoting mitochondrial biogenesis and respiration. Our lab has also recently demonstrated that ROS augment PGC-1α promoter activity and mRNA expression in murine myotubes, an effect that was abolished when the antioxidant, *N*-acetylcysteine was present (65). Furthermore, current work has shown that AMPK must phosphorylate PGC-1α prior to SIRT1 deacetylation for the full activation of PGC-1α and its downstream target genes, indicating that the different signaling pathways work in concert to activate PGC-1α (21).

The post-translational modifications of PGC-1α lead to its activation/repression, and its sub-cellular redistribution. In the nucleus, PGC-1α coactivates transcription factors regulating mitochondrial biogenesis, including NRF-1/2, ERRα, PPARs, and MEF2 (137). These transcription factors regulate the promoters of genes involved in electron transport chain (ETC) function and fatty acid oxidation. Endurance exercise facilitates this coactivator function by causing a shift in the subcellular localization of

PGC-1α from the cytoplasm to the nucleus. This occurs prior to any exercise-induced increase in PGC-1α protein expression (153). Moreover, it has recently been reported that endurance exercise mediates the translocation of PGC-1α to the mitochondria where it may serve as a co-activator for mitochondrial transcription factor A (Tfam) on mitochondrial DNA (mtDNA; (120). PGC-1α also increases the expression of Tfam through the co-activation of NRF-1/2. Tfam is required for mediating the transcription of 13 ETC subunits, 2 rRNAs and 22 tRNAs encoded by the mtDNA, and also controls mtDNA copy number and maintenance (38). In this manner, PGC-1α contributes to the coordinated regulation of the nuclear and mitochondrial genomes during mitochondrial biogenesis.

Studies conducted using PGC-1α transgenic and knock-out (KO) mice also support an important role for PGC-1α in the regulation of mitochondrial function and exercise adaptations. Muscle-specific PGC-1α over-expression results in similar adaptations as those seen with endurance exercise training, including higher mitochondrial content, enhanced fat oxidation and lower glycogen utilization during exercise, as well as an increased endurance capacity (147). Research conducted in our lab has illustrated that mice deficient in PGC-1α have reduced basal mitochondrial content and impaired respiratory function (4), yet the endurance exercise-induced adaptive gene response is not abolished in these mice (82; 117). Using a cell culture model of chronic contractile activity, we confirmed that PGC-1α was necessary for most, but not all, of the exercise-mediated changes culminating in mitochondrial biogenesis (138). Together, these data imply redundancy in the molecular mechanisms regulating metabolic

homeostasis, such that other members of the PGC-1 family, or novel transcription factors such as p53, play a vital part in exercise-induced mitochondrial synthesis.

2.1.5. Evidence for systemic effects of exercise

Endurance exercise is a powerful physiological inducer of mitochondrial oxidative capacity in skeletal muscle. The regulatory conduits that lead to mitochondrial biogenesis with exercise have been studied extensively, and considerable advances in our understanding of how exercise mediates local muscular adaptations have been made. Since depleted oxidative capacity in skeletal muscle is associated with insulin resistance, type 2 diabets, obesity and aging, it is intuitive to circumvent pathology by increasing skeletal muscle mitochondrial quality and capacity, and thereby improving organismal health. More recently, there has been a growing appreciation for endurance exercise to induce mitochondrial adaptations in tissues beyond skeletal muscle, including adipose tissue (130), liver (18), brain (18; 30), and kidney (18).

There is ample evidence that endurance exercise has wide-spread systemic metabolic effects, and confers cellular and phenotypic protection from age-related disease in organs beyond skeletal muscle and the cardiovascular system. Endurance exercise has been shown to lower disability scores and all-cause mortality (24; 152), along with reducing death due to cancer (37) and improving glucose control in type 2 diabetes (7). In addition, long-term endurance exercise in the elderly appears to attenuate a reduction in whole-body aerobic capacity, and completely preserve insulin sensitivity (49; 53; 79; 80). In support of human epidemiological data, exercise training in rodents attenuates the

effects of age on mitochondrial and antioxidant enzyme activities, and oxidative damage in several non-muscle tissues (e.g. kidney, brain, liver; (18; 119). Additionally, exercise training in elderly induces the expression of mitochondrial and nuclear DNA repair proteins associated with aging (114; 115). While the protective effect of exercise is a *fait accompli*, the molecular mechanisms underlying the systemic effects of exercise remain unclear. Recent work stipulates the release of myokines from contracting muscle as a possible means for achieving the systemic adaptations.

2.2. p53-mediated mitochondrial biogenesis

2.2.1. Introduction to the tumor suppressor protein p53

The tumor suppressor protein p53 is a master stress response factor well known for its pleiotropic effects on cell cycle arrest, apoptosis, pro- and anti-oxidant activity, angiogenesis, DNA repair, differentiation, fertilization, aging and senescence (83). In addition to serving as the "Guardian of the Genome" (78) under stressful conditions, mounting evidence indicates that p53 plays a vital role in the maintenance of energy homeostasis in non-stressful environments. Notably, p53 has been demonstrated to be involved in the maintenance of mitochondrial biogenesis (92). Lack of p53 impairs aerobic capacity and exercise performance, and results in greater fatigue in p53 KO mice highlighting a role for p53 in skeletal muscle health (122). While the complex mechanisms underlying this effect have not been fully elucidated, tangible progress has been made in illustrating how p53 contributes substantially to the maintenance of mitochondrial content and function.

2.2.2. Structure, activation and regulation of p53

The p53 gene consists of eleven exons with two translation start sites in exons 2 and 4 (105). Human p53 protein is composed of 393 amino acid residues. At the N-terminus, the protein is composed of two transcriptional activation domains (TAD1, TAD2) which are required for the transcriptional induction of the target genes, and an overlapping proline-rich domain (PRD) necessary for protein-protein interactions (100; 105; 156). The central region of the protein houses the DNA binding domain (DBD),

which is the most mutated site in the p53 protein (100; 105; 128; 156). The DBD is followed by the tetramerization motif (TET), which promotes the oligomerization of p53, and the nuclear export signal (NES) (100; 105; 156). The TET motif allows p53 to exist in its wild-type tetrameric conformation, made by two p53 homo-dimers (100; 105; 156). At the C-terminus, the protein has a C-terminal regulatory domain (CTD) which also contains three nuclear localization signals (NLS; (100; 156)). p53 can undergo post-translational modifications at all of these domains leading to variable effects on protein stability and degradation.

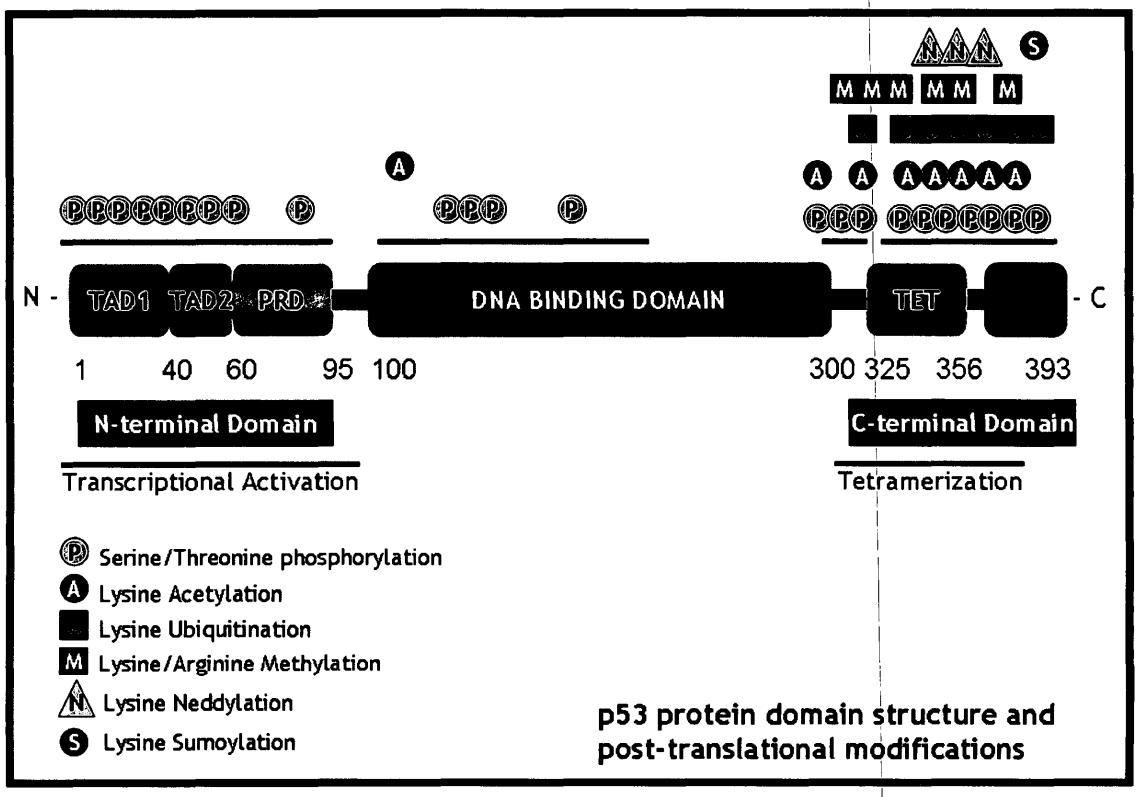

Figure 3. p53 protein domain structure and post-translational modifications. Schematic illustration of the 393 amino acid domain structure of p53 showing important sites for post-translational modifications including phosphorylation, acetylation, ubiquitination, methylation, neddylation and sumoylation. Figure adapted from (91).

p53 can be activated by mild, as well as by more severe genotoxic stress signals, leading to the understanding that p53 plays an important role during normal health and development. Studies of cells in culture subject to nutrient (i.e. glucose) deprivation demonstrated an increase in the activation of AMPK, leading to the downstream phosphorylation of p53 at serine 15 ($p53Ser^{15}$). This is a post-translational modification associated with an increased stability and activity of p53 (32). Moreover, $p53Ser^{15}$ is a bona fide target of p38 MAPK under conditions of cellular stress (83). Interestingly, we have previously shown that an acute bout of contractile activity results in concomitant increases in AMPK, p38 MAPK and $p53Ser^{15}$ phosphorylation (122). This suggests the possibility that p53 may be involved in endurance exercise-induced mitochondrial biogenesis upon post-translational modifications induced by AMPK and/or p38 MAPK signaling cascades.

p53 can be regulated by numerous post-translational modifications including phosphorylation, acetylation, glycosylation, ubiqutination, sumolyation, neddylation, ribosylation, *O*-GlcNAcylation and lysine methylation (63; 64; 81; 105; 118; 140; 156). It has around 10 phosphorylation sites grouped together in the N-terminal part and a few more at the carobxy terminal (81; 156). Post-translational modification of p53 fluctuates with the cellular stress levels and correlates with its downstream effect. For example, phosphorylation of p53 lowers its affinity for mdm2, its negative regulator, consequently reducing degradation (105; 140). Phosphorylation dominates the post-translational modification milieu as p53 can be targeted by a multitude of different protein kinases including ATM, ATR, the checkpoint kinases (Chk1, Chk2), Jun NH_2-terminal kinase

(JNK), p38, ERK (MAPK) and AMP-activated protein kinase (AMPK; (20; 81; 118)). Evidence suggests that acetylation of lysine residues near the C-terminal region can improve DNA binding by p53 and acetylation of different lysine residues can have differential effects on target gene expression (105; 140).

In response to stress signals, p53 levels can be rapidly up-regulated. Evidence suggests that this occurs primarily via decreased protein degradation rather than increased transcription (140). Subcellular localization of p53 plays a role in regulating its activity. Mono-ubiqutination of p53 by mdm2, exposes its nuclear export signal (NES) and promotes p53 to be shuttled out of the nucleus and to the cytoplasm (63; 105). Once in the cytoplasm, monoubiqutinated p53 can undergo polyubiqutination and consequently proteolytic degradation or it can be trafficked to the mitochondria (63). Moll UM recently illustrated in an elegant study that monoubiqutinated p53 can be targeted to the mitochondrial outer membrane where it is rapidly deubiquitinated by the mitochondrial herpesvirus associated ubiquitin-specific protease (HAUSP; (63; 94)).

2.2.3. Mitochondrial function is dependent on p53

A commonly used indicator of mitochondrial content is cytochrome *c* oxidase (COX) activity. The COX complex, made up of thirteen subunits that are both nuclear- and mtDNA-encoded, plays a crucial role in aerobic respiration by catalyzing the transfer of electrons from reduced cytochrome *c* to molecular oxygen. Ablation of a functional p53 protein manifests as reduced COX activity (97; 122). This decrease in COX enzyme activity has been attributed to the transcriptional and post-transcriptional control exerted

by p53 on the expression of COX subunit I and COX subunit II (159) respectively. In addition, p53 transcriptionally regulates the synthesis of cytochrome *c* oxidase 2 (SCO2), a protein required for the assembly of mtDNA-encoded COX II subunit into the COX complex (97). SCO2 defects have been causally linked to aerobic respiratory failure and terminal cardioencephalomyopathy (97). The lower rate of mitochondrial respiration observed in p53 KO cell lines was rescued by the transient transfection of SCO2 (97), indicating that this is an important pathway through which p53 promotes optimal mitochondrial biogenesis.

A number of studies have also provided direct evidence for the presence of p53 response elements in the promoter regions of nuclear-encoded mitochondrial proteins related to metabolism, such as apoptosis inducing factor (AIF), heat shock protein 70 (hsp70) and hsp90, and Tfam. While AIF is known for its role in the induction of apoptosis, during basal conditions AIF contributes to efficient oxidative phosphorylation by promoting the proper assembly and function of mitochondrial respiratory complex I. Hsp70 and hsp90 are cytosolic chaperones that assist in the targeting of mitochondrial-destined proteins to the mitochondria. As well, Park and colleagues (106) demonstrated that the presence of p53 is a determinant of both Tfam expression and mtDNA content. This suggests that p53 uses the regulation of Tfam as a conduit through which it can control the process of mitochondrial biogenesis.

2.2.4. p53 and mitochondrial DNA

The small number of proteins encoded by the mtDNA are vital for ETC function. Mutations in mtDNA have a range of pathological consequences, with ~250 known disease-causing mutations now reported. Furthermore, mutations in the nuclear DNA-transcribed proteins affecting mtDNA mutations are even more frequent, thus increasing the genetic load of mtDNA dysfunction. p53 can be imported into mitochondria, and several studies have concluded that it may be involved in modulating mtDNA-encoded gene expression and stability (2; 76; 106; 157). The presence of a putative p53 response element in mtDNA indicates the possibility that p53 could be directly involved in the transcription of mtDNA (55). Indeed, p53 KO mouse embryos displayed a deficiency in mtDNA-encoded 16S rRNA transcripts (31). p53 also promotes and maintains mitochondrial genomic stability directly 1) via its inherent 3' → 5' exonuclease activity (9), 2) by physically interacting with Tfam and regulating the binding of Tfam to damaged mtDNA (157), and 3) by enhancing the function of mtDNA polymerase gamma (pol γ), the only DNA polymerase in the mitochondria that is responsible for mtDNA replication and repair (2). Clearly, p53 has a validated and significant impact on maintaining mtDNA transcription and integrity.

2.2.5. Effect of p53 on glycolysis

Endurance exercise training is known to favor an increased reliance on aerobic metabolism, rather than glycolysis, as means of energy production in muscle. Coincidentally, along with its effect on promoting optimal mitochondrial function and

mtDNA integrity, p53 appears to suppress glycolysis as well. A novel p53-transactivated gene called TP53-induced glycolysis and apoptosis regulator (TIGAR) lowers fructose-2,6-bisphosphate levels in cells, resulting in an inhibition of glycolysis (13). Additionally, p53 can induce the degradation of phosphoglycerate mutase, leading to decreased glycolytic flux (92). As one of the hallmarks of cancer is a shift from aerobic to glycolytic metabolism, a process known as the Warburg effect (92), the p53-mediated suppression of glycolysis is in line with its role in cancer suppression. It remains to be seen whether the exercise-induced suppression of glycolysis, and enhanced reliance on mitochondrial metabolism, is in part relayed by the recruitment and activation of p53.

2.2.6. p53, mTOR and autophagy

p53 is either a positive or a negative regulator of autophagy, depending on the cellular localization of the protein and cellular stress conditions (93). During cellular stress when p53 is localized in the nucleus, it promotes the expression of target genes that induce autophagy. Some of the genes identified include AMPK subunits β1 and β2, tuberous sclerosis complex 2 (TSC2), Sestrin2, damage-related autophagy modulator (DRAM), and death-associated protein kinase 1 (DAPK-1; (93). In particular, AMPK, TSC2 and Sestrin2 are able to exert a negative influence over the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) pathway that participates in cell growth and metabolism, and negatively regulates autophagy (35; 93). Therefore, nuclear-localized p53 can increase autophagic flux by suppressing mTORC1, a classic inhibitor of autophagy.

Conversely, during basal, non-stressful conditions, a large fraction of p53 resides in the cytoplasm. This cytoplasmic pool of p53 appears to inhibit autophagy (133). For example, an elevated basal content of autophagosomes is evident in p53 KO mice, suggesting that autophagy is stimulated in the absence of p53, and there is no further increase in these autophagy vesicles upon starvation, as is commonly manifest in WT mice (133). Thus, the absence of cytoplasmic p53 may allow autophagy to proceed at a maximal rate. It therefore appears that p53 may serve as a rheostat of autophagic signaling, involved in adjusting the rate of autophagy depending on its localization, and the presence of absence of cell stress. Further research on the coordinated interplay between p53 and mTORC1 on the rate of autophagy, and the subsequent affect on mitochondrial turnover, is warranted.

2.2.7. Role of p53 in mitochondrial fission and fusion

Mitochondria are dynamic organelles, capable of adapting to energy perturbations within the cell. The maintenance of mitochondrial morphology depends on equilibrium between the states of fission and fusion (25). Fusion involves the merging of the inner and outer membranes, as well as the mixing of mitochondrial material. On the other hand, fission divides the mitochondrial network into smaller organelles. Disruptions in either of these opposing processes can lead to developmental defects and disease, suggesting that proper maintenance of mitochondrial morphology is critical for normal cell function (25).

p53 has been linked to the regulation of both mitochondrial fission and fusion proteins. Knockdown of p53 has been reported to reduce mitochondrial fission, likely due

to the ability of p53 to transcriptionally upregulate the expression of the fission protein Drp1 (84). On the other hand, a consensus p53 binding site has been identified within the promoter region of Mfn2, an important fusion protein. p53 has been illustrated to positively modulate the promoter activity, and protein expression of Mfn2 (143). To date, there is no evidence that a net increase or decrease in mitochondrial network formation occurs in the absence of p53. However, we have previously noted that lack of p53 results in altered cristae formation in SS mitochondria, and reduced reticular network in the IMF mitochondria isolated from p53 KO mice (122). This alludes to the involvement of p53 in determining muscle mitochondrial morphology, and further study is required to fully understand the role of p53 in mitochondrial structural dynamics.

2.2.8. Role of p53 in modulating exercise-induced adaptations

The disruption of p53 expression carries significant physiological repercussions, as evident by the greater fatigability and reduced exercise capacity observed in p53 KO animals (97; 106; 122). In order to implicate p53 in endurance exercise-induced mitochondrial adaptations, it is likely that: 1) p53 should be post-translationally modified by contractile activity, and/or 2) its sub-cellular localization be modified as a result of an exercise stimulus. We have shown that contractile activity exercise induces an increase in p53Ser^{15} phosphorylation (122), classically linked to the increased stability and activity of the protein. This enhanced phosphorylation could be due to a combined effect of the activation of p38MAPK and AMPK, both of which are canonical exercise-activated kinases. This may confer a change in p53 cellular localization to enter the nucleus or the

mitochondrion, though this remains to be demonstrated. These changes in localization could have a profound impact on the expression of nuclear and mitochondrial genes.

p53 does appear to have an influence on the expression of PGC-1α, a coactivator which is important for normal mitochondrial adaptations to contractile activity. Our earlier work identified a putative p53 response element in the promoter region of PGC-1α identified through *in silico* analysis (66). PGC-1α protein expression was also reduced in muscle of p53 KO animals (122). Thus, this suggests that p53 is a positive regulator of PGC-1α gene expression. In distinct contrast, a recent study has reported that telomere dysfunction elicits increased expression and activation of p53, which then consequently binds to the both the PGC-1α and PGC-1β promoters, and represses their expression in mouse embryonic fibroblasts (MEFs, (121). Deleting germline p53 in these telomerase deficient mice restored PGC-1α/β expression, and rescued the mitochondrial oxidative pathology present in these mice (121). These results are in complete opposition to those which indicate the importance of p53 for the maintenance of mitochondrial integrity. It may be that the effect of p53 on PGC-1α/β expression observed in this study is tissue-specific, or that the signaling millieu in cells from the telomere dysfunctional animals varies significantly from a normal mouse. It is clear from studies using KO animals that the loss of p53 does not result in higher PGC-1α/β expression, nor does it improve mitochondrial function. Thus, further work is clearly required to fully unravel the relation between p53 and PGC-1α, and the resultant effects on mitochondrial biogenesis.

2.2.9. Integrative role of p53 in exercise-induced mitochondrial biogenesis – Future Directions

Despite a growing appreciation for a widening role for p53 in mitochondrial biogenesis and function, there is very limited literature available which has analyzed the necessity of p53 for endurance exercise-induced mitochondrial biogenesis in muscle, as most of the research on p53 has been conducted in cancer cell lines. We have shown that in response to eight weeks of wheel running, p53 KO mice have a similar relative increase in COX activity (~26%) as WT mice, despite running 5-fold lower distances, suggesting that p53 may be important, but not required, for exercise-mediated increases in muscle oxidative capacity (122). Park et al. (106) reported that five weeks of treadmill training improved peak oxygen consumption, work capacity and blood lactate levels in WT and not p53 KO mice, underscoring the value of p53 for the accrued benefits of endurance exercise.

As illustrated in figure 4, p53 affects mitochondrial biogenesis by exerting control over a plethora of different cellular pathways. p53 can transcriptionally activate important factors such as PGC-1, Tfam, AIF and SCO2 that lead to mitochondrial biogenesis. p53 can also translocate to the mitochondrion and interact with mtDNA, Tfam and Polγ, contributing to mtDNA-mediated transcription and ensuring the integrity of the genome. Clearly, the cellular localization of p53 is important in determining the consequences of its actions. Thus, it would be particularly interesting to deduce whether exercise induces a post-transcriptional modification in p53, culminating in its cellular re-localization. The presence of p53 in the nucleus or extranuclear regions also dictates its effect on

autophagy. The autophagic destruction of dysfunctional or fragmented mitochondria is an important pathway for the maintenance of a constant mitochondrial content. We hypothesize that p53 may play an important role in the removal of damaged mitochondria with endurance exercise. Furthermore, it may be that an exercise-induced activation of p53 could lead to the transcription of both Mfn2 and Drp1, thus maintaining optimal rates of fission and fusion in the cell which are required to maintain a healthy mitochondrial pool. Further research in these areas is required to prove/disprove these hypotheses.

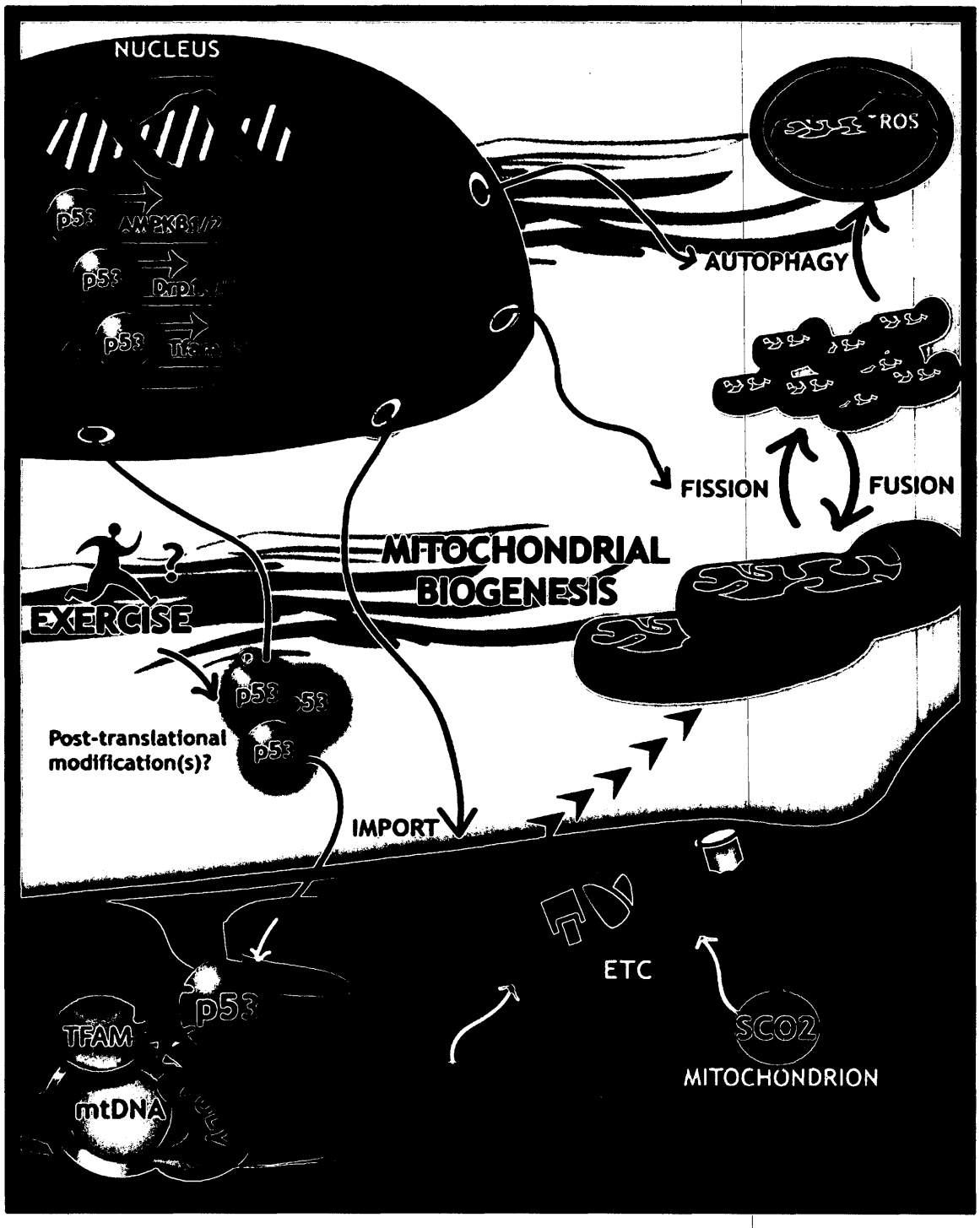
NUCLEUS
ROS
AUTOPHAGY
FISSION
FUSION
MITOCHONDRIAL BIOGENESIS
EXERCISE
?
Post-translational modification(s)?
IMPORT
ETC
TFAM
mtDNA
SCO2
MITOCHONDRION

Figure 4. Regulation of mitochondrial biogenesis by p53. As a conventional nuclear transcription factor, p53 can induce the transcription of proteins involved in mitochondrial turnover (autophagy, fission and fusion) and mitochondrial synthesis. p53 transcribes pro-autophagic proteins such as AMP-activated protein kinase (AMPK) subnits β1 and β2, tuberous sclerosis complex 2 (TSC2), and others (see the text) to induce autophagy. It also evokes the transcription of mitofusin 2 (Mfn2) and dynamin-related protein 1 (Drp1) that contribute to mitochondrial fusion and fission, respectively. Autophagy of dysfunctional mitochondria and mitochondrial fission/fusion flux may work in concert to ensure efficient mitochondrial turnover and maintenance of healthy mitochondria. Furthermore, p53 also transcribes genes involved in controlling mitochondrial synthesis such as mitochondrial transcription factor A (Tfam), synthesis of cytochrome c oxidase 2 (SCO2) and apoptosis inducing factor (AIF), among others. These proteins are subsequently imported into the mitochondria where they perform specific functions. Tfam is responsible for mtDNA replication and transcription. SCO2 is necessary for the proper assembly of subunit II in complex IV in the electron transport chain (ETC), and AIF contributes to the optimal assembly and function of complex I within the ETC. Lastly, p53 translocates into the mitochondria and positively affects mtDNA transcription and genome integrity by interacting with Tfam and the mtDNA repair enzyme polymerase γ (POLγ). p53 also has inherent DNA repair activity. Whether exercise causes post-translational modification(s), or changes in the subcellular localization of p53 (?) to trigger the nuclear- and/or mitochondrial-specific effects, has yet to be elucidated.

2.2.10. Conclusion

Regularly performed endurance exercise exerts a plethora of adaptive effects leading to a multitude of health benefits, such as reduced risk of cardiovascular disease, obesity, type 2 diabetes and cancer. These therapeutic effects are mediated by the activation of various transcription factors and cellular signaling pathways positively regulating the overall health of the organism. p53 has emerged as a relatively new player in this field, as it is now established to have an important impact on mitochondrial function and content. Several epidemiological studies have firmly indicated that regular endurance exercise results in a reduced risk of cancer (146). Whether endurance exercise potentiates the systemic activation of the tumor suppressor protein p53 in multiple tissues, and thereby retards cancer incidence/growth is not known. However, it is intriguing to speculate that exercise-activated p53-dependent regulation of mitochondrial function and content could rescue the loss of oxidative capacity in muscle wasting conditions such as cancer cachexia. Clearly, the integration of p53 within the regulatory network that contributes to endurance exercise-mediated metabolic and therapeutic adaptations in muscle is an exciting new avenue for study, and form the basis of my PhD dissertation. Further work in this area will significantly contribute to existing knowledge on exercise-induced mitochondrial biogenesis, and provide impetus for future investigations into the metabolic aberrations that underlie cancer progression, with the hope of deciphering innovative therapies.

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CHAPTER 3:

PhD DISSERTATION OBJECTIVES & HYPOTHESES

Based on the literature review, it is clear that endurance exercise-mediated benefits are manifold and ubiquitous, and modulated by a plethora of transcriptional- and non-transcriptional- regulators, of which the tumor suppressor protein p53 is an important player. Many questions remain unanswered including the mechanism(s) by which p53 modulates endurance exercise-mediated adaptations in skeletal muscle and the scope of its activation and involvement in the signalling milieu post-exercise. Since an increase in mitochondrial content is one of the major adaptations to endurance exercise, we specifically evaluated the role of p53 in the context of mitochondrial biogenesis. Thus, the objectives of my dissertation were three-fold:

OBJECTIVE #1 (Chapter 4): To investigate the role of p53 on mitochondrial protein import and Complex IV assembly.

Hypotheses:

1) We hypothesized that mitochondria from p53 knockout mice will display impaired import kinetics and reduced expression of proteins involved in mediating the process.

2) We posited that lack of p53 would impair the assembly of Complex IV since p53 is previously shown to regulate an assembly factor of Complex IV.

OBJECTIVE #2 (Chapter 5): To assess whether p53 is regulated by an acute bout of endurance exercise at the level of mRNA, protein, post-translational modification and sub-cellular localization.

Hypotheses:

1) We hypothesized that acute endurance exercise would activate p53 via post-translational modifications such as serine phosphorylation, and increase its mRNA and protein content.
2) We also expected that acute exercise would lead to enhanced nuclear and mitochondrial expression of p53, wherein it would exert its pro-metabolic effect through nuclear and/or mtDNA transcriptional regulation.

OBJECTIVE #3 (Chapter 6): To delineate the importance of p53 for the exercise-induced alterations in cellular signalling with respect to mitochondrial biogenesis and autophagy/mitophagy.

Hypotheses:

1) We expected that absence of p53 would ameliorate or dampen the magnitude of the adaptive response to exercise when characterizing changes in gene expression and signalling involved in inducing mitochondrial biogenesis.
2) Given the differential effects of p53 on autophagy we hypothesized that lack of p53 would impair both autophagy and mitophagy, leading to the build up dysfunctional mitochondria characteristic of p53 knockout mice.

CHAPTER 4:

Effect of p53 on mitochondrial protein import and complex IV assembly

Ayesha Saleem[1,2], Sobia Iqbal[1,2], Yuan Zhang[1,2] and David A. Hood[1,2,3]

[1]School of Kinesiology and Health Science, [2]Muscle Health Research Centre, and [3]Department of Biology, York University, Toronto, Ontario, M3J 1P3, Canada

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Author Contributions

AS and DAH conceived and designed the experiments, interpreted the data and wrote the manuscript. AS collected and analyzed all the data except cytosolic protein expression immunoblottting experiments which were performed by SI. YZ provided technical assistance during the study.

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Abstract

The purpose of this study was to investigate whether p53 regulates mitochondrial function by affecting mitochondrial protein import and complex IV activity and assembly. Mitochondrial fractions isolated from skeletal muscle of p53 wild-type (WT) and knockout (KO) mice were probed for differences in the expression of proteins involved in import, *in vitro* rates of import, complex IV activity and assembly. OCT import into SS or IMF mitochondria was unaffected in the absence of p53, despite protein- and mitochondrial subfraction-specific reductions in Tom20, Tim23, mtHsp70 and mtHsp60 in mitochondria from the KO mice. Complex IV (COX) activity in isolated mitochondria was also unchanged in KO mice. Using two-dimensional BN-PAGE we measured the assembly of complex IV, and found it to be reduced in the IMF fractions from KO mice, in tandem with lower levels of Surf1, a protein involved in the assembly process. This observed defect in complex IV assembly may explain the previously documented impairment in mitochondrial function in p53 KO mice.

Introduction

The endosymbiont theory suggests that mitochondria originated when a protobacterium was engulfed by a primordial eukaryotic cell about 1.5-2 billion years ago (11). If this were true, then the ancient eukaryotic cell made an adaptive transformation as today mitochondria are the primary source of energy production in our cells, and are pivotal to a variety of cellular processes, such as fatty acid oxidation, amino-acid metabolism, calcium storage, iron-sulfur cluster synthesis, and apoptosis (30). A human cell can contain hundreds of mitochondria, and each mitochondrion contains multiple copies of mitochondrial DNA (mtDNA), a circular 16,559bp large, double-stranded DNA molecule. mtDNA transcribes 13 subunits of the electron transport chain (ETC), as well as the 22 tRNA and 2 rRNA species necessary for mtDNA-encoded proteins translation. The ETC is comprised of five complexes situated in the inner mitochondrial membrane, and each complex is composed of both nuclear- and mtDNA-encoded proteins, with the exception of complex II which is entirely nuclear DNA-transcribed. Since a single mitochondrion is comprised of nearly 1600 proteins, and mtDNA encodes only < 1% of the mitochondrial proteins (30), this necessitates the presence of a fully functional protein import system.

Mitochondrially-destined proteins are synthesized by ribosomes in the cytosol and are guided to the mitochondrion by cytosolic chaperones such as heat shock protein 70 (Hsp70), Hsp90 and mitochondrial import stimulating factor (MSF, (19; 20; 40). These chaperones deliver the proteins directly to the translocase of the outer mitochondrial membrane (TOM) complex where the import process begins. Tom20 is one of the

receptor proteins on the TOM complex that recognizes and binds to cytosolic preproteins (3; 22; 25). Once through the TOM complex, the precursor proteins can be directed towards different pathways depending on whether the protein is targeted to the outer mitochondrial membrane, inner membrane, intermembrane space or the matrix (30). Precursor proteins destined for the matrix are imported by the translocase of the inner membrane (TIM) complex, through the channel-forming Tim23 protein (3; 22; 39), with the assistance of the presequence translocase-associated motor (PAM). The core of PAM is composed of mitochondrial heat shock protein 70 (mtHsp70) which actively pulls the unfolded preprotein into the matrix in an ATP- and $\Delta\Psi$-dependent manner (3; 22). The precursor proteins undergo processing and are folded into their final configurations with the assistance of mtHsp60 and chaperonin 10 (cpn10, (13).

Of the many factors that control mitochondrial function, p53 is a relatively new and important addition. It has been shown to be significant in maintaining optimal mitochondrial content and function, and absence of the protein is detrimental for endurance capacity (26; 27). The tumor suppressor protein p53 was first identified to affect oxidative capacity via its ability to transcriptionally regulate synthesis of cytochrome c oxidase (SCO2), an important accessory factor in mitochondrial complex IV assembly (18). Subsequently, we and others demonstrated lower complex IV activity in whole muscle homogenates, along with several impaired indices of mitochondrial function evident in the p53 KO mice (24; 26). Like many other complexes of the electron transport chain in the mitochondria, COX is made up of both mtDNA- and nuclear DNA-encoded proteins, rendering mitochondrial protein import as an important determining

factor in the biogenesis of the complex. We therefore hypothesized that the observed impairment in COX activity and mitochondrial respiration (24; 26) in p53 KO mice may be, in part, due to impaired mitochondrial import, in conjunction with decreased assembly of nuclear-encoded proteins into complexes such as complex IV of the ETC.

Methods

Animal Breeding

p53 mice (9), were obtained from Taconic labs (New York, USA). Heterozygous p53 mice were bred and treated experimentally in accordance with principles of the York Animal Care Committee. Each progeny of the breeding pair was genotyped as follows. An ear clipping obtained from each animal was used for a crude DNA extraction. Extracted DNA was added to a PCR tube containing DNA Taq Polymerase (Sigma Jumpstart REDtaq Ready Mix PCR Reaction Mix) and forward and reverse primers for the wild-type (WT) p53 gene or the knockout (KO) p53 gene. Differences in the genome were detected using polymerase chain reaction (PCR) amplification. The reaction products were separated on a 2% agarose gel at 90 volts for 2-2.5 hours and visualized with the use of ethidium bromide.

Experimental Design

At around 3 months of age, p53 KO and their WT littermates (N=9/group) were cervically dislocated, and the *quadriceps femoris*, and *gastrocnemius* muscle groups were excised and placed in ice-cold buffer for cytosolic and mitochondrial fractionation. The

protein content of cytosolic and mitochondrial extracts was determined using the Bradford method (2).

Mitochondrial and cytosolic fractionation

Freshly isolated *quadriceps femoris*, and *gastrocnemius* was minced, homogenized and subjected to differential centrifugation to isolate the subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial subfractions, as described previously (4; 17; 34). Mitochondria were suspended in resuspension medium (100 mM KCl, 10 mM MOPS and 0.2% BSA). After the isolation procedure, SS and IMF mitochondria were used for analyses of mitochondrial protein import, two-dimensional Blue Native PAGE (2D BN-PAGE), cytochrome c oxidase enzyme activity and immunoblotting. Cytosolic fractions were prepared from freshly isolated skeletal muscle using a commercially available extraction kit (Pierce NE-PER, Rockford, IL, USA). Briefly, 50-75 mg of skeletal muscle was minced and homogenized in CER-I buffer containing protease inhibitor cocktail Complete, EDTA-free (Roche Applied Sciences, Manheim, Germany). After a series of wash steps, the cytosolic fraction was obtained as the supernate following centrifugation at 100,000 × *g* at 4 °C for 60mins.

Cytochrome c Oxidase (COX) Enzyme Activity

COX activity was measured as previously detailed (6). Briefly, mitochondrial extracts were added to a test solution containing fully reduced cytochrome c. Enzyme activity was determined as the maximal rate of oxidation of fully reduced cytochrome c measured by the change in absorbance at 550 nm in a Synergy HT microplate reader at 30 °C.

DNA isolation and In vitro transcription

The full-length cDNA clone for ornithine transcarbamylase (OCT), a matrix-bound mitochondrial protein, was a generous gift from Dr. Gordon Shore (McGill University, Montreal, Canada). The vector containing the OCT cDNA was linearized using *Sac* I at 37°C for 2hours. Plasmid DNA was subsequently phenol extracted and ethanol precipitated overnight at -80°C. The DNA was diluted to a concentration of 0.8 μg/μl, and was transcribed at 40°C for 90 min using SP6 RNA polymerase. The resulting mRNA pellet was resuspended to a final concentration of 2.8 μg/μl and aliquots were stored at -80C for use in import assays.

In vitro translation and import

The pOCT mRNA underwent *in vitro* translation in the presence of [^{35}S]methionine within a rabbit reticulocyte lysate system. Briefly, isolated SS and IMF mitochondria were allowed to equilibrate by preincubation for 10 min at 30°C before the import assay. Translation mix (12 μl per 50 μg of mitochondrial protein) was added to the mitochondria, and the import incubation was allowed to proceed at 30°C for 20 min. After 20 min, import was stopped by applying the mitochondrial translation mix to an ice-cold sucrose cushion (in mM: 600 sucrose, 100 KCl, 20 HEPES, and 2 $MgCl_2$). Mitochondria were pelleted by centrifugation for 15 min at 16,000 *g* (4°C) and resuspended in 20 μl of ice-cold breaking buffer (600 mM sorbitol, 20 mM HEPES, pH 7.4). The samples were denatured for 5 min and electrophoresed through a 12% SDS-polyacrylamide gel. Gels were processed and dried with a vacuum gel dryer. Radiolabelled precursor and mature

proteins were detected using phosphorimaging (Pharos FX, Bio-Rad) and quantified with electronic autoradiography (Quantity One, Bio-Rad, Hercules, CA). The percentage of protein import was calculated based on the ratio of the intensity of the mature OCT and total OCT (sum of mature and precursor bands). A translation lane mixture was run on each gel as a control for the *in vitro* translation and import reaction.

Two-Dimensional Blue Native PAGE (2D BN-PAGE)

Separation of electron transport complexes was performed using BN-PAGE according to the protocol described by Schagger et al. (28; 29). Mitochondrial fractions (200 μg) were solubilized with η dodecyl β-D maltoside in a buffer composed of 750 mM ε-amino-η-caproic acid, 50 mM Bis–Tris, pH 7.0, at 4 °C. Following solubilization, samples were centrifuged for 25 min at 100,000 *g* at 4 °C. Coomassie G-250 was added to the resulting supernatants using a detergent-to-dye ratio of 4:1 (w/w). The supernatants were applied on a 5–13% gradient BN PAGE gel and electrophoresed overnight for 16h at 4 °C and 23V. Blots were normalized for loading using VDAC in the first dimension. To perform 2D BN-PAGE, strips from the first dimension were cut, rotated 90° and subjected to further separation in the second dimension on a 12% denaturing SDS gel. After electrophoresis, proteins were electroblotted onto PVDF membranes and subsequently probed with COX-I (complex IV) antibody as described below.

Immunoblotting

Mitochondrial and cytosolic extracts were separated by performing 10%-15% SDS-PAGE and subsequently transferred onto a nitrocellulose membrane, washed, blocked

and immunoblotted overnight at 4°C with primary antibody directed against Tom20 (Santa Cruz), Tim23 (BD Biosciences), mtHsp70, mtHsp60, cpn10 (Assay Designs), hsp70, hsp90 (Stressgen Bioreagent), MSF-L (gift from Dr. K. Mihara, Kyushu University), Surfeit locus protein 1 (Surf1), mitochondrial electron transport chain cocktail (Mitosciences), COX-I, porin (Invitrogen) and aciculin. Membranes were washed 3x with tris-buffered-saline-tween-20 (TBST) solution containing 25 mM Tris-HCl (pH 7.5), 1 mM NaCl and 0.1% Tween 20. Membranes were then incubated with appropriate secondary antibody coupled to horseradish peroxidase at room temperature for 60 minutes. Following incubation, membranes were then washed 3x in TBST, developed using an enhanced chemiluminescence (ECL) kit and quantified via densitometric analysis of the intensity of signal using Sigma Scan Pro v.5 software (Jandel Scientific, San Rafael, CA).

Statistical analysis

Data were analyzed using Graph Pad 4.0 software and values are reported as means ± SE unless otherwise indicated. Mitochondrial protein import and COX activity were analyzed using a two-way ANOVA and Bonferroni post-tests. All other data were analyzed using Student's t-test. Significance levels were set at $p < 0.05$.

Results

Absence of p53 reduces the expression of several mitochondrial protein import machinery components.

Tom20, Tim23, mtHsp70, mtHsp60 and cpn10 are intimately associated with the import and subsequent processing of matrix-destined proteins. Expression of these factors was determined in isolated SS (Fig. 1A, C) and IMF (Fig. 1B, D) mitochondrial fractions. In the SS mitochondria, lack of p53 induced a significant reduction in Tom20 (39%), Tim23 (36%), mtHsp70 (56%) and mHsp60 (32%) content, whereas cpn10 expression remained unchanged (Fig. 1A, C). We also observed a significant decrease in the protein levels of Tim23, mtHsp70 and mtHsp60 by ~71%, ~56%, and ~45%, respectively, in the IMF fractions isolated from p53 KO mice when compared to their WT counterparts (Fig. 1B, D). Cpn10 and Tom20 expression were not altered in the IMF mitochondrial pool in p53 KO mice. VDAC was used as a loading control, and it was not affected by the absence of p53.

Expression of cytosolic chaperones involved in mitochondrial protein import remains unchanged in p53 KO animals.

Cytosolic chaperones are known to affect the import of mitochondrial proteins with an N-terminal pre-sequence or internal localization signal. We investigated the expression of Hsp70, Hsp90 and MSF-L in cytosol isolated from p53 WT and KO mice. There was no alteration in the expression of the cytosolic chaperones in p53 KO muscle when compared to WT counterparts (Fig. 2A, B).

Import of matrix-destined preproteins into SS and IMF mitochondria is not impaired in p53 KO mice.

The import of the matrix-destined precursor protein pOCT into isolated SS and IMF mitochondria is illustrated as a representative autoradiogram with its quantification below (Fig. 3A, B). The rate of protein import was not significantly altered between SS or IMF mitochondria from p53 KO, when compared to WT mice (Fig 3A, B). As has been shown previously (34), the overall rate of pOCT import into SS mitochondrial subfraction was lower than that observed in the IMF mitochondria.

Mitochondrial COX activity and expression of electron transport chain (ETC) machinery components were unaffected in muscle in the absence of p53.

Using isolated SS and IMF mitochondria from p53 WT and KO mice, we measured no change in the rate of cytochrome c oxidase (COX) activity (Fig. 4A). In addition, the expression of components of the ETC machinery such as complex V ATP synthase subunit α (V), complex III subunit core 2 (III), complex IV subunit I (IV), complex II subunit 30 kDa (II), and complex I subunit NADH-ubiquinone oxidoreductase 1β subcomplex (I) did not differ between the two genotypes in either mitochondrial subfractions (Fig. 4B, C).

Impaired assembly of the cytochrome c oxidase (COX) complex in IMF mitochondria derived from p53 KO mice.

We used a two-dimensional BN-PAGE technique to specifically analyze the assembly of complex IV (COX) in SS (Fig. 5B) and IMF mitochondria (Fig. 5D) from p53 WT and KO mice. While assembly and synthesis of mature complex IV (S4) was not altered in the SS fraction (Fig. 5B), it was decreased by 39% in the IMF mitochondria ($p < 0.05$,

Fig. 5D). This was accompanied by a significant 41% decrease in the expression of Surf1, (Fig. 5C), an accessory assembly factor that facilitates the assembly of COX in mitochondria. Surf1 expression was not different between the two genotypes in SS mitochondria (Fig. 5A).

Discussion

Mitochondrial synthesis is a highly regulated process that involves the coordination of both the nuclear and the mitochondrial genomes. Nearly 99% of mitochondrial proteins are imported into the mitochondria, rendering this process crucial for the synthesis and function of the organelle. Rates of mitochondrial protein import can be regulated by alterations in 1) mitochondrial respiration, 2) expression of protein import machinery (PIM) components, and 3) changes in mitochondrial membrane potential ($\Delta\Psi m$). Our previous work has illustrated the adaptability of the protein import pathway in skeletal muscle mitochondria to various physiological perturbations such as denervation (32), chronic contractile activity (10), aging (8; 14), thyroid hormone treatment (5; 7; 31) and muscle differentiation (12). While the regulation of protein import into the mitochondria and its response during exercise remains enigmatic, it is clear that an improved ability to import more proteins would allow for a more streamlined process of mitochondrial assembly, therefore improve organelle function.

The diverse effects of p53 on mitochondrial function and oxidative capacity have only begun to be investigated in skeletal muscle. While no attempts have been made to assess the impact of p53 in regulating the protein import process, the literature indicates

that p53 affects the underlying determinative indices of protein import such as respiration, and Ψm (16; 26; 37). We have previously illustrated that respiration was impaired in the IMF mitochondrial sub-fraction from p53 KO muscle (26). This in turn likely affects ATP production, thus rendering an important conduit by which mitochondrial protein import is regulated, to be defective in p53 KO mice. Others have also illustrated that Ψm is dependent on the presence of p53 (16; 37). No prior studies exist that have examined the effect of p53 on the expression of protein import machinery components. Additionally, p53 transcribes SCO2, an important assembly factor for complex IV of the ETC (18). Thus we also determined if lack of p53 impairs COX assembly in mitochondria.

First we investigated whether the protein levels of select PIM components vital to the import of matrix-destined proteins are dependent on p53 expression. Our analyses revealed a protein- and mitochondrial fraction-specific effect on content. Tom20, Tim23, mtHsp70 and mtHsp60 were all significantly reduced in the SS mitochondria derived from p53 KO mice when compared to WT controls. In contrast, only Tim23, mtHsp70 and mtHsp60 levels were lower in the IMF mitochondria in p53 KO animals. We subjected the upstream 2000bp of the *mus musculus* promoter regions of these PIM components to promoter analysis using the Patch Software http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi. Bioinformatic analysis revealed the presence of putative p53 response elements in the promoter regions of Tom20 (at nucleotide positions +1158, +1872), Tim23 (-1824), cpn10 (-824, -1271, -1422, -1663, -1965) and mtHsp60 (+35, -105, -240, -259, +480, -480, -1495, +1812). No

putative p53 binding sites were observed in the promoter of mtHsp70, which was nevertheless significantly reduced in the KO mitochondrial samples. Likewise, the cpn10 promoter possessed several p53 response motifs, yet the expression was not affected in the KO mice. Further work is required to fully elucidate whether p53 affects the expression of these proteins either directly via transcription, or indirectly via changes in post-transcriptional mechanisms. Alterations in the expression of PIM components, in conjunction with impaired respiration and membrane potential, would theoretically limit mitochondrial protein import. Thus, we next determined the rate of import of a matrix-destined protein in isolated mitochondrial sub-fractions. As shown previously (34), IMF mitochondrial import rates were elevated when compared to SS mitochondria, likely due to the higher respiration kinetics of the IMF mitochondrial pool. To our surprise, we observed no difference in the percent import of mOCT in either subfraction between p53 WT and KO mice. In intact cells *in vivo*, mitochondrial preproteins also interact with cytosolic chaperones that primarily mediate the unfolding and transport of newly synthesized proteins. Thus, it is entirely plausible that within the intact cellular environment, mitochondrial protein import rates in p53 KO skeletal muscle maybe under another locus of regulation which could affect the rate of import. However, no changes were observed in the expression of cytosolic chaperones (Hsp90, Hsp70 and MSF-L) that coordinate the import of mitochondrial proteins. Clearly, the mitochondrial capacity for protein import into the matrix is not impaired in the absence of p53 in skeletal muscle, despite a decrease in the expression of selected PIM components. It is still possible that import into other sub-mitochondrial localizations such as the intermembrance space, and

inner/outer membranes may still be affected. Further research is warranted to fully elucidate the effect of p53 on mitochondrial import mechanics.

We next explored mitochondrial complex IV activity and the expression of ETC components in mitochondria from WT and KO mice. COX activity was not different between the two genotypes in SS or IMF mitochondria. We had hypothesized that COX activity would be impaired due to previously reported defects in mitochondrial respiration, and elevated ROS production in isolated mitochondria from skeletal muscle of KO animals (18; 24; 26). The results of this experiment, though contrary to our expectations, are not necessarily contradictory to defective respiration rates, as mitochondrial oxidative phosphorylation is the culmination of the activity of the entire ETC, whereas COX activity reflects the function of complex IV in isolation from the other complexes. In the current study, we also noted no difference in the expression of representative ETC subunits in p53 KO muscle SS and IMF mitochondria. However, this does not preclude any changes in the activity of the native ETC complexes in the KO animals, which could be evaluated using complex-specific enzymatic assays or through BN-PAGE in gel activity techniques.

The COX complex is composed of 13 subunits, two copper centers, two hemes and several cytochromes. The assembly of COX is thought to be a linear process which initiates around a seed formed by subunit I, subsequently assisted by a number of nuclear-encoded ancillary factors (33). The process involves four sub-complex assembly intermediate stages, and the final mature complex (S4) dimerizes to result in the functional COX holoenzyme. To date, most disorders that involve COX deficiency are

attributed to mutations in ancillary factors such as COX10 and COX15, which are important for heme A biosynthesis (1; 36), LRPPRC (21) and TACO1 (38) that mediate the expression of COX subunits, Surf1, essential for formation of early assembly intermediates (41), and SCO1/2 which are required for COX copper metallation (15; 23; 35). Interestingly, despite documentation of p53-SCO2-dependent regulation of oxidative capacity in liver and cancer cell lines, SCO1/2 levels do not decrease in the absence of p53 in skeletal muscle, and may be relatively unimportant for the observed mitochondrial defect present in skeletal muscle of p53 KO animals, as shown recently (24). Thus, we analyzed the expression of the assembly factor Surf1, which contains a putative p53 response element in its promoter region (at nucleotide position -809) as revealed by *in silico* analysis done on the promoter region of the *mus musculus* Surf1 gene using the Patch Software (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi). Furthermore, Surf1 has been shown to be vital in the biogenesis of complex IV (41). Our results show that Surf1 expression was significantly decreased in the IMF mitochondria of KO animals. This decrement occurred in tandem with a decrease in the assembly of complex IV (S4) in the same mitochondrial pool. It is intriguing to speculate whether this delayed assembly process contributes to the oxidative defect present in mitochondria from p53 KO muscle, in spite of the unaffected complex activity and import kinetics.

In summary, while p53 plays an indispensible role in determining oxidative capacity and mitochondrial function, the absence of the protein does little to impair matrix-destined mitochondrial protein import. The reduced assembly of complex IV may

be another course by which p53 determines the oxidative potential of mitochondria. However, more research is needed to comprehensively elucidate the mechanistic grid underlying the decrease in several components of the import and assembly pathway, culminating in reduced COX assembly in the absence of p53.

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Figure Legends

Figure 1. Effect of p53 on expression of Protein Import Machinery (PIM) components in SS and IMF mitochondria. Representative western blots of Tom20, Tim23, mtHsp70, mtHsp60 and cpn10 in SS (A, C) and IMF (B, D) mitochondria isolated from muscle of p53 wildtype (WT) and knockout (KO) mice, with the respective graphical representation below. VDAC was unchanged between the two genotypes and was used as a loading control. Values are expressed as fold changes (KO/WT) ± SEM. *p < 0.05, KO vs. WT, N = 4-9.

Figure 2. Expression of cytosolic chaperones in p53 WT and KO animals. Representative western blots of Hsp70, Hsp90, and MSF (A) and the respective graphical representation below (B). Aciculin content was unaffected between the two genotypes and was used as a loading control. Values are expressed as fold changes (KO/WT) ± SEM, N = 3-6.

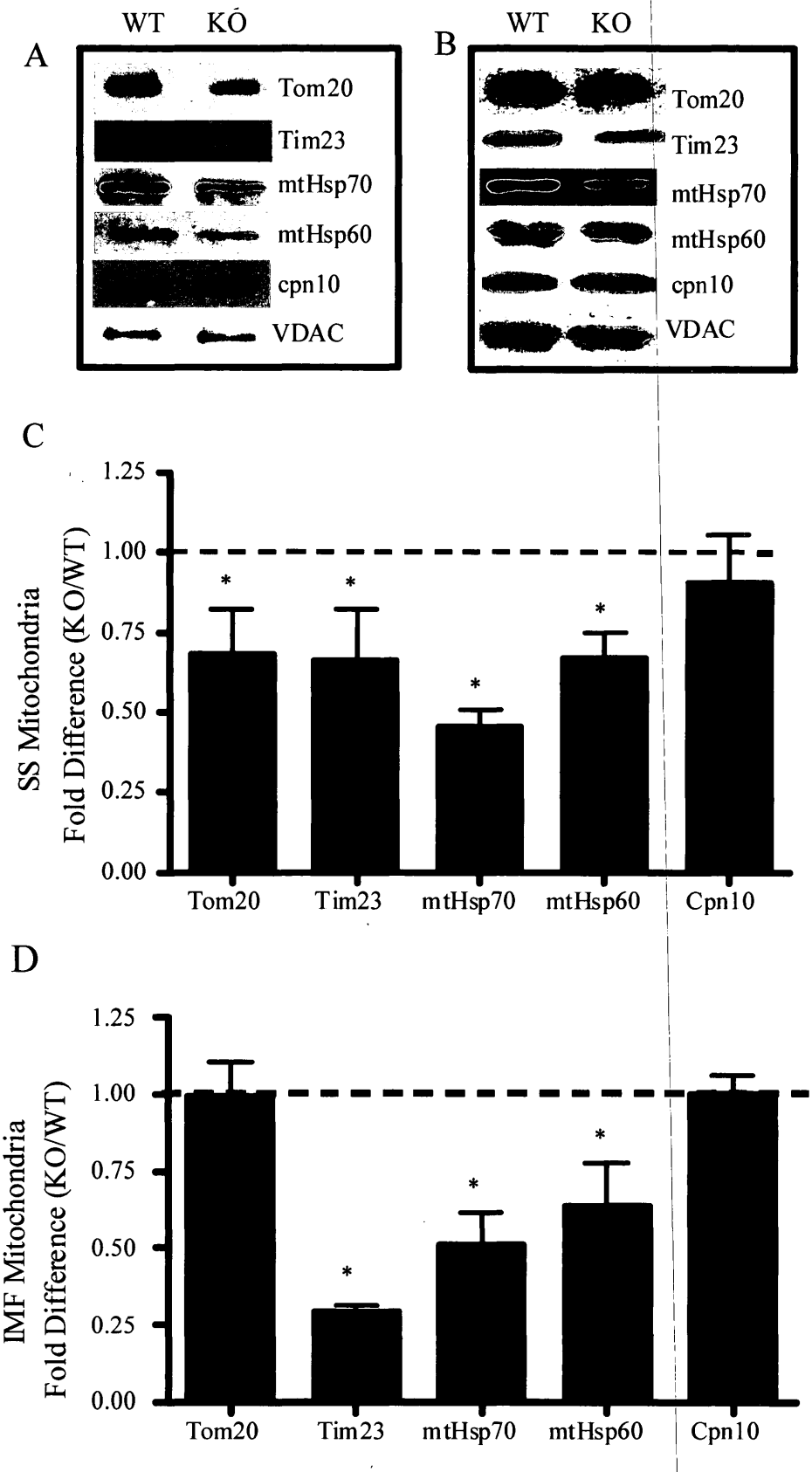
Figure 3. Mitochondrial protein import of matrix-destined OCT protein in SS and IMF mitochondria. Representative autoradiograms of protein import in the two mitochondrial populations from p53 WT and KO mice (A). Quantification of protein import was determined as a ratio of mature OCT (mOCT) to total OCT [precursor OCT (pOCT) + mOCT] (B). TL, 5µl of translation lane product that was not incubated with

any mitochondria was run with each import reaction and used to verify translational efficiency. *p < 0.05, SS vs. IMF mitochondrial sub-fraction, $N = 6\text{-}8$.

Figure 4. COX activity and expression of ETC protein components in mitochondria from p53 WT and KO mice. COX activity was measured in isolated SS and IMF mitochondrial fractions from the two genotypes (A).Values are expressed as means ± SEM, $N = 8$. Representative western blots (left) and the corresponding graphical representation (right) of components of ETC complexes in SS (B) and IMF (C) mitochondria from WT and KO samples. VDAC was used as a loading control. Values are expressed as means ± SEM, $N = 4$.

Figure 5. Expression of Surf1 and mitochondrial COX assembly. Western blots along with graphical summaries depicting the expression of Surf1 in SS (A) and IMF mitochondria (C). VDAC was used as a loading control. Values are expressed as means ± SEM, *p < 0.05, WT vs. KO, N=5-7. BN-PAGE gels were used to analyze the assembly of complex IV in SS (B) and IMF (D) mitochondria from WT vs. KO animals. S4 refers to the final assembly product and densitometric analysis revealed it to be deficient in the IMF mitochondria from KO mice ($p < 0.05$), $N = 4$.

Figure 1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WT
KO
Tom20
Tim23
mtHsp70
mtHsp60
cpn10
VDAC
B
WT
KO
Tom20
Tim23
mtHsp70
mtHsp60
cpn10
VDAC
C
SS Mitochondria
Fold Difference (KO/WT)
1.25
1.00
0.75
0.50
0.25
0.00
Tom20
Tim23
mtHsp70
mtHsp60
Cpn10
D
IMF Mitochondria
Fold Difference (KO/WT)
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1.00
0.75
0.50
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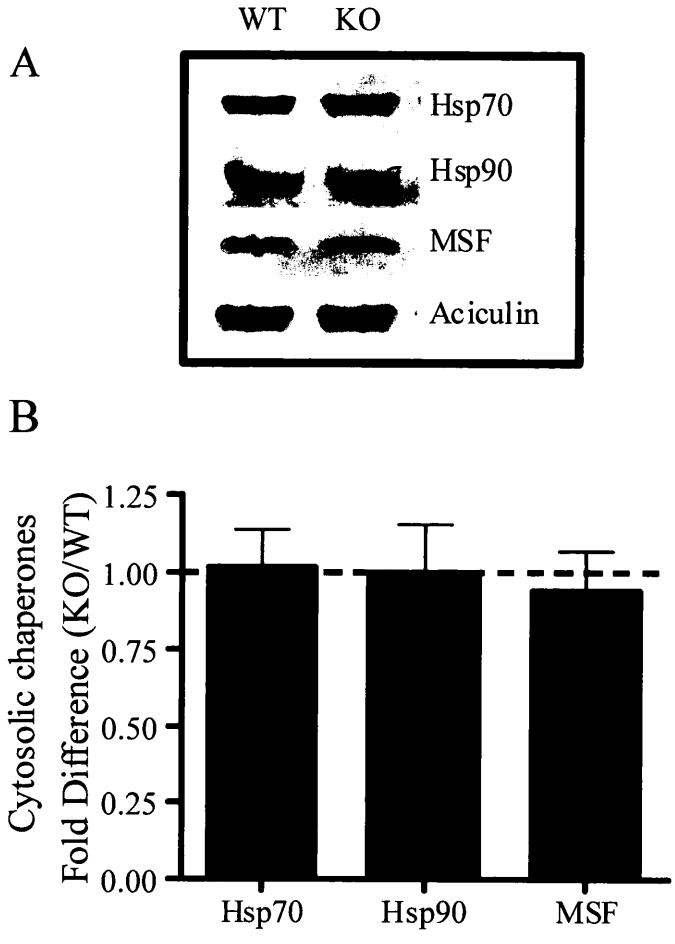
0.00
Tom20
Tim23
mtHsp70
mtHsp60
Cpn10

Figure 2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WT
KO
Hsp70
Hsp90
MSF
Aciculin
B
Cytosolic chaperones
Fold Difference (KO/WT)
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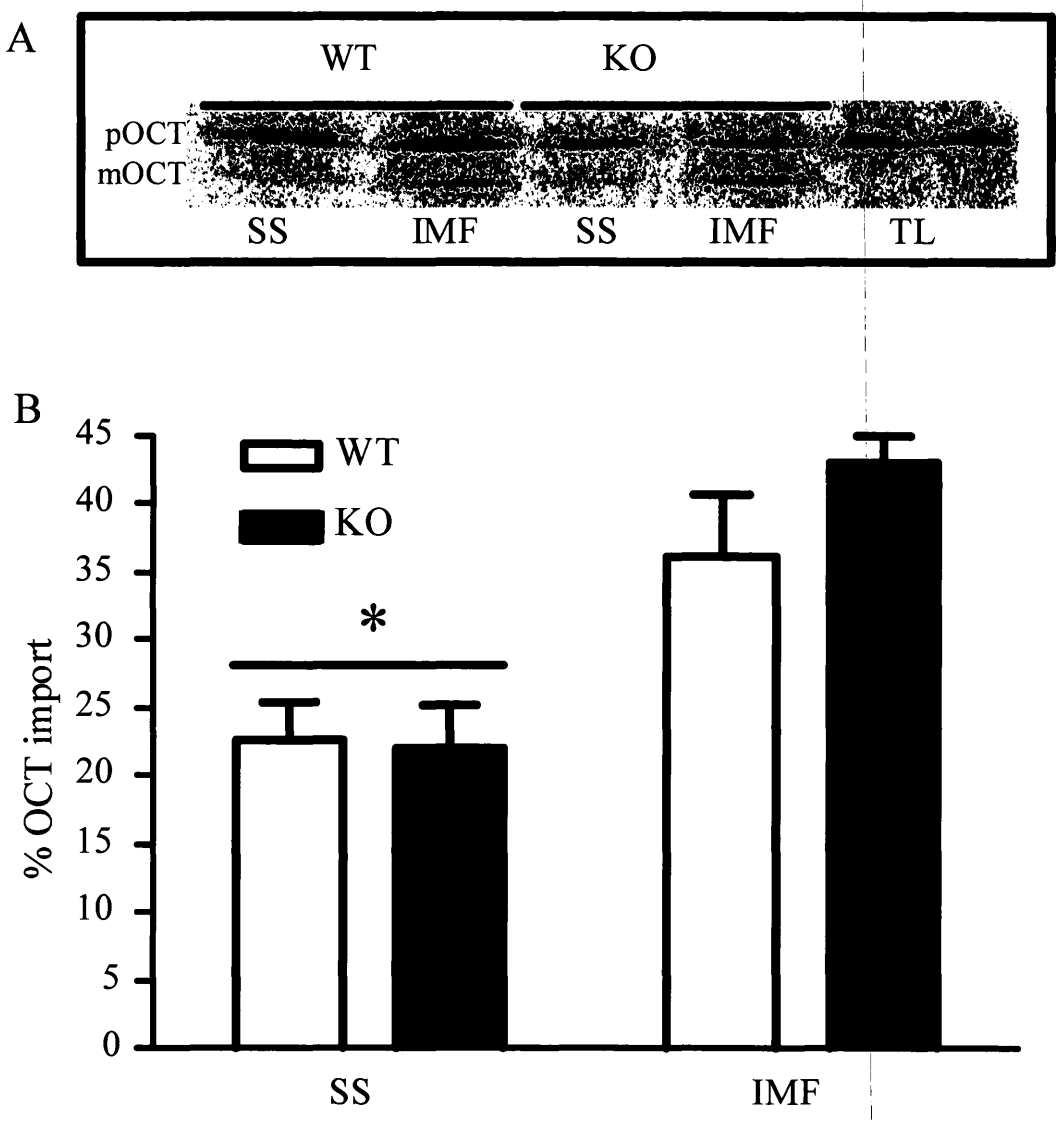
0.75
0.50
0.25
0.00
Hsp70
Hsp90
MSF

Figure 3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WT
KO
pOCT
mOCT
SS
IMF
SS
IMF
TL
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35
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5
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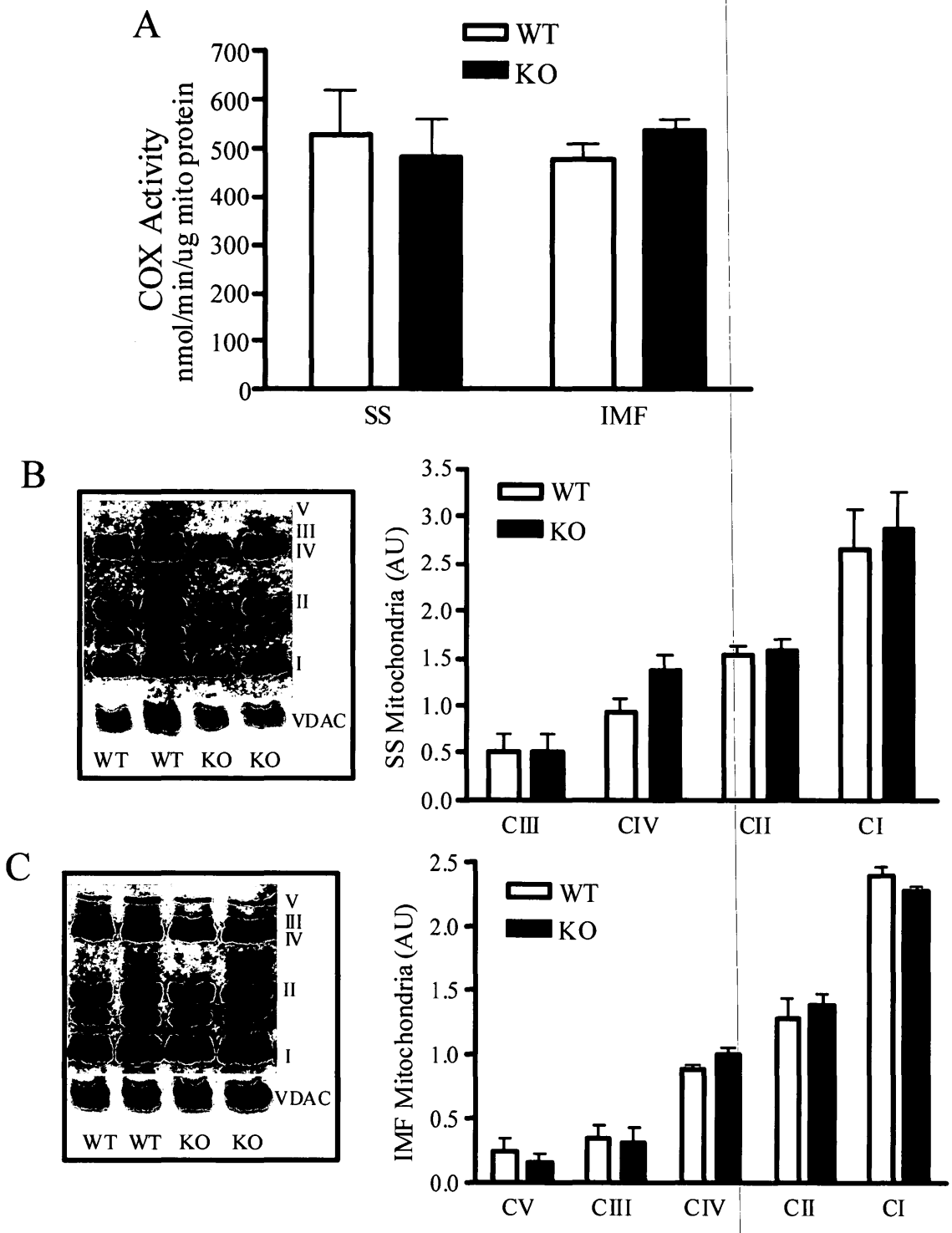
% OCT import
WT
KO
*
SS
IMF

Figure 4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600
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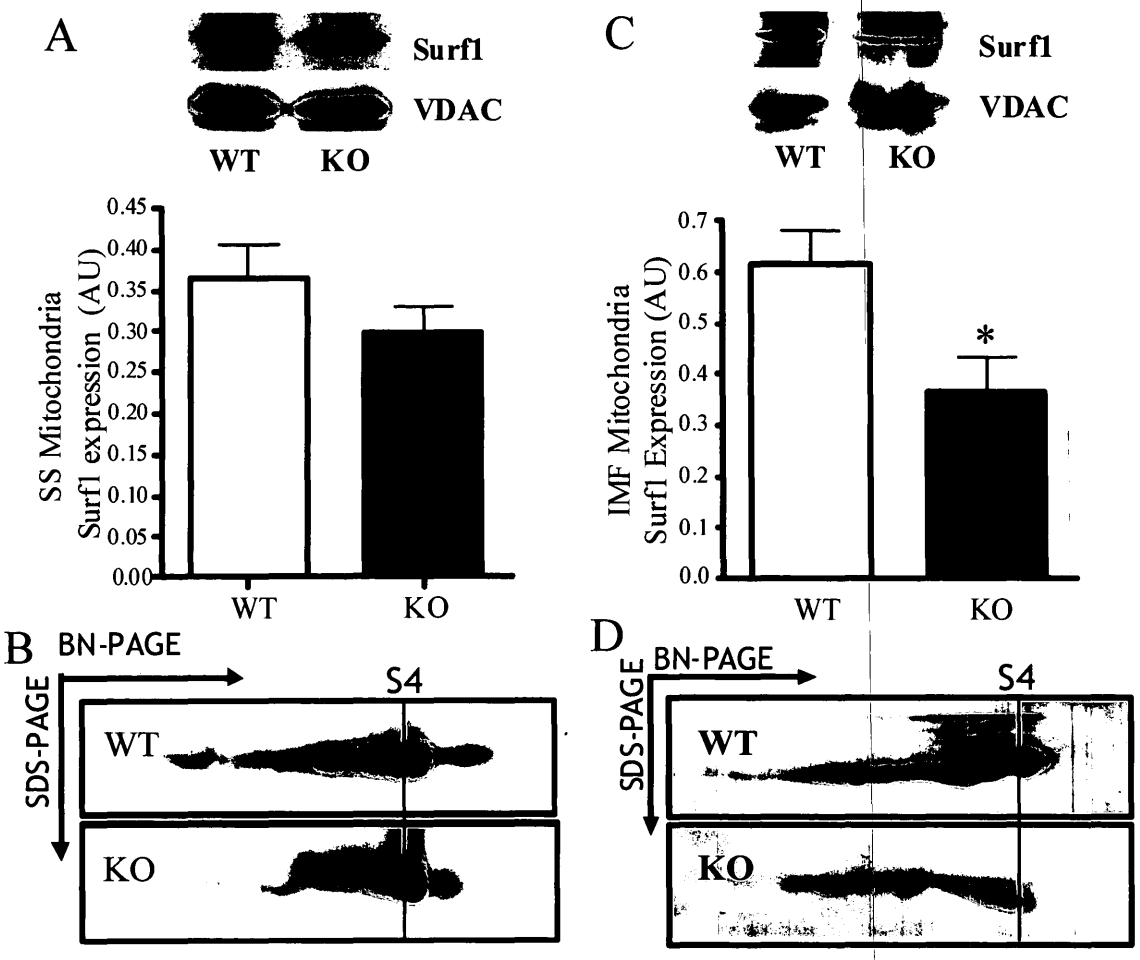
400
300
200
100
0
SS
IMF
B
V
III
IV
II
I
VDAC
WT WT KO KO
SS Mitochondria (AU)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CIII
CIV
CII
CI
C
IMF Mitochondria (AU)
CV
CIII
CIV
CII
CI

Figure 5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Surf1
VDAC
WT
KO
SS Mitochondria
Surf1 expression (AU)
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0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
WT
KO
C
Surf1
VDAC
WT
KO
IMF Mitochondria
Surf1 Expression (AU)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
WT
KO
B
BN-PAGE
SDS-PAGE
S4
WT
KO
D
BN-PAGE
SDS-PAGE
S4
WT
KO

CHAPTER 5:

Acute Exercise induces p53 translocation to the mitochondria and promotes a p53-Tfam-mtDNA complex in skeletal muscle

Ayesha Saleem[1,2] and David A. Hood[1,2]

[1]School of Kinesiology and Health Science and [2]Muscle Health Research Centre, York University, Toronto, Ontario, M3J 1P3, Canada

Running title: Exercise and mitochondrial p53

Keywords: acute exercise, mitochondrial biogenesis, p53, Tfam, ChIP

Author Contributions

AS and DAH conceived and designed the experiments, interpreted the data and wrote the manuscript. AS collected and analyzed all the data. Both authors approved the final version of the manuscript.

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Abstract

The major tumor suppressor protein p53 plays an important role in maintaining mitochondrial content and function in skeletal muscle. p53 has been shown to reside in the mitochondria complexed with mitochondrial DNA (mtDNA), however the physiological repercussions of mitochondrial p53 remain unknown. We endeavoured to elucidate whether an acute bout of endurance exercise could mediate an increase in mitochondrial p53 levels. C57Bl6 mice (n=6/group) were randomly assigned to sedentary (SED), acute exercise (AE, 15m/min for 90mins) or acute exercise+3h recovery (AER) groups. Exercise concomitantly increased the mRNA content of nuclear-encoded (PGC-1α, Tfam, NRF-1, COX-IV, CS) and mtDNA-encoded (COX-I) genes in the AE group, and further by ~5-fold in AER group. Nuclear p53 protein levels were reduced in the AE and AER groups, while in contrast, the abundance of p53 was drastically enhanced by ~2.4-fold and ~3.9-fold in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, respectively, in the AER conditions. Within the mitochondria, the interaction of p53 with mtDNA at the D-loop and with Tfam was elevated by ~4.6-fold and ~3.6-fold, respectively, in the AER group. In the absence of p53, the enhanced COX-I mRNA content observed with AE and AER was abrogated. This study is the first to indicate that endurance exercise can signal to localize p53 to the mitochondria where it may serve to positively modulate the activity of the mitochondrial transcription factor Tfam. Our findings help us understand the mechanisms underlying the effects of exercise as a therapeutic intervention designed to trigger the pro-metabolic functions of p53.

Introduction

The concept of utilizing physical exercise as a viable therapeutic modality for the treatment and management of age-associated chronic diseases often defined by dysregulated metabolism such as obesity, type 2 diabetes, cardiovascular diseases, dementia and cancer, is fast gaining acceptance (Warburton *et al.*, 2010). While the overall health benefits of exercise are undeniable, the underlying molecular mechanisms mediating the protection conferred by exercise is only beginning to be amassed. Although a multitude of cellular conduits are involved, a fundamental adaptation is the increase in the mitochondrial oxidative capacity post-exercise training. This increase in mitochondrial biogenesis brought about by endurance exercise is orchestrated by a host of transcriptional factors and co-activators that contrive together to coordinate improvements in oxidative capacity.

Affectionately known as the 'Guardian of the Genome' for its role in inducing cell-cycle arrest or cell death upon genotoxic stress signals, p53 is now reputed to play a vital role during cell metabolism, growth and development, and can be activated by physiological stressors to elicit an adaptive response (Saleem *et al.*, 2011). p53 participates in regulating metabolism, mobilizing cellular anti-oxidant defence against physiological oxidative stress, and orchestrating a balance between the anabolic and catabolic pathways within the cell (Vousden & Lane, 2007). Interestingly, the cellular fate in response to p53 activation often hinges on its subcellular localization. For example, within the cytoplasm p53 inhibits autophagy, whereas in the nucleus it serves to activate autophagy through direct transcriptional activation of effector genes that promote

autophagy (Maiuri *et al.*, 2010). Similarly, *in vitro* studies have shown that within the mitochondrial matrix, p53 specifically binds to mtDNA polymerase γ ensuring mtDNA genomic integrity and maintenance (Achanta *et al.*, 2005). p53 has also been purported to play a role in mtDNA transcription and translation by either binding directly (Heyne *et al.*, 2004;Kulawiec *et al.*, 2009) or indirectly via Tfam to mtDNA (Yoshida *et al.*, 2003). p53 is not the only nuclear transcription factor to have been found interacting with the mitochondrial genome. Another important nuclear protein for mitochondrial biogenesis, PPAR γ co-activator-1α (PGC-1α), also reportedly resides within the mitochondria in a complex with mtDNA, where it may be involved in facilitating mtDNA transcription (Aquilano *et al.*, 2010;Safdar *et al.*, 2011). In addition, retinoic acid X recepor (RXR) and estrogen receptors ERα/β also localize to the mitochondria, and have been implicated in mtDNA transcription (Casas *et al.*, 2003;Chen *et al.*, 2004). Clearly, the presence of bona fide nuclear proteins within mitochondria warrants further investigation into the physiological role of these proteins stationed in this organelle.

While much work has been done on p53 in cancer cell lines and in response to cell damaging signals, the physiological function of p53 within skeletal muscle and in response to physiologically-relevant signals such as exercise remains unknown. We have previously demonstrated an increase in p53ser15 phosphorylation content in response to acute contractile activity (Saleem *et al.*, 2009). Here, we further investigated whether a physiological alteration in the cellular milieu, represented by an acute bout of exercise, could induce a change in the subcellular localization of p53 in murine skeletal muscle. Our findings indicate that p53 levels decreased substantially in the nucleus with exercise

and recovery. We further illustrate that exercise preferentially shuttled p53 into the skeletal muscle mitochondria where it forms a complex at the D-loop region of mtDNA. These data suggest that the pro-metabolic/survival function of p53 in skeletal muscle can be differentially regulated in response to exercise.

Methods

Ethical Approval

C57Bl/6J mice, bred in an institutional central animal facility (York University), were housed in micro-isolator cages in a temperature- and humidity- controlled room and maintained on a 12-h light-dark cycle with food and water *ad libitum.* All animal care protocols were submitted to the York University Animal Care Committee and were approved in accordance with the guidelines set forth by the Canadian Council on Animal Care. Animals were sacrificed via cervical dislocation at the end of each experiment. p53 wild-type (WT) and knockout (KO) mice were acquired from Taconic Labs (Germantown, NY).

Animal breeding and Experimental Design.

At 3 months of age, C57Bl/6J mice (N = 6/group), were matched for sex and body weight, and randomly assigned to sedentary (SED), acute bout of exercise (AE), or acute exercise followed by three hours of recovery (AER) group. All mice were acclimatized to the treadmill two weeks prior to the beginning of the experiment. The animals in both the AE and AER groups were then selected and subjected to an acute bout of treadmill

running at 15 m/min for 90 min. All of the mice subjected to treadmill exercise were visibly exhausted at the end of the exercise as determined by their ability to withstand air and shock stimuli for greater than 5 seconds. The AE exercise group was sacrificed immediately following exercise. The SED was euthanized by cervical dislocation at the same time as the AER group. Quadriceps femoris muscle was extracted from all mice and ~70 mg was immediately snap frozen and stored at -80°C for subsequent mRNA expression. The remaining ~200 mg of fresh quadriceps femoris was utilized for nuclear, cytosolic and mitochondrial fractionation. To assess the importance of p53, WT and p53 KO mice (N=4/group) were subjected to the same acute exercise challenge as documented above. Quadriceps femoris muscles were extracted and frozen for subsequent mRNA analysis.

RNA Isolation.

Total RNA was isolated from ~70 mg of frozen muscle using Tri-reagent (Invitrogen) according to manufacturer's instructions. RNA concentration and quality was measured using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and further verified with RNA gels.

mRNA Expression Analyses.

The mRNA expression of peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC-1α), mitochondrial transcription factor A (Tfam), nuclear respiratory factor-1 (NRF1), cytochrome *c* oxidase subunits I and IV (COX-I, COX-IV), citrate synthase (CS), glyceraldehye phosphate dehydrogenase (GAPDH) and p53 was quantified using

7500 Real-time PCR System (Applied Biosystems Inc., Foster City, CA, USA) and SYBR® Green chemistry (PerfeCTa SYBR® Green Supermix, ROX, Quanta BioSciences, Gaithersburg, MD, USA). First-strand cDNA synthesis from 2 μg of total RNA was performed with primers using SuperscriptIII transcriptase (Invitrogen) according to manufacturer's directions. Forward and reverse primers (Table 1) for the aforementioned genes were designed based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) using the MIT Primer 3 designer software (http://wi.mit.edu/cgi- bin/primer3/primer3_www.cgi), and were confirmed for specificity using the basic local alignment search tool (www.ncbi.nlm.nih. gov/BLAST/). β-2 microglobulin was used as a control housekeeping gene, the expression of which did not alter between groups. All samples were run in duplicate simultaneously with negative controls that contained no cDNA. Melting point dissociation curves generated by the instrument were used to confirm the specificity of the amplified product. Primer efficiency curves were generated for each set to ensure 100±2% efficiency.

Mitochondrial Fractionation.

Briefly, ~150 mg of skeletal muscle was minced, homogenized and subjected to differential centrifugation as previously documented to yield subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial fractions (Saleem *et al.*, 2009). The mitochondria were re-suspended in a small volume of resuspension buffer (100 mM KCl, 10 mM MOPS, and 0.2% BSA, pH 7.4, supplemented with protease inhibitor cocktail Complete, ETDA-free [Roche Applied Science, Manheim, Germany]). All centrifugation steps were

carried out at 4°C. Mitochondrial homogenates were analyzed for protein content using the Bradford assay, and subsequently frozen at -80°C for further biochemical analysis including mitochondrial co-immunoprecipitation assay, mitochondrial chromatin immunoprecipitation (ChIP) assay and immunoblotting.

Mitochondrial Co-Immunoprecipitation Assay.

Mitochondrial co-immunoprecipitation assay was performed on isolated IMF mitochondrial fractions using Pierce Co-Immunoprecipitation Kit (Pierce, Rockford, IL, USA) according to manufacturer's instructions. Briefly, mitochondrial fractions were homogenized in lysis buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4) supplemented with protease inhibitor cocktail Complete, ETDA-free (Roche Applied Science, Manheim, Germany). Mitochondrial protein fraction (1 mg) was pre-cleared by incubation with control agarose resin to minimize non-specific binding. Anti-p53 (FL-393, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 40 μg) was covalently coupled onto an amine-reactive resin. The pre-cleared lysates were subsequently incubated with antibody-coupled beads overnight at 4°C. Co-immunoprecipitates were, washed, eluted and subsequently collected by centrifugation, boiled in 50 μL of lane marker sample buffer, and used for immunoblot analysis for Tfam. Anti-IgG antibody was used as a non-specific control.

mtDNA Chromatin Immunoprecipitation (ChIP) Assay.

ChIP assay was performed using an EZ-ChIP^{TM} kit (Millipore, Billerica, MA, USA) according to manufacturer's instructions. Mitochondrial fractions (1 mg) were cross-

linked in 1% formaldehyde for 10 min at room temperature, and 10X glycine was added to stop fixation. Samples were then homogenized in 1 mL of SDS lysis buffer supplemented with protease inhibitor cocktail Complete, ETDA-free (Roche Applied Science, Manheim, Germany). Chromatin was sheared by sonicating each sample on ice (output 20%, 4 times for 20 sec, with a 20 sec pause each time). Following centrifugation at 10,000 × *g* at 4 °C for 10 min, the supernate containing 1 mg of protein was diluted to 1 mL with ChIP dilution buffer. Anti-p53 (FL393, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 10 μg) or anti-IgG antibody was added per sample and incubated overnight at 4 °C. Anti-IgG antibodies were used as non-specific controls. Protein G-agarose (60 μL) was added and the sample was mixed for 1 h at 4 °C with rotation. Precipitated samples were washed, and then eluted in 100 μL of elution buffer, and cross-linking was reversed by the addition of 8 μL of 5 M NaCl per sample followed by incubation at 65 °C for 12 h. Co-immunoprecipitated DNA was purified according to the manufacturer's instructions. mtDNA D-loop region (Table 1) was quantified using 7500 Real-time PCR System (Applied Biosystems Inc., Foster City, CA, USA) and SYBR® Green chemistry (PerfeCTa SYBR® Green Supermix, ROX, Quanta BioSciences, Gaithersburg, MD, USA).

Nuclear and Cytosolic fractionation.

Nuclear and cytosolic fractions were prepared from freshly isolated skeletal muscle using a commercially available nuclear extraction kit (Pierce NE-PER, Rockford, IL, USA). Briefly, 50-75 mg of skeletal muscle was minced and homogenized in CER-I buffer

containing protease inhibitor cocktail Complete, EDTA-free (Roche Applied Sciences, Manheim, Germany). After a series of wash steps, nuclear proteins were extracted in high salt NER buffer supplemented with protease-inhibitors. The cytosolic fraction was spun at 100,000 × *g* at 4 °C for 60mins to obtain pure cytosolic fraction.

Immunoblotting.

Proteins were resolved on 8% or 12% SDS-PAGE gels depending on the molecular weight of the protein of interest. The gels were transferred onto ECL nitrocellulose membranes, followed by blocking with 1-3% milk in TBST overnight at 4 °C. Immunoblotting was carried out using mouse p53 (1:20, pAb421, kind gift from Dr. Sam Benchimol), rabbit Tfam antibody (1:500, in house), rabbit p53ser^{15} (1:500, R&D systems, AF2887) and rabbit PGC-1α (1:500, Millipore, AB3242). With regard to PGC-1α, we have previously validated the use of this particular PGC-1α antibody in myotubes (Uguccioni & Hood, 2011), and it has also been used by other muscle physiology laboratories to detect PGC-1α (Hancock *et al.*, 2008; Philp *et al.*, 2011;Williams *et al.*, 2009). We also further ascertained the antibody specificity by performing western blotting analysis using quadriceps femoris muscle from PGC-1α KO mice. Membranes were then incubated with the appropriate secondary antibody (Abcam) coupled to horseradish peroxidase (HRP) at room temperature for 2 hours. After incubation, membranes were washed three times in TBST, developed with an enhanced chemiluminescence (ECL) kit, and quantified via densitometric analysis of the intensity of signal with Sigma Scan Pro v.5 software (Jandel Scientific, San Rafael, CA). VDAC

and Histone 2 B (H2B) were used as loading controls for mitochondrial and nuclear fractions, respectively.

Statistical Analyses.

Data were analyzed using one-way analysis of variance (ANOVA), except for Fig 5D which was analyzed using a two-way ANOVA, using Graph Pad 4.0 software. For all one-way ANOVA analyses, a Tukey's post-hoc test was utilized to identify individual differences when statistical significance was observed. Statistical significance was established at a $P \leq 0.05$. Data are presented as mean ± standard error of the mean (SEM).

Results

Purity of cellular fractions, antibody specificity, and cellular distribution of p53.

The purity of nuclear and mitochondrial fractions and specificity of p53 antibody are of extreme importance for the subsequent subcellular localization analyses of p53 carried out in this study. We have previously established that the isolated mitochondrial subfractions are pure and functionally intact (Cogswell *et al.*, 1993). To further assess the purity of the nuclear and mitochondrial fractions, we immunoblotted for the mitochondrial protein cytochrome *c* oxidase IV (COX-IV, Fig 1A) and the nuclear protein histone 2B (H2B, Fig 1B). COX-IV and H2B were only detected in mitochondrial and nuclear fractions, respectively. Furthermore, no cytosolic contamination was apparent in the nuclear and mitochondrial fractions as measured by GAPDH (Fig 1C). We also confirmed the specificity of p53 antibody against the protein by measuring p53

expression in whole muscle homogenates from wildtype (WT) and p53 knockout (KO) mice (Fig 1D). p53 was not detected in the muscle homogenates from KO mice (Fig 1D) illustrating the specificity of the p53 antibody utilized in this study. The subcellular distribution of p53 in skeletal muscle was measured by probing for p53 in cytosolic, mitochondrial and nuclear fractions (Fig 1E). p53 content was highest in the cytosolic fraction, followed by the nuclear and mitochondrial compartments in muscle (Fig 1E).

Validation of PGC-1α antibody

To ascertain the specificity of the PGC-1α antibody, we performed immunoblotting using quadriceps femoris muscle from PGC-1α KO (Fig. 1F, lane 1), and WT mice (Fig. 1F, lane 2). We also included a soleus muscle homogenate as a positive control (Fig. 1F, lane 3), as it clearly depicts an increased abundance of PGC-1α in a slow, oxidative tissue such as soleus as opposed to a primarily fast glycolytic muscle such as quadriceps femoris. The ~94kDa band is absent in lane 1 confirming the antibody specificity. There is slight immunoreactivity in lane 1, but it is present at a higher molecular weight than the band in the WT lanes. We suspect it to be non-specific binding.

mRNA content of genes related to energy metabolism increases following an acute bout of endurance exercise.

The transcript levels of nuclear DNA-encoded (PGC-1α, Tfam, NRF-1, COX-IV, CS) and mtDNA-encoded (COX-I) genes involved in mitochondrial biogenesis increased immediately following an acute bout of exercise ($p<0.05$, Fig 2). This enhancement in

mRNA expression of these transcripts was maintained or further exacerbated after three hours of recovery following the exercise ($p<0.05$, Fig 2). p53 mRNA levels decreased with acute exercise, whereas GAPDH transcript levels remained relatively unchanged with acute exercise (AE), and acute exercise with 3hrs recovery (AER, Fig 2).

Acute exercise reduces nuclear, and enhances mitochondrial abundance of p53

p53 content decreased steadily after exercise by 56% in the nuclear fraction from the AER group ($p<0.05$, Fig 3A). In contrast, an elevation of PGC-1α expression was evident in the nucleus in the AE and AER conditions ($p<0.05$, Fig 3B), in line with previous literature (Wright *et al.*, 2007). H2B served as a loading control and remained unchanged with the exercise treatments. A gradual increase in p53 levels within mitochondria was observed by ~2.4-fold ($p<0.05$, Fig 4A) in the subsarcolemmal (SS) mitochondrial fraction, and by ~3.9-fold ($p<0.05$, Fig 4B) in the intermyofibrillar (IMF) mitochondrial fraction. The increase in p53 content occurred concomitantly with a gradual increase in p53ser15 phosphorylation in mitochondrial fractions (Fig 4C). Both the mtDNA transcription factor Tfam, as well as VDAC expression remained unchanged with the exercise treatments (Fig 4D).

Mitochondrial p53 forms a complex with Tfam and binds to mtDNA at the D-loop region

Given the apparent translocation of p53 to the mitochondria with exercise, we posited that acute exercise would positively enhance this association of p53 with Tfam and mtDNA. To test our hypothesis we performed co-immunoprecipitation assays with the SS

mitochondrial fraction from SED, AE and AER groups (Fig 5A). Congruent with previous *in vitro* findings (Wong *et al.*, 2009;Yoshida *et al.*, 2003), we observed that p53 forms a complex with Tfam in skeletal muscle mitochondria (Fig 5A). Since Tfam is primarily found in the mitochondrial matrix, this observation also confirmed the matrix-specific localization of p53 in mitochondrial fractions (Fig 5A and B). Furthermore, we observed that the p53-Tfam complex increased progressively upon an acute bout of exercise by ~3.6-fold in the AER group ($p<0.05$, Fig 5A). We also performed mtDNA co-immunoprecipitation assays to determine if p53 was bound to the D-loop of mtDNA, and whether this interaction was positively modified by acute exercise. We observed that in skeletal muscle mitochondria, p53 forms a complex with mtDNA within the D-loop region, and that this association is enhanced by ~4.6-fold in the AER group in IMF mitochondria ($p<0.05$, Fig 5B).

Increase in mtDNA-derived COX-I mRNA post-exercise is dependant upon p53 expression

The transcript levels of mtDNA-encoded COX-I subunit increased immediately following an acute bout of exercise, and further with recovery in the WT mice ($p<0.05$, Fig 5D) as shown earlier. Remarkably, this exercise-induced adaptation in COX-I mRNA was completely abolished in the p53 KO mice ($p<0.05$, Fig 5D).

Discussion

The disruption of p53 expression carries grave physiological repercussions, as evident by aberrant mitochondrial bioenergetic efficiency, reduced mitochondrial biogenesis, greater fatigability and reduced exercise capacity observed in p53 KO animals (Matoba *et al.*, 2006;Park *et al.*, 2009;Saleem *et al.*, 2009). If p53 plays a role in potentiating endurance exercise-induced mitochondrial adaptations, it is likely that p53 is post-translationally modified in response to exercise which may differentially regulate its sub-cellular localization post-contractile activity. We and others have previously reported elevated p53ser15 phosphorylation levels in response to acute contractile activity (Saleem *et al.*, 2009;Bartlett *et al.*, 2012), a modification that is classically linked to the increased stability and activity of the protein. Interestingly, here we demonstrate that p53ser15 phosphorylation levels increase progressively with AE and AER conditions in the mitochondrial fractions, in line with the enhanced phosphorylation status of p53 as previously reported in whole muscle homogenates (Saleem *et al.*, 2009;Bartlett *et al.*, 2012). It is entirely plausible that the measured increase in Ser15 phosphorylation is a due to the combined effect of the activation of p38 MAPK and AMPK, both of which are bona fide exercise-activated kinases (Akimoto *et al.*, 2005;Ljubicic & Hood, 2009) and are known to post-translationally modulate p53 (Jones *et al.*, 2005;She *et al.*, 2000). In a recent paper, Bartlett et al. (Bartlett *et al.*, 2013) provided further suggestive evidence of a link between AMPK signalling and p53 phosphorylation in human skeletal muscle. Furthermore, since p53 is subject to a host of other post-translational modifications such as acetylation, ubiquitination, sumolaytion, and neddylation, it is possible that any of

these may assist in trafficking p53 out of the nucleus and into the mitochondria. In fact, it has been previously demonstrated that mono-ubiquitinated p53 is shuttled out of the nucleus in a CRM1-dependent manner (Lohrum *et al.*, 2001). Subsequent to this, mono-ubquitinated p53 is rapidly trafficked to the mitochondria where it undergoes deubiquitination by resident deubiquitinases such as HAUSP (Marchenko *et al.*, 2007). Additionally, proteomic analyses have revealed that a large number of mitochondrial destined nuclear-encoded proteins lack canonical N-terminal mitochondrial targeting signal, but instead have cryptic internal signals. There is no discernable consistent pattern to the nature and location of these cryptic signals. Similar to many other proteins such as PGC-1α, RXR and ERα/β that have been identified within mitochondria, p53 also does not contain a canonical mitochondrial targeting signal, but it appears to possess a cryptic signal (Boopathi *et al.*, 2008). Clearly, further research is required to carefully distil which post-translational modification is pivotal to the change in the sub-cellular address of p53 and its subsequent import into the mitochondria.

AIF, SCO2 and Tfam are important transcriptional targets of p53 through which it regulates cell metabolism. While AIF is known for its role in the induction of apoptosis, during basal conditions AIF contributes to efficient oxidative phosphorylation by promoting the proper assembly and function of mitochondrial respiratory complex I (Stambolsky *et al.*, 2006). SCO2 has been shown to be vital for the proper assembly of subunit I of the mitochondrial cyctochrome *c* oxidase complex, and re-expression of SCO2 quickly rescued the oxidative impairment in p53 KO cells in cancer cells (Matoba *et al.*, 2006). A multitude of studies (Bourdon *et al.*, 2007;Kulawiec *et al.*,

2009;Lebedeva *et al.*, 2009;Park *et al.*, 2009;Yoshida *et al.*, 2003) have demonstrated that the presence of p53 is a determinant of both Tfam expression and mtDNA content. Since p53 clearly targets the promoter regions of these genes and positively up-regulates their expression, we posited that p53 levels would increase in the nucleus upon an exercise stimulus in an effort to increase the transcription of the aforementioned gene targets. However, p53 content reduced drastically with exercise, and even more so in the recovery period in the nuclear fraction. This is at odds with the expression pattern of the classic activator of mitochondrial biogenesis, PGC-1α, wherein the content of PGC-1α steadily increased with exercise and recovery in the nuclear fraction as shown here, and by others (Wright *et al.*, 2007). We hypothesize that the decrease in p53 content occurred so as to remove p53 from the vicinity of the plethora of gene targets that can induce cell death, or senescence, reactive outcomes often dictated by p53 in times of stress. Additionally, a recent study (Sahin *et al.*, 2011) has indicated that p53 can transcriptionally suppress PGC-1α and PGC-1β expression, co-activators that are known to induce mitochondrial biogenesis. Therefore it is possible that the decrease in nuclear p53 content ensures that p53 does not suppress the activation of PGC-1α/β, so as not to impede the process of mitochondrial biogenesis.

It should also be noted that despite the apparent exodus of p53 from the nucleus, a basal level of p53 is still present in nuclei post-exercise which may be sufficient to carry on its pro-metabolic role. Moreover, the idea that p53 is a transcriptional inhibitor of PGC-1α is not universally accepted. A recent study reported p53 binds to the promoter of PGC-1α and up-regulates its expression during times of mild oxidative and nutritional

stress (Aquilano *et al.*, 2012). Thus, the interaction between p53 and PGC-1α appears to be specific to the cellular milieu, and it remains to be seen how exercise affects this relationship.

Another intriguing finding was the decrease in the mRNA level of p53, which was in vivid contrast to the significant elevations in the transcripts of other genes involved in mitochondrial biogenesis such as Tfam, NRF-1, PGC-1α, CS, COX I and COX IV. This could be due to the exquisite control exerted by p53 on its own mRNA expression. As illustrated previously (Mosner *et al.*, 1995), with increasing p53 protein expression, there is a subsequent decrease in its mRNA expression as p53 binds to the 5′ untranslated region (UTR) of its own mRNA and causes it to be degraded. Thus the decrease in mRNA expression that was observed in this study could simply be due to the ability of p53 to fine-tune its own expression.

The binding of p53-Tfam, and p53 with mtDNA post-exercise is an exciting finding. It is possible that p53 acts as an accessory factor that may augment Tfam activity, an association highly dependent on altered energy demands such as during exercise. Tfam primarily exists as a mitochondrial transcription factor, but also functions to maintain mtDNA integrity and repair. We observed increasing amounts of Tfam bound to p53 in the mitochondria with exercise and 3hrs recovery, despite a lack of change in the amount of total Tfam in the mitochondria. It is known that the majority of Tfam within the organelle is closely associated with mtDNA (Park & Larsson, 2011), at the D-loop region to allow Tfam to regulate mtDNA transcription (Larsson *et al.*, 1998). We have previously shown that chronic contractile activity results in increased Tfam binding

to mtDNA D-loop region, indicative of the ability of chronic exercise to modify mtDNA transcription (Gordon *et al.*, 2001). Interestingly, p53 has been shown to increase the binding of Tfam to damaged DNA (Wong *et al.*, 2009;Yoshida *et al.*, 2003) and it is entirely plausible that it potentiates binding of Tfam to the D-loop as well. We also observed an elevated amount of p53 bound to the D-loop of mtDNA, either directly or via Tfam, in the mitochondrial fractions following exercise and recovery. Therefore, we hypothesize that p53 may function as a mitochondrial transcription factor, positively modulating mtDNA-encoded gene expression either directly, or via modulation of Tfam activity at the D-loop region. To assess the plausibility of this hypothesis, we subjected the D-loop region 15400bp-16299bp of the *mus musculus* mitochondrial genome (NCBI reference number: NC_005089.1) to a patch analysis using the Consite software http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite. Bioinformatic analysis revealed putative p53 response elements (RE), which were identified and plotted against the mtDNA D-loop sequence. This is in line with previous studies documenting the interaction of p53 with mtDNA (Heyne *et al.*, 2004;Kulawiec *et al.*, 2009), and corresponds with several reports that have identified common nuclear transcription factors and co-activators such as SIRT1, ERα/β, retinoid X receptor and PGC-1α that also reside in the mitochondria, and are involved in regulating mtDNA transcription (Aquilano *et al.*, 2010;Casas *et al.*, 2003;Chen *et al.*, 2004;Safdar *et al.*, 2011). Further support for a significant role of p53 in regulating mtDNA transcription can be acquired from observing the large increase in COX-I mRNA expression in wild-type mice, which was completely abrogated in the p53 knockout animals. This indicates that the increase

in intra-mitochondrial p53 with exercise is indeed functional in facilitating mtDNA transcription. In addition, p53 can mediate mtDNA genomic stability directly, via its inherent base excision repair activity, and indirectly by 1) promoting the binding of Tfam to damaged mtDNA, and 2) by enhancing the function of mtDNA polymerase γ, the sole mtDNA repair enzyme (Saleem *et al.*, 2011). Therefore, it is possible that exercise induces p53-Tfam-mediated maintenance and repair of mtDNA, exclusively or concomitantly with enhanced mtDNA transcription.

Thus, the nuclear depletion and mitochondrial accumulation of p53 subsequent to an acute bout of exercise highlights the fascinating diverse transcriptional and non-transcriptional modes of regulating oxidative metabolism as commandeered by p53 in a physiological setting. Clearly, the action of p53 on the mitochondrial genome may represent an important conduit by which it facilitates the coordinated expression of the nuclear and mitochondrial genomes in promoting mitochondrial biogenesis and mtDNA stability in muscle. The tumor suppressor protein p53 plays an indispensible role in halting tumorigenesis, is important in mediating oxidative metabolism, and as indicated by our data, responds to an exercise stimulus. Together with the current awareness of exercise as a feasible, cost-effective and safe therapy to reduce the incidence and progression of cancer (Friedenreich & Orenstein, 2002;Warburton *et al.*, 2010) and other metabolic diseases, our results may carry important clinical implications. Further work that delineates the signalling mechanisms involved, and that focuses on elucidating the effect of exercise training on p53 function and regulation is clearly warranted.

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Tables

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

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
PGC-1α	TTCCACCAAGAGCAAGTAT	CGCTGTCCCATGAGGTATT
Tfam	GAAGGGAATGGGAAAGGTAGA	AACAGGACATGGAAAGCAGAT
NRF-1	ATCCGAAAGAGACAGCAGACA	TGGAGGGTGAGATGCAGAGTA
p53	CCGACCTATCCTTACCATCATC	TTCTTCTGTACGGCGGTCTC
COX-I	CTAGCCGCAGGCATTACTAT	TGCCCAAAGAATCAGAACAG
COX-IV	CTCCAACGAATGGAAGACAG	TGACAACCTTCTTAGGGAAC
CS	GCATGAAGGGACTTGTGTA	TCTGGCACTCAGGGATACT
GAPDH	AACACTGAGCATCTCCCTCA	GTGGGTGCAGCGAACTTTAT
β-2 microglobulin	GGTCTTTCTGGTGCTTGTCT	TATGTTCGGCTTCCCATTCT
mtDNA D-loop	AGCCCATGACCAACATAACTG	AGACTGTGTGCTGTCCTTTCA

Figures and legends

Figure 1. Sub-cellular fraction purity, cellular distribution of p53, and antibody specificity of p53 and PGC-1α. (A) COX-IV, (B) Histone 2B and (C) GAPDH were used as indicators of the purity of mitochondrial, nuclear and cytosolic fractions, respectively. (D) Specificity of p53 antibody as demonstrated by measuring p53 expression in whole muscle homogenates from p53 wild-type (WT) and knockout (KO) mice. (E) Sub-cellular pattern of p53 expression in cytosolic (C), mitochondrial (M) and nuclear (N) fractions. (F) PGC-1α expression in KO quadriceps femoris (KO-Q, lane 1), WT quadriceps femoris (WT-Q, lane 2) and WT soleus (WT-S, lane 3) muscle homogenates. The molecular weight in kDa is shown on the left for (D), (E), and (F).

Figure 2. Changes in nuclear and mitochondrial DNA-encoded mRNA transcripts. Transcriptional regulators of mitochondrial biogenesis (PGC-1α, NRF-1 and Tfam), components of the electron transport chain (COX-IV and COX-I), and mitochondrial enzyme citrate synthase (CS) mRNA expression increased in the AE and AER conditions. Glycolytic enzyme GAPDH did not change and p53 mRNA levels decreased with the experimental conditions. β2-microglobulin was used as a house keeping control gene. *$p<0.05$ AE vs. SED, †$p<0.05$ AER vs. SED. Data are presented as a fold-increase over SED mice. Error bars represent SEM values, N=6/group.

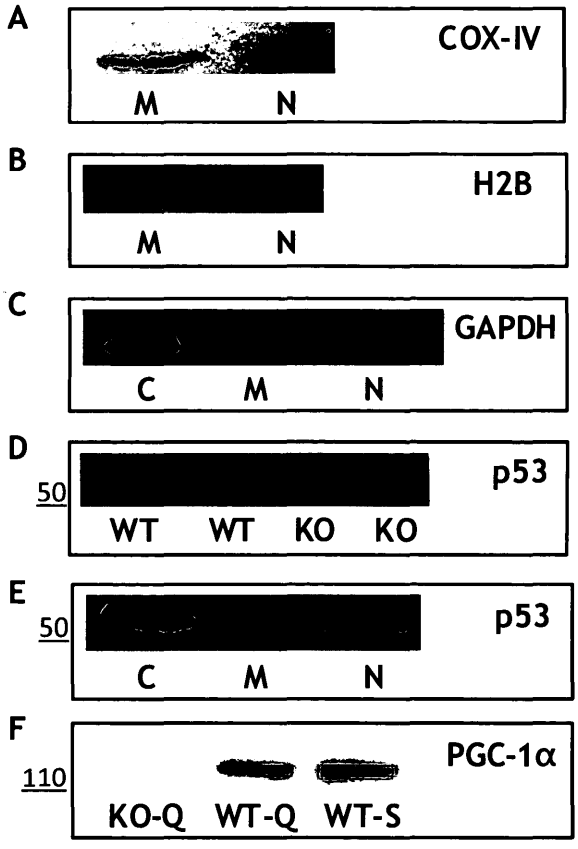
Figure 3. Alterations in nuclear expression of p53 and PGC-1α with acute exercise. Nuclear content of (A) p53 steadily decreased, and that of (B) PGC-1α progressively increased in the AE and AER animal groups. Histone 2B was used as a loading control and did not change during the conditions. *p<0.05 AE vs. SED, †p<0.05 AER vs. SED. ¶p<0.05 AER vs. AE. Data are presented as mean ± SEM, *N*=6/group.

Figure 4. Changes in mitochondrial Tfam and p53 content upon acute exercise. p53 content in (A) SS mitochondria and (B) IMF mitochondria increases significantly in the AER group. (C) p53 phosphorylation at serine 15 displayed a gradual increase in AE and AER conditions in SS mitochondria compared to SED control (representative blot from n=2). (D) Tfam content did not alter immediately after AE, and nor in the AER group. VDAC was used as loading control and did not alter between the experimental conditions. *p<0.05 AER vs. SED. ¶p<0.05 AER vs. AE. Data are presented mean ± SEM values, *N*=6/group.

Figure 5. Acute exercise enhances the association of p53 with Tfam inside the mitochondria and promotes p53-mtDNA D-loop complex formation. (A) p53 was immunoprecipitated (IP) followed by immunoblotting (IB) for Tfam in isolated mitochondrial fractions from SED, AE, and AER mice. p53-Tfam complex increased significantly in the AER group. *p<0.05 AER vs. SED. IgG was used as a non-specific control for the co-immunoprecipitation assay. VDAC was used to ensure that equal amounts of mitochondrial fractions were used for co-IP experiments from each group of

mice. Data is presented as a fold-increase over SED values. Error bars refer to SEM values, *N*=6/group. (B) There is a significant increase in the p53 complexed at the D-loop region of the mtDNA in the AER group. *p<0.05 AER vs. SED. Anti-IgG antibody was used as a non-specific control. Real-time amplification of signal from IgG group was below detectable limit. Data is presented as a fold-increase over SED values. Error bars refer to SEM values, *N*=6/group. (C) Schematic illustrating the D-loop sequence of mtDNA and putative p53 response elements (RE). The position of the forward (F′) and reverse (R′) primers used in mtDNA-ChIP analysis is identified, and the sequences of the three putative p53 RE within the amplicon amplified by the PCR reaction are shown. (D) mRNA content of mtDNA-encoded COX-I subunit in wild-type (WT) and p53 knockout (KO) mice in SED, AE and AER conditions. β2-microglobulin was used as a housekeeping control gene. *p<0.05 WT vs. KO, †p<0.05 AE and AER vs. SED in WT mice. Data are presented as a fold-increase over SED mice. Error bars represent SEM values, *N*=4/group.

Figure 1.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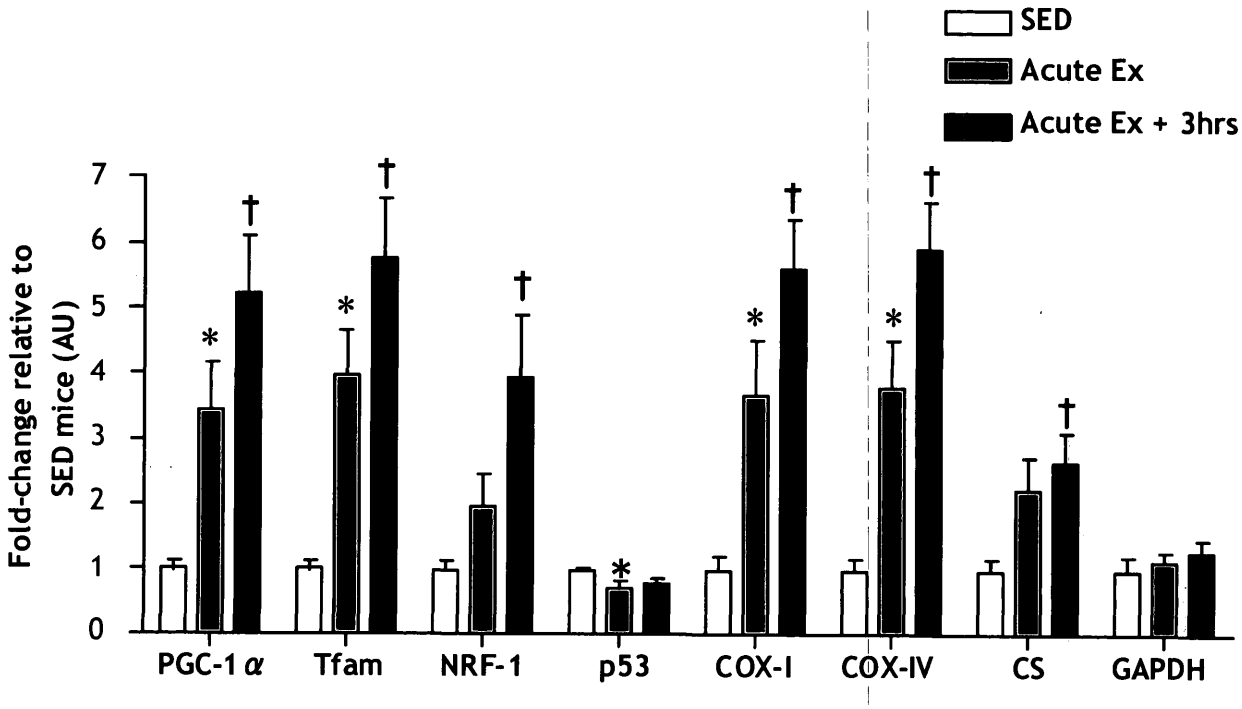
WT-Q
WT-S

Figure 2.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Acute Ex + 3hrs
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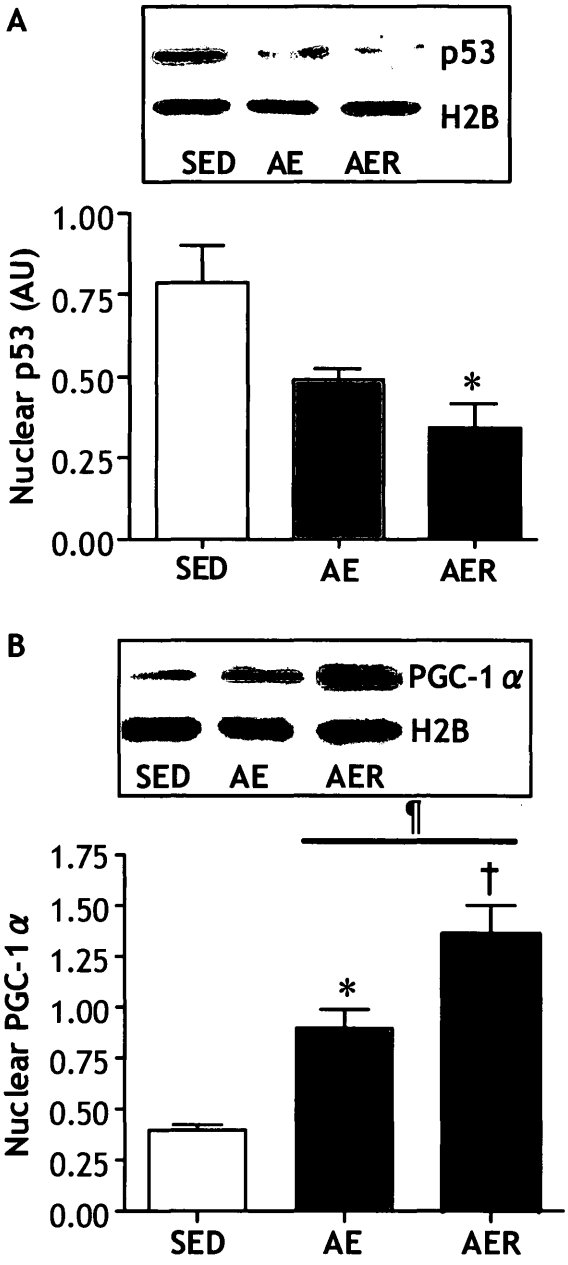
Tfam
NRF-1
p53
COX-I
COX-IV
CS
GAPDH

Figure 3.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Nuclear PGC-1 α
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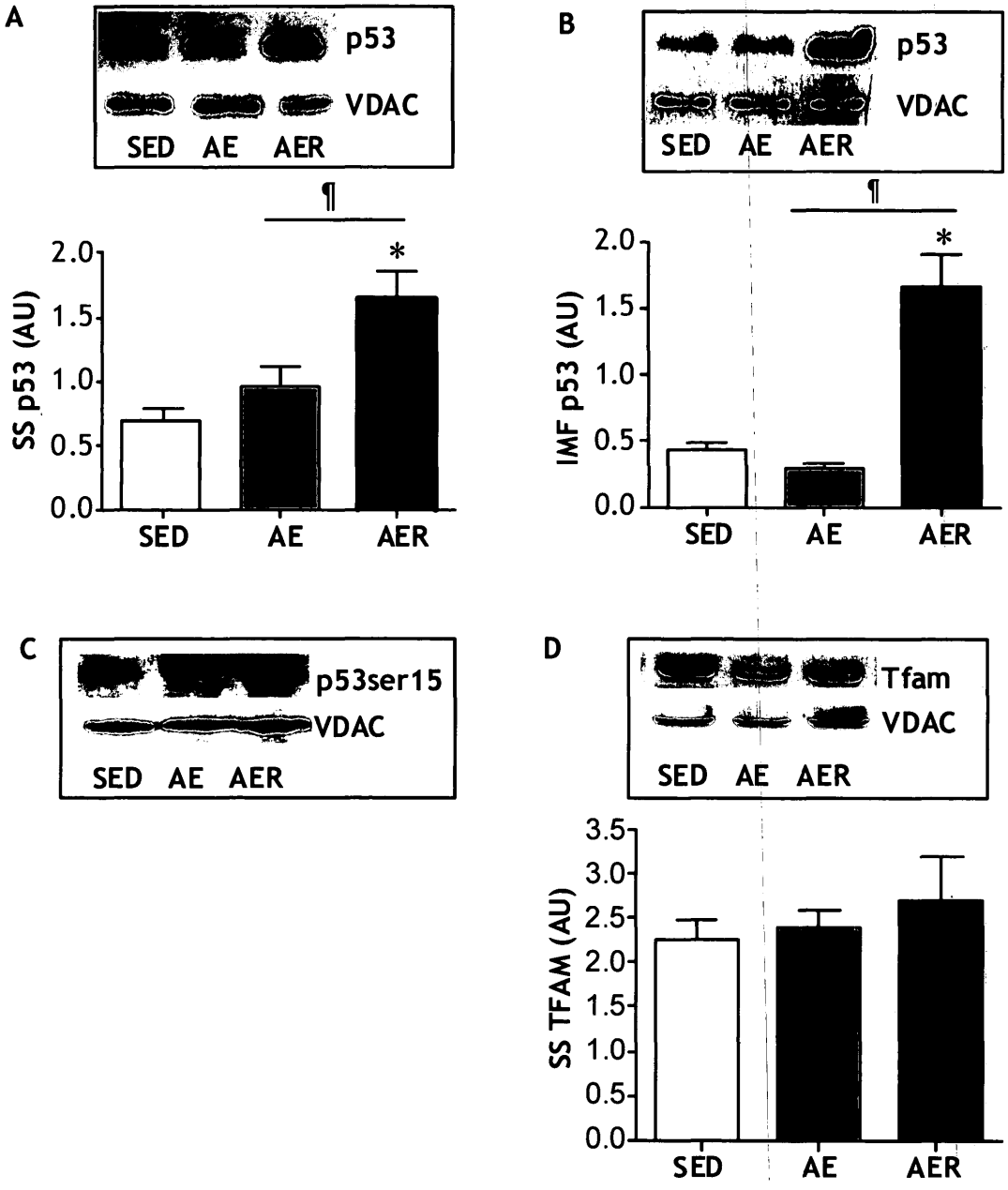
Figure 4.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D
Tfam
VDAC
SED AE AER
SS TFAM (AU)
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AE
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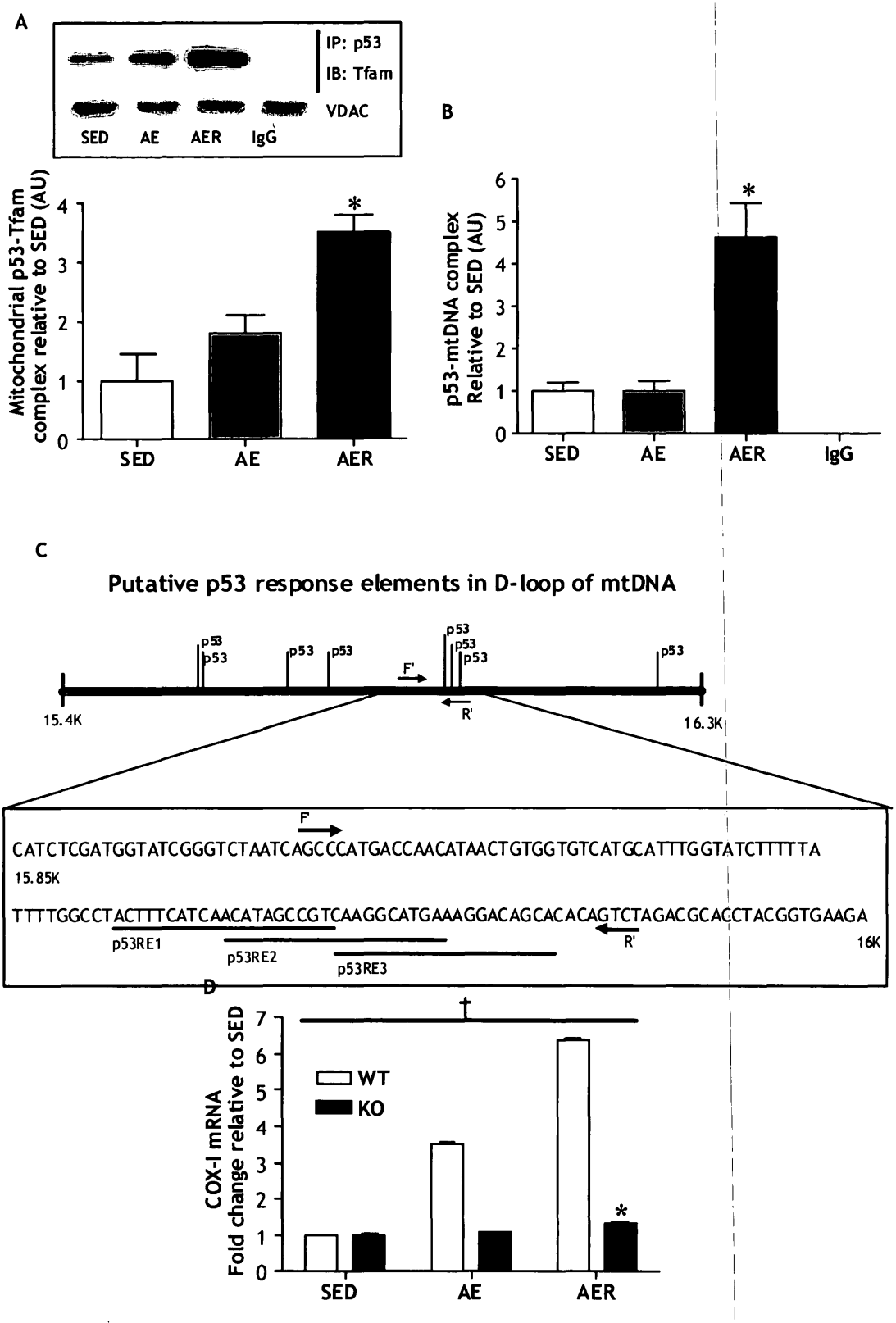
Figure 5.


A
IP: p53
IB: Tfam
VDAC
SED
AE
AER
IgG
Mitochondrial p53-Tfam complex relative to SED (AU)
B
p53-mtDNA complex Relative to SED (AU)
C
Putative p53 response elements in D-loop of mtDNA
15.4K
16.3K
F'
R'
CATCTCGATGGTATCGGGTCTAATCAGCCCATGACCAACATAACTGTGGTGTCATGCATTTGGTATCTTTTTA
15.85K
TTTTGGCCTACTTTCATCAACATAGCCGTCAAGGCATGAAAGGACAGCACACAGTCTAGACGCACCTACGGTGAAGA
p53RE1
p53RE2
p53RE3
16K
D
COX-I mRNA Fold change relative to SED
WT
KO

CHAPTER 6:

p53 is necessary for the adaptive changes in the cellular milieu subsequent to an acute bout of endurance exercise

Ayesha Saleem[1,2], Heather Carter[1,2] and David A. Hood[1,2,3]

[1]School of Kinesiology and Health Science, [2]Muscle Health Research Centre, and [3]Department of Biology, York University, Toronto, Ontario, M3J 1P3, Canada

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Author Contributions

AS and DAH conceived and designed the experiments, interpreted the data and wrote the manuscript. AS collected and analyzed all the data except autophagy and mitophagy western blotting experiments which were completed by HC. All authors approved the final version of the manuscript.

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Abstract

An acute bout of exercise activates downstream signaling cascades that ultimately result in mitochondrial biogenesis. In addition to recruitment of factors that lead to net mitochondrial synthesis, endurance exercise also triggers the removal of damaged cellular material via autophagy, and of dysfunctional mitochondria through mitophagy. Here we investigated the necessity of p53, an important regulator of mitochondrial content/function, oxidative capacity and autophagy, to the changes that transpire within the muscle upon an imposed metabolic and physiological challenge such as a bout of endurance exercise. We randomly assigned age and gender-matched C57BL6 (wildtype, WT) and p53 knockout (KO) mice (N=6 each) to control, acute exercise (90mins at 15m/min, AE) and acute exercise + 3hr recovery (AER) group, and measured downstream alterations in markers of mitochondrial biogenesis, autophagy and mitophagy. In the absence of p53, exercise-induced activation of p38 MAPK was abolished, whereas CAMKII and AMPK phosphorylation levels did not increase in the AE group, but displayed an attenuated enhancement in the AER group compared to WT mice. The translocation of PGC-1α to the nucleus was diminished and only observed in the AER group, and the subsequent increase in mRNA transcripts related to mitochondrial biogenesis with exercise and recovery was absent in the p53 KO animals. Whole muscle autophagic and lysosomal markers did not respond to exercise irrespective of the genotype of the exercised mice, with the exception of increased ubiquitination observed in KO mice with exercise. Since non-exercised p53 KO muscle displays an impaired ability to undergo autophagy in basal conditions, this could be a compensatory

response to elevate autophagy back to WT levels. Markers of mitophagy were elevated in response to AE and AER conditions in both WT and p53 KO runners. The data suggest that p53 is important for the exercise-induced activation of mitochondrial synthesis, and is integral in regulating autophagy during control conditions, but not in response to acute exercise.

Introduction

Skeletal muscle is a highly adaptable tissue that undergoes numerous metabolic and morphological adaptations in response to regular physical activity. In particular, endurance exercise training has been illustrated to induce beneficial physiological alterations that help extend life expectancy, reduce morbidity in disease states such as obesity, cardiovascular disease, type 2 diabetes, metabolic syndrome, cancer, as well as physical disability in later life (33). While an extensive amount of information is available on the benefits of exercise, complete insight into the molecular mechanisms underlying these changes is lacking. Elucidating the causes and molecular signaling events behind the adaptations in response to endurance exercise carries great significance for the treatment of physical inactivity-related diseases.

Muscle contraction results in rapid cellular changes that subsequently activate downstream signaling kinases. Upon each bout of muscle contraction, there are variations in the AMP:ATP ratio, increases in intracellular calcium (Ca^{2+}), and generation of reactive oxygen species (ROS) that can activate AMP-activated protein kinase (AMPK, (36), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII (23), and p38 mitogen-activated protein kinase (p38 MAPK, (1), respectively. These bonafide exercise-responsive signaling kinases then initiate more widespread changes in the muscle by recruiting regulators of mitochondrial biogenesis such as peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC-1α). PGC-1α is a transcriptional co-activator and a critical regulator of the transcription of nuclear genes encoding mitochondrial proteins, and it has been implicated as a master mediator of the adaptive

response to exercise in skeletal muscle (18; 22). Both acute and chronic exercise can activate PGC-1α and cause it to localize to the nucleus (35) where it can directly or indirectly control the transcription of its target genes including nuclear respiratory factor 1 (NRF-1), cytochrome *c* oxidase subunit IV (COX IV), mitochondrial transcription factor Tfam among others (32), and thus enhancing mitochondrial biogenesis.

In addition to triggering the synthesis of mitochondria, exercise has recently been recognized to play a part in the removal of damaged or dysfunctional mitochondria, thereby maintaining mitochondrial homeostasis (10; 11; 15). Autophagy refers to the process where damaged cellular materials are marked, encapsulated and delivered to the lysosomes for degradation. Mitophagy is the selective degradation of dysfunctional mitochondria often tagged by enhanced ubiquitination of mitochondrial proteins, a consequence of elevated ROS accumulation, or dissipation of the mitochondrial membrane potential (9). A multitude of proteins have been identified to be a part of this process, including Beclin1, Atg7, p62, and LC3II that participate at the various stages in the process of autophagy(8; 9; 12). Beclin1 and Atg7 are involved in vesicle nucleation and LC3 maturation, p62 and LC3II recognize ubiquitinated proteins, and LC3II is now commonly used as a marker of autophagy as it necessary for the construction of the autophagosome (3).

The tumor suppressor protein p53 has an established role in modulating mitochondrial content, and subsequently oxidative capacity (20; 26; 27). Its transcriptional control over many vital factors involved in mitochondrial biogenesis such as PGC-1α, Tfam, and synthesis of cytochrome *c* oxidase 2 (SCO2), an important

assembly factor in mitochondrial electron transport chain complexes, renders the expression of p53 to be of significance with respect to mitochondrial adaptations in response to exercise training (27). However, it is unknown, whether p53 is necessary for the physiological changes that occur subsequent to an acute bout of exercise. Incidentally, p53 also serves as a dual regulator of autophagy, a positive enforcer via transcriptional regulation of genes that induce autophagy (19), and a negative moderator when it is present in the cytoplasm through a hitherto uncharacterized mechanism (31). Considering the role of p53 in mediating oxidative capacity, autophagy and its recognition as a target of AMPK and p38 MAPK (16; 29), we hypothesized that the absence of p53 will result in a diminished adaptive cellular response to exercise.

Methods

Animal Breeding

p53 mice (5) were obtained from Taconic labs (New York, USA). Heterozygous p53 mice were bred and treated experimentally in accordance with principles of the York Animal Care Committee. Each progeny of the breeding pair was genotyped as follows. An ear clipping obtained from each animal was used to produce a crude DNA extract. Extracted DNA was added to a PCR tube containing DNA Taq Polymerase (Sigma Jumpstart REDtaq Ready Mix PCR Reaction Mix) and forward and reverse primers for the wild-type (WT), or the knockout (KO) p53 gene. Differences in the genome were detected using polymerase chain reaction (PCR) amplification. The reaction products

were separated on a 2% agarose gel at 90 volts for 2-2.5 hours and visualized with the use of ethidium bromide.

Exercise Performance Test

WT and KO mice were subjected to a graded treadmill exercise test to determine maximum exercise capacity. Mice were acclimatized to the treadmill one week prior to the test. Animals commenced running at 5 m/min on a 0% incline for 5 mins, followed by 10 m/min for 10 min. Running speed was increased by 1 m/min every min until mice reached exhaustion, defined as the point whereby mice remained at the back of the treadmill on an electric shock pad for 5s. The Work performed was calculated by the formula where, Work (J) = Force [body weight (kg) X 9.8 m/sec^2] X Vertical distance [speed (m/min) x time (min)] (21).

Experimental Design

As no differences were observed in the endurance capacity of the two genetic strains of mice, p53 WT and KO mice (N = 6/exercise condition, 18/genotype) at ~2 months of age were matched for sex and body weight, and randomly assigned to control (CON), acute exercise (AE), or acute exercise followed by three hours of recovery (AER) groups. All mice were acclimatized to the treadmill one week prior to the beginning of the experiment. The animals in both the AE and AER groups were then selected and subjected to an acute bout of treadmill running at 15 m/min for 90 min. All of the mice subjected to treadmill exercise were visibly exhausted at the end of the exercise, as

determined by their ability to withstand air and electrical shock stimuli for greater than 5 seconds. The AE exercise group was sacrificed immediately following exercise. The animals in the CON group were euthanized by cervical dislocation at the same time as the AER group. Quadriceps femoris muscle was extracted from all mice, and ~70 mg was immediately snap frozen and stored at -80°C for subsequent mRNA expression and whole muscle western blotting purposes. The remaining ~200 mg of fresh quadriceps femoris was utilized for nuclear, and mitochondrial fractionation. Frozen muscle samples were pulverized into a powder with a stainless steel mortar cooled to the temperature of liquid nitrogen. Powdered tissue was resuspended in RIPA buffer, sonicated, centrifuged, and stored in liquid nitrogen until further use. The protein content of extracts was determined using the Bradford method (4).

RNA Isolation.

Total RNA was isolated from ~70 mg of frozen muscle using Tri-reagent (Invitrogen) according to manufacturer's instructions. RNA concentration and quality were measured using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and further verified with RNA gels.

mRNA Expression Analyses.

The mRNA expression of PGC-1α, Tfam, NRF1, COX-IV, and SCO2 was quantified using 7500 Real-time PCR System (Applied Biosystems Inc., Foster City, CA, USA) and SYBR® Green chemistry (PerfeCTa SYBR® Green Supermix, ROX, Quanta BioSciences,

Gaithersburg, MD, USA). First-strand cDNA synthesis from 2 μg of total RNA was performed with primers using Superscript III transcriptase (Invitrogen) according to manufacturer's directions. Forward and reverse primers (Table 1) for the aforementioned genes were designed based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) using the MIT Primer 3 designer software (http://wi.mit.edu/cgi- bin/primer3/primer3_www.cgi), and were confirmed for specificity using the basic local alignment search tool (www.ncbi.nlm.nih. gov/BLAST/). β-2 microglobulin was used as a control housekeeping gene, the expression of which did not change between groups. All samples were run in duplicate simultaneously with negative controls that contained no cDNA. Melting point dissociation curves generated by the instrument were used to confirm the specificity of the amplified product. Primer efficiency curves were generated for each set to ensure 100±2% efficiency.

Mitochondrial Fractionation.

Briefly, ~150 mg of fresh skeletal muscle was minced, homogenized and subjected to differential centrifugation as previously documented to yield the subsarcolemmal (SS) mitochondrial fraction (26). The mitochondria were re-suspended in a small volume of resuspension buffer (100 mM KCl, 10 mM MOPS, and 0.2% BSA, pH 7.4, supplemented with protease inhibitor cocktail Complete, ETDA-free [Roche Applied Science, Manheim, Germany]). All centrifugation steps were carried out at 4°C. Mitochondrial homogenates were analyzed for protein content using the Bradford assay, and subsequently frozen at -80°C for further biochemical analysis.

Nuclear fractionation.

Nuclear fractions were prepared from freshly isolated skeletal muscle using a commercially available nuclear extraction kit (Pierce NE-PER, Rockford, IL, USA). Briefly, 50-75 mg of skeletal muscle was minced and homogenized in CER-I buffer containing protease inhibitor cocktail Complete, EDTA-free (Roche Applied Sciences, Manheim, Germany). After a series of wash steps, nuclear proteins were extracted in high salt NER buffer supplemented with protease-inhibitors.

Immunoblotting

Proteins were resolved on 8% or 12% SDS-PAGE gels depending on the molecular weight of the protein of interest. The gels were transferred onto ECL nitrocellulose membranes, followed by blocking with 1-3% milk in TBST overnight at 4 °C. Immunoblotting was carried out using rabbit PGC-1α (Millipore), phospho and total AMPK, p38 MAPK, CaMKII (Cell Signalling), lysosomal markers: cathepsin-D (CTS-D, Santa Cruz), lysosomal-associated membrane protein2 (lamp2, Abcam), autophagy markers: ubiqutin (Ub, Assay Designs), p62 (Sigma), light chain 3 II (LC3II, Cell Signalling), autophagy-related protein 7 (Atg7, Sigma), and Beclin1 (Cell Signalling. Membranes were then incubated with the appropriate secondary antibody coupled to horseradish peroxidase (HRP) at room temperature for 2 hours. After incubation, membranes were washed three times in TBST, developed with an enhanced chemiluminescence (ECL) kit, and quantified via densitometric analysis of the intensity

of signal with Sigma Scan Pro v.5 software (Jandel Scientific, San Rafael, CA). Aciculin, voltage-dependent anion channel (VDAC, Mitosciences) and Histone 2 B (H2B, Cell Signalling) were used as loading controls for whole muscle, mitochondrial and nuclear fractions, respectively.

Statistical analysis

Data were analyzed using Graph Pad 4.0 software and values are reported as means $\pm$ SE unless otherwise indicated. Data were analyzed using a two-way ANOVA and Bonferroni post-tests. All other data were analyzed using Student's t-test. Significance levels were set at $p < 0.05$.

Results

Maximum treadmill exercise capacity is similar in WT and p53 KO mice.

To estimate the maximum aerobic running potential of p53 KO mice, we subjected p53 WT and KO mice to an acute bout of graded treadmill exercise to exhaustion. p53 KO animals attained a maximum speed comparable to that of their WT counterparts (Table 2), as reported by Park et al. previously (21). Interestingly, p53 KO mice, though previously demonstrated to have impaired respiration (20; 21; 26) and exercise performance in wheel running (26) and swimming tests (20; 21), displayed no deficits in maximum distance run, or work performed (Table 2). Thus, both p53 WT and KO mice were subjected to the same acute exercise protocol for 90 minutes at 15m/min, as no basal differences in exercise capacity were apparent.

Exercise-induced kinase activation is suppressed in p53 KO animals.

While a plethora of signals are triggered upon exercise, the transient activation of p38 MAPK, AMPK and CaMKII in skeletal muscle are known modifications in response to contractile activity. As expected, there was a large increase in p38 MAPK phosphorylation in the AE group, which returned to control levels with recovery in WT mice (Fig. 1A). In contrast, p38 MAPK phosphorylation upon exercise was abolished in p53 KO mice (Fig. 1A). Similarly, AMPK (Fig. 1B) and CaMKII (Fig. 1C) phosphorylation was enhanced by ~2.5-fold in AE and AER groups in WT mice. As opposed to this, p53 KO mice displayed a delayed activation of AMPK and CaMKII phosphorylation subsequent to endurance exercise. AMPK phosphorylation increased by ~2-fold (Fig. 1B), and CaMKII activation was elevated by ~1.5-fold (Fig. 1C), but this only occurred in the AER group in p53 KO mice. Interestingly, while no differences were documented between the basal phosphorylation status of p38 MAPK and AMPK in non-exercised muscle, levels of CaMKII phosphorylation tended to be higher in the KO mice when compared to their WT counterparts in the CON condition ($p=0.09$, data not shown).

Nuclear translocation of PGC-1α with acute exercise is attenuated in p53 KO mice.

PGC-1α, considered to be an important transcriptional co-factor, has been previously illustrated to rapidly translocate to the nucleus upon an exercise bout (35). We assessed the accumulation of PGC-1α in the nuclear fractions isolated from sedentary control (CON), acute exercise (AE) and acute exercise with 3 hours recovery (AER) group from p53 WT and KO mice (Fig. 2A). PGC-1α increased by 1.5-2-fold in WT animals with

AE and AER (Fig. 2A). In contrast, PGC-1α nuclear content was not elevated in p53 KO mice in the AE group, and displayed only a slight increase in the AER group. The level of nuclear PGC-1α content in KO mice under non-exercised, basal conditions tended to be lower than that of WT, but this did not reach statistical significance.

mRNA levels of genes related to energy metabolism increase following acute exercise in p53 WT, but not KO mice.

The transcript levels of genes involved in mitochondrial biogenesis such as PGC-1α, Tfam, NRF-1, and COX-IV increased immediately following an acute bout of exercise in the WT mice (Fig. 2B, 3). The enhancement in mRNA expression of these transcripts was maintained, or further increased, after three hours of recovery following the exercise in WT animals (Fig. 2B, 3A-C). Surprisingly, the only transcript which increased as a result of exercise or recovery in p53 KO mice was PGC-1α (Fig. 2B). The response of other transcripts to exercise, and exercise with recovery, remained relatively unchanged in the KO animals (Fig. 3A-C). The mRNA levels of SCO2, a well-known target of p53 that was initially illustrated to be pivotal for p53-mediated mitochondrial biogenesis in cells in culture, did not respond to the acute exercise stimulus in either WT or KO animals (Fig. 3D).

Muscle autophagic and lysosomal markers are unchanged in response to acute exercise in p53 WT and KO mice.

We immunoblotted for lysosomal and autophagic proteins intimately associated with the induction of the autophagic process. Basal levels of Cts-D and lamp2 in non-exercised muscle were lower in KO animals compared to their WT littermate controls (Fig. 4B), suggesting a lower lysosomal capacity in p53-deficient animals. In contrast, LC3-II content was similar, and p62 expression was enhanced in the KO animals compared to WT controls. Thus, p53 exerts specific effects on the expression of autophagy and lysosomal proteins in muscle. Exercise and recovery had no effect on the level of any of these proteins in muscle. Beclin1, and Atg7 levels were comparable in WT and KO mice at rest, and also did not respond to the exercise stimulus (Fig. 4A). Ubiquitination, a post-translational modification that most commonly earmarks cellular material for proteosomal or autophagic degradation, was also measured in whole muscle homogenates (Fig. 4B, C). Steady-state levels of ubiquitination were reduced in p53 KO mice (Fig. 4B), and while WT ubiquitination levels remained unchanged with exercise, the KO animals displayed a significant increase in ubiquitinated proteins in the AE and AER groups when compared to WT controls (Fig. 4C, D). This effectively raised the level of ubiquitination in KO tissue back to the level observed in WT animals.

Mitophagy signaling is activated by acute exercise in p53 WT and KO mice.

We measured the amount of lipidated LC3, p62, and ubiquitination in isolated mitochondrial fractions from WT and KO mice as indicators of mitophagy (Fig. 5). Levels of mitochondrial LC3-II (Fig. 5B inset) were increased ($p < 0.05$), as well as organelle ubiquitination ($p=0.06$, Fig. 5D inset) in the KO mice, when compared to their

WT counterparts in control non-exercised conditions, suggesting a heightened drive for mitophagy in the KO muscle. Basal p62 levels remained unchanged in mitochondria from KO compared to WT animals (data not shown). A significant main effect of exercise and recovery on all three mitophagy indicators was evident in both WT and KO animals (Fig. 5).

Discussion

Steady-state mitochondrial content is determined by the interplay between mitochondrial biogenesis and mitophagy. We, and others, have extensively characterized the synthesis arm of mitochondrial turnover, but there are only a few studies that have investigated exercise and its effects on mitophagy. Thus, the purposes of this study were to explore how mitochondrial biogenesis and mitophagy are regulated in response to acute endurance exercise in skeletal muscle, and the role executed by p53 in this context.

PGC-1α is intimately involved in regulating mitochondrial biogenesis, due to its ability to rapidly translocate to the nucleus and co-activate the transcription of numerous downstream mediators of mitochondrial biogenesis upon an exercise signal (18). Interestingly, p53 exerts a significant degree of control over the transcription of PGC-1α. We had earlier identified a putative p53 response element in PGC-1α promoter (13), which was subsequently demonstrated to both suppress (25), and activate transcription of PGC-1α (2). Clearly, the interaction is dependent on the cellular milieu, and the experimental model in which it is investigated. We here report that while there is no effect on basal nuclear PGC-1α localization between the two genotypes, the kinetics of its

movement into the nucleus upon exercise are impaired in the absence of p53. This is important as the delayed increase in nuclear PGC-1α may suppress the onset of mitochondrial biogenesis. Indeed, the expected augmentation in the transcript levels of PGC-1α, Tfam, NRF-1 and COX-IV that occurs with exercise and recovery in WT animals, was either attenuated or abolished in the KO mice. It is quite possible that there is an eventual increase in the mRNA content of the different transcripts in the p53 KO animals, requiring either a longer recovery period (> 3 hours), or multiple bouts of exercise to gain the same adaptation as WT mice. It is important to note that the p53-transcribed gene SCO2, involved in the assembly of complex IV of the electron transport chain, was unresponsive to exercise in either WT or KO mice.

To understand the mechanisms of reduced PGC-1α translocation to the nucleus, we measured the activation of upstream kinases. p38 MAPK directly phosphorylates PGC-1α at three sites which releases it from its repressor binding protein known as p160 and leads to its stabilization (6). AMPK activates PGC-1α by phosphorylating it on the threonine-177 and serine-538 residues (14). Our data indicate that the absence of p53 results in a lack of, or delay in the phosphorylation of p38 MAPK, and AMPK, respectively, in KO mice subjected to acute exercise. This may be the underlying reason behind the sluggish response of PGC-1α translocation into the nucleus upon exercise in p53 KO animals.

The diminished AMPK activity in the KO mice is not likely attributed to reduced levels of the allosteric activator AMP in the muscle of p53 KO animals during exercise. This is because muscle of p53 KO animals have a reduced oxidative capacity, and thus

more likely to increase cellular AMP levels, as well as the activation of AMPK (17). However, p53 KO cells have been shown to maintain comparable cellular ATP levels basally, and higher ATP levels during conditions of metabolic stress such as glucose deprivation when compared to WT cells (20; 31). It is possible that the lower AMPK activity may be due to the transcriptional control exerted by p53 over the β1 accessory subunit of the AMPK complex (7). AMPK exists as a heterotrimeric complex, with one catalytic α subunit, and two regulatory β, and γ subunits. The β subunit functions as a scaffold for the other two, and has been shown to modulate AMPK localization and activity (34). Thus it is likely that, in the absence of p53, reduced β subunit expression somehow manifests as a delayed increase in AMPK activity upon acute exercise.

p38 MAPK can be activated by a plethora of different oxidative, DNA damage, and inflammatory stress stimuli, and is a bona fide activator of p53, as well as being subject to negative regulatory feedback regulation by p53-inducible genes (30). p38 MAPK phosphorylation in response to acute exercise was completely abrogated in the KO mice, although basal levels were unchanged. We expected higher basal p38 MAPK activity in the KO mice, having previously reported elevated reactive oxygen species levels in these animals (26), and also because of the negative correlation that exists between oxidative capacity and activation of p38 kinase activity (17). We do not know why p38 activation is attenuated in control non-exercised muscle from p53 KO mice, but given that p38 MAPK is an integral component of many cellular processes, further research is warranted to investigate this observation more closely.

The effect of p53 on autophagy is potentially ambiguous, depending on its cellular location. Cytoplasmic p53 can inhibit autophagy during non-stressful cellular conditions, whereas the accumulation of nuclear p53 upon a cellular insult results in the p53-mediated transcriptional up-regulation of autophagy genes including AMPK β1, death associated protein kinase 1 (DAPK-1), damage-regulated autophagy modulator (DRAM), proapoptotic Bcl-2 proteins (e.g. Bad, Bax, Bnip3, and Puma), sestrin 2, and tuberous sclerosis protein 2 (TSC2, (19; 27). In this study we have totally abrogated p53 activity and expression, and examined the consequence on the expression of autophagy and autophagy-related proteins. Our results revealed attenuated lysosomal capacity as illustrated by lower CtsD and lamp2 protein expression in the KO animals at rest. Concomitantly, there was decreased ubiquitination, and an accumulation of p62 indicative of a lower autophagy flux in the resting muscle of KO mice. LC3II, Beclin1 and Atg7 expression remained unchanged in the absence of p53. Interestingly, the mitochondrial localization of LC3II and ubiquitin content was higher in the KO, compared to the WT animals, indicative of an underlying basal drive to remove dysfunctional mitochondria during non-exercised conditions, but limited by a lower lysosomal capacity for degradation. Consistent with this, it has been shown that liver, kidney and pancreas from p53 KO mice had higher basal levels of LC3 puncta and autophagosomal accumulation in whole cell homogenates (31), characteristic of an impaired autophagic process in the absence of p53.

Since both autophagy and mitophagy have been recently implicated to be activated in response to acute endurance exercise in muscle (10; 11; 15), we next

investigated whether this avenue of mitochondrial turnover is dependent on the presence of p53. He et. al (11) previously reported an increase in the autophagosomal membrane-associated lipidated form of LC3II, and degradation of autophagy substrate p62 with acute exercise in WT mice. We investigated of a number of autophagy and lysosomal markers, and did not observe any changes in the WT mice with the exercise protocol employed, despite the considerable induction of gene expression. It is likely that our exercise protocol, while commonly used to successfully invoke mitochondrial synthesis (24; 35), and physiologically equivalent to running at ~75% VO_2 max (28), is not as intense or long enough as that used by others (11) to induce macroautophagy in WT mice. Indeed, a recent study showed the up-regulation of autophagy proteins in humans that could be observed after an ultra-endurance exercise bout of exercise lasting 24 hours (15). Clearly, exercise dosage is of paramount importance as it dictates the magnitude of the downstream activation of autophagic signaling. The increase in whole muscle ubiqutination evident only in p53 KO mice may play a therapeutic role in raising the attenuated level of this process up to healthy, control levels in the absence of p53. Exercise-induced ubiquitination is commonly used to tag cellular debris for removal by the proteosome or for selective autophagy, therefore it is possible that this change in ubiquitin content is upstream of an activation of the autophagic process in the KO mice. With respect to mitophagic signaling initiation, both WT and KO animals displayed an equal propensity to enhance mitochondrial LC3II, p62 and ubiquitin content with exercise. Thus, acute exercise promotes mitophagy by enhanced targeting of LC3II and

p62 to the mitochondria, and by ubiquitinating mitochondrial proteins even when p53 is absent.

This study documents a comprehensive analysis of the necessity of p53 for exercise-induced changes in cellular signaling and gene expression in skeletal muscle. Exercise has been touted as a viable means to decrease the incidence and progression of cancer, as well as many inactivity-related pathologies such as obesity, type 2 diabetes, and cardiovascular disease. We have previously identified that endurance exercise can directly activate and re-localize p53 to the mitochondria where it affects mitochondrial DNA (mtDNA) transcription (Saleem, J Physiol 2013, in press). Here, we demonstrate that the presence of p53 is requisite for the activation, and subsequent initiation of mitochondrial biogenesis after an acute bout of exercise. In addition, lack of p53 seems to be important for the basal expression and recruitment of autophagic proteins, but has no effect on the induction of mitophagic signaling after an acute bout of exercise. Further research is warranted in order to fully appreciate the interplay between autophagy and mitophagy, and the dependence of these cellular processes on p53 expression in skeletal muscle after exercise. These findings add considerably to the literature on muscle adaptations to exercise, and they suggest that individuals with decreased or ablated p53 expression, may require a greater exercise dose in order to benefit from the mitochondrial adaptations which normally accompany the pursuit of a regular program of physical activity.

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Table 1. Primer sequences used for RT-PCR analysis.

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
PGC-1α	TTCCACCAAGAGCAAGTAT	CGCTGTCCCATGAGGTATT
Tfam	GAAGGGAATGGGAAAGGTAGA	AACAGGACATGGAAAGCAGAT
NRF-1	ATCCGAAAGAGACAGCAGACA	TGGAGGGTGAGATGCAGAGTA
COX-IV	CTCCAACGAATGGAAGACAG	TGACAACCTTCTTAGGGAAC
SCO2	TCCCTTCACCCTTCGCTGAAC	CAGTAGCATCGTGGACCTGAA
β-2 microglobulin	GGTCTTTCTGGTGCTTGTCT	TATGTTCGGCTTCCCATTCT

Table 2. Body weight and treadmill exercise performance.

Genotype	Body Weight (g)	Speed (m/min)	Distance (km)	Work (kJ)
WT (N=4)	17.6 ± 1.52	42.2 ± 1.44	0.99 ± 0.06	172.4 ± 24.04
KO (N=3)	19.6 ± 0.17	44.0 ± 1.73	1.06 ± 0.08	204.1 ± 13.0

Figure and Table legends

Table 1. List of forward and reverse primer sequences used for real time PCR.

Table 2. Body weight and treadmill exercise performance. p53 WT and KO mice were subjected to a graded treadmill exercise challenge until exhaustion. No difference was observed in maximum speed attained, distance covered or work performed between the WT and KO mice. *N*=3-4.

Figure 1. Exercise-induced activation of signaling kinases. (A) p38 MAPK phosphorylation increased significantly with exercise and recovery in the WT mice, and not in the KO animals ($p<0.05$). (B) AMPK phosphorylation levels were enhanced by ~2 - 2.5-fold in AE and AER conditions in WT mice, respectively ($p<0.05$). p53 KO group displayed a delayed ~2-fold elevation in phosphorylated AMPK in the AER group ($p<0.05$). (C) Phospho-CaMKII content was higher by ~2.5-fold in WT mice in AE and AER, and by ~1.5-fold in AER condition in the KO counterparts ($p<0.05$). †$p<0.05$ main effect of AE and AER vs. CON, *$p<0.05$ main effect of WT vs. KO, ¶$p<0.05$ main interaction effect. Data are presented as a fold-increase over CON values. Error bars represent SEM values. *N*=6/exercise condition.

Figure 2. Alterations in expression of PGC-1α protein and mRNA. (A) PGC-1α nuclear content increased by 1.5 – 2-fold in WT mice with AE and AER ($p<0.05$), and this effect was nearly absent in the KO mice. Histone 2 B was used as a nuclear loading

control (B) As expected, a large up-regulation of PGC-1α mRNA was evident in WT mice with exercise and recovery. On the other hand, p53 KO exercised mice displayed an attenuated increase in PGC-1α mRNA content. †$p<0.05$ main effect of AE and AER vs. CON, *$p<0.05$ main effect of WT vs. KO. Data are presented as a fold-increase over CON values. Error bars represent SEM values. *N*=6/exercise condition.

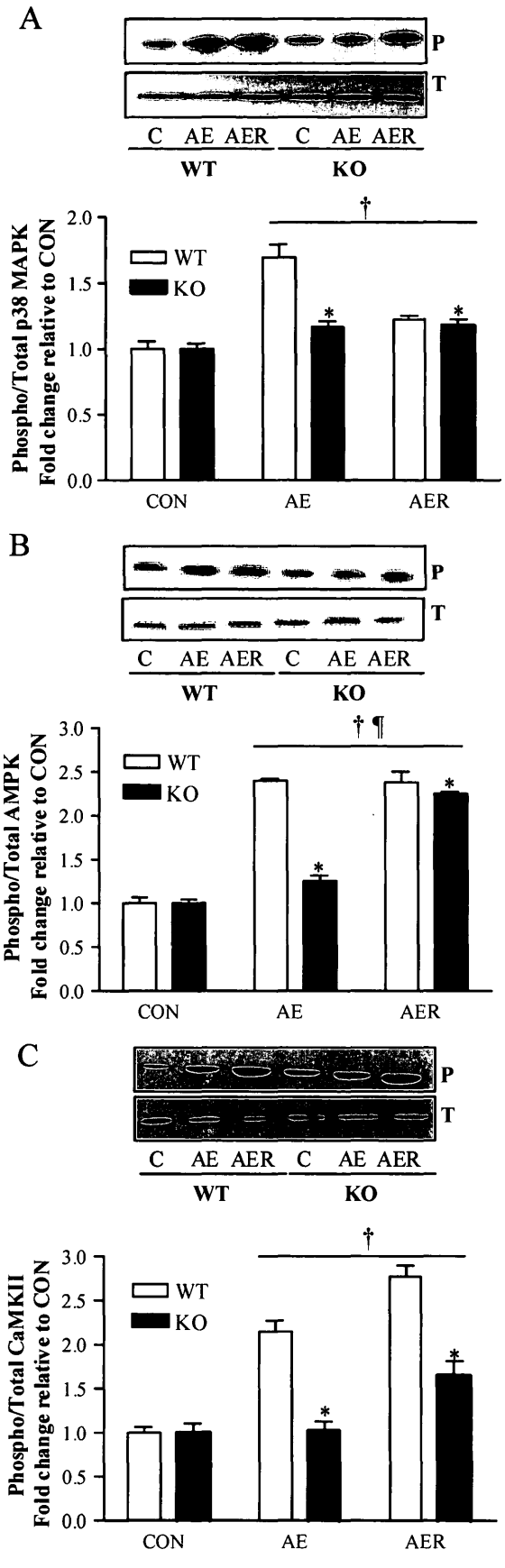
Figure 3. Changes in mRNA transcripts related to mitochondrial biogenesis. The transcript levels (A) Tfam, (B) NRF-1, and (C) COX-IV increased immediately following acute exercise, and was further elevated in recovery in the WT mice ($p<0.05$). This increase in mRNA levels was abolished in the KO mice. (D) SCO2, a transcriptional target of p53 involved in maintaining mitochondrial function, did not respond to exercise in the WT mice, and its levels decreased in the absence of p53 ($p<0.05$). β-2 microglobulin was used as an internal housekeeping gene. †$p<0.05$ main effect of AE and AER vs. CON, *$p<0.05$ main effect of WT vs. KO. Data are presented as a fold-increase over CON values. Error bars represent SEM values. *N*=4/exercise condition.

Figure 4. Response of whole muscle autophagic and lysosomal markers to acute exercise. (A) Immunoblots depicting the expression of lysosomal (CTSD, Lamp2) and autophagic (p62, LC3II, Beclin1, ATG7) markers in WT and KO samples in CON, AE and AER conditions. No changes in protein content were observed in either genotype with exercise. (B) Non-exercised control levels of LC3II were unchanged, CTSD, Lamp2 and ubiquitin levels were lower, and p62 content was higher in p53 KO mice compared to

littermate WT controls ($p<0.05$). AU refers to arbitrary units. *$p<0.05$ WT vs. KO. Data is presented as mean ± SEM values. (C) Representative western blot for ubiquitinated proteins and (D) its graphical illustration illustrates an exercise-induced elevation of ubiqutin content in p53 KO mice only ($p<0.05$). Aciculin was used as a loading control. *$p<0.05$ main effect of WT vs. KO. Data is presented as a fold-increase over CON values. Error bars represent SEM values. *N*=6/exercise condition.

Figure 5. Activation of mitophagy signaling post-acute bout of exercise. (A) Lipidated LC3, p62, and ubiquitination in isolated mitochondrial fractions from WT and KO mice. VDAC was used as a loading control. Graphical illustration depicting an increase in (B) LC3II, (C) p62, and (D) ubiquitinated protein expression with exercise in mitochondria isolated from both WT and p53 KO mice. (B, inset) Increased amounts of LC3II ($p<0.05$), concomitantly with (D, inset) higher ubiqutination ($p=0.06$) were documented in the mitochondria from mice with p53 ablation. †$p<0.05$ main effect of AE and AER vs. CON, *$p<0.05$ main effect of WT vs. KO. Data are presented as a fold-increase over CON values. Error bars represent SEM values. AU refers to arbitrary units. *N*=6/exercise condition.

Figure 1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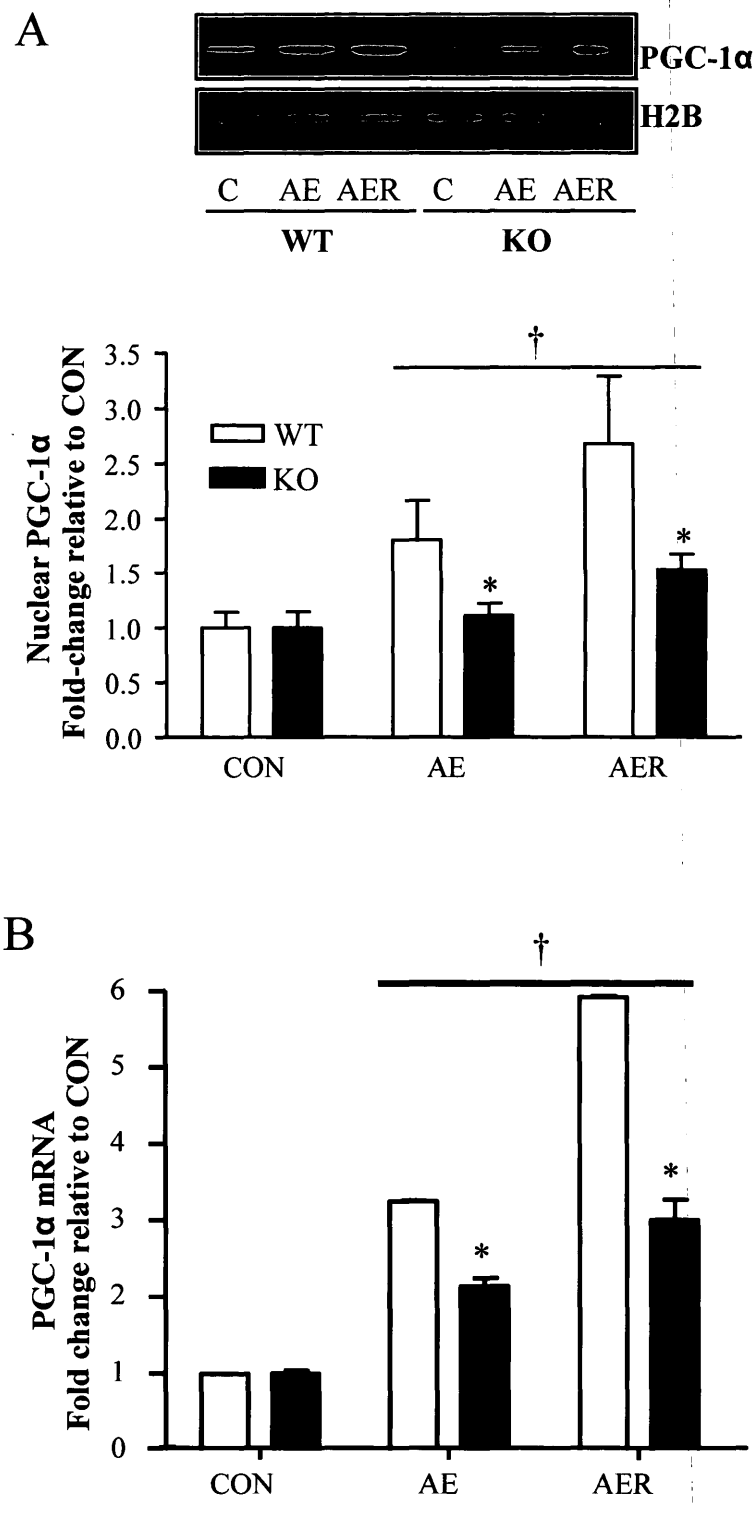
P
T
C AE AER C AE AER
WT KO
Phospho/Total p38 MAPK
Fold change relative to CON
WT
KO
CON AE AER
B
Phospho/Total AMPK
Fold change relative to CON
C
Phospho/Total CaMKII
Fold change relative to CON

Figure 2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PGC-1α
H2B
C AE AER C AE AER
WT
KO
†
Nuclear PGC-1α
Fold-change relative to CON
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3.0
2.5
2.0
1.5
1.0
0.5
0.0
WT
KO
*
*
CON
AE
AER
B
†
PGC-1α mRNA
Fold change relative to CON
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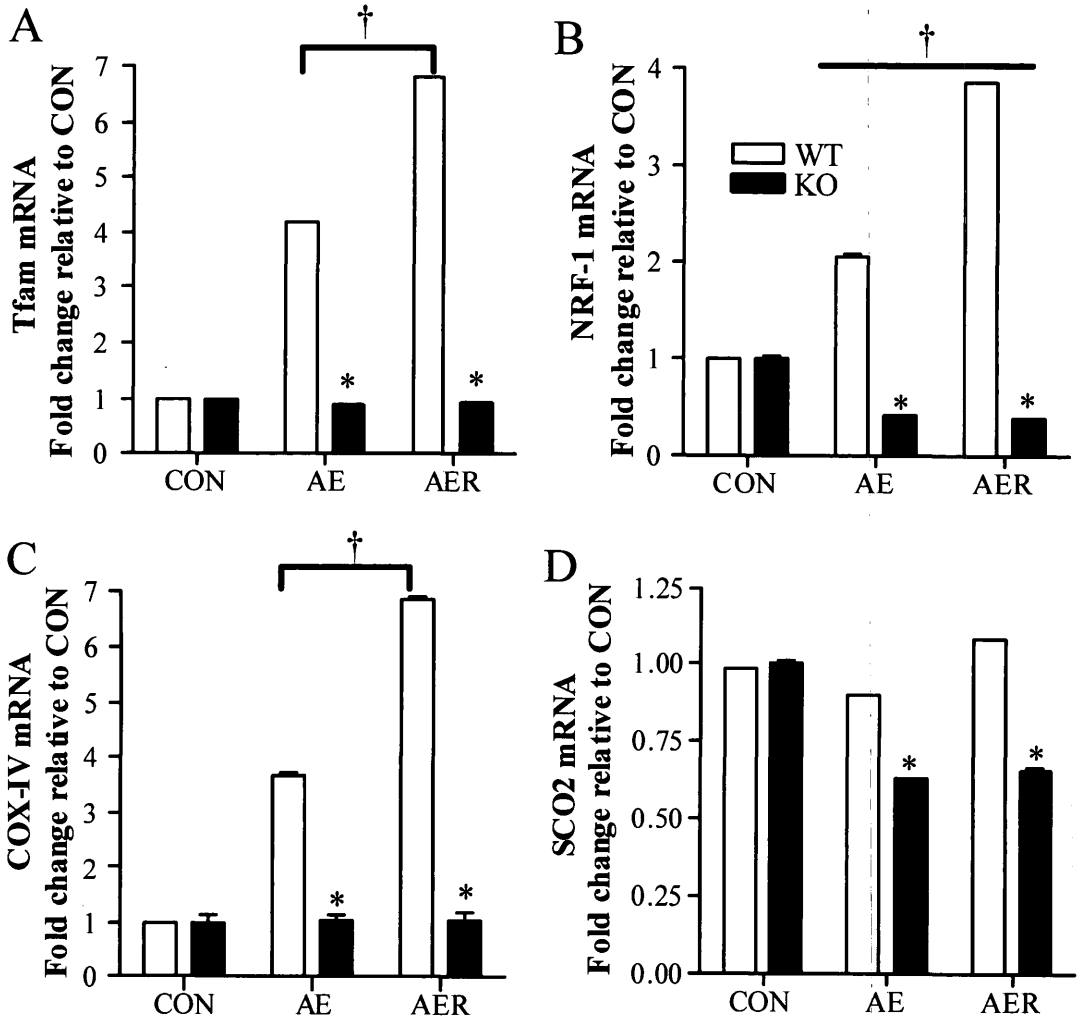
5
4
3
2
1
0
*
*
CON
AE
AER

Figure 3


A
Tfam mRNA
Fold change relative to CON
0
1
2
3
4
5
6
7
†
*
*
CON
AE
AER
B
NRF-1 mRNA
Fold change relative to CON
0
1
2
3
4
WT
KO
†
*
*
CON
AE
AER
C
COX-IV mRNA
Fold change relative to CON
0
1
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4
5
6
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*
CON
AE
AER
D
SCO2 mRNA
Fold change relative to CON
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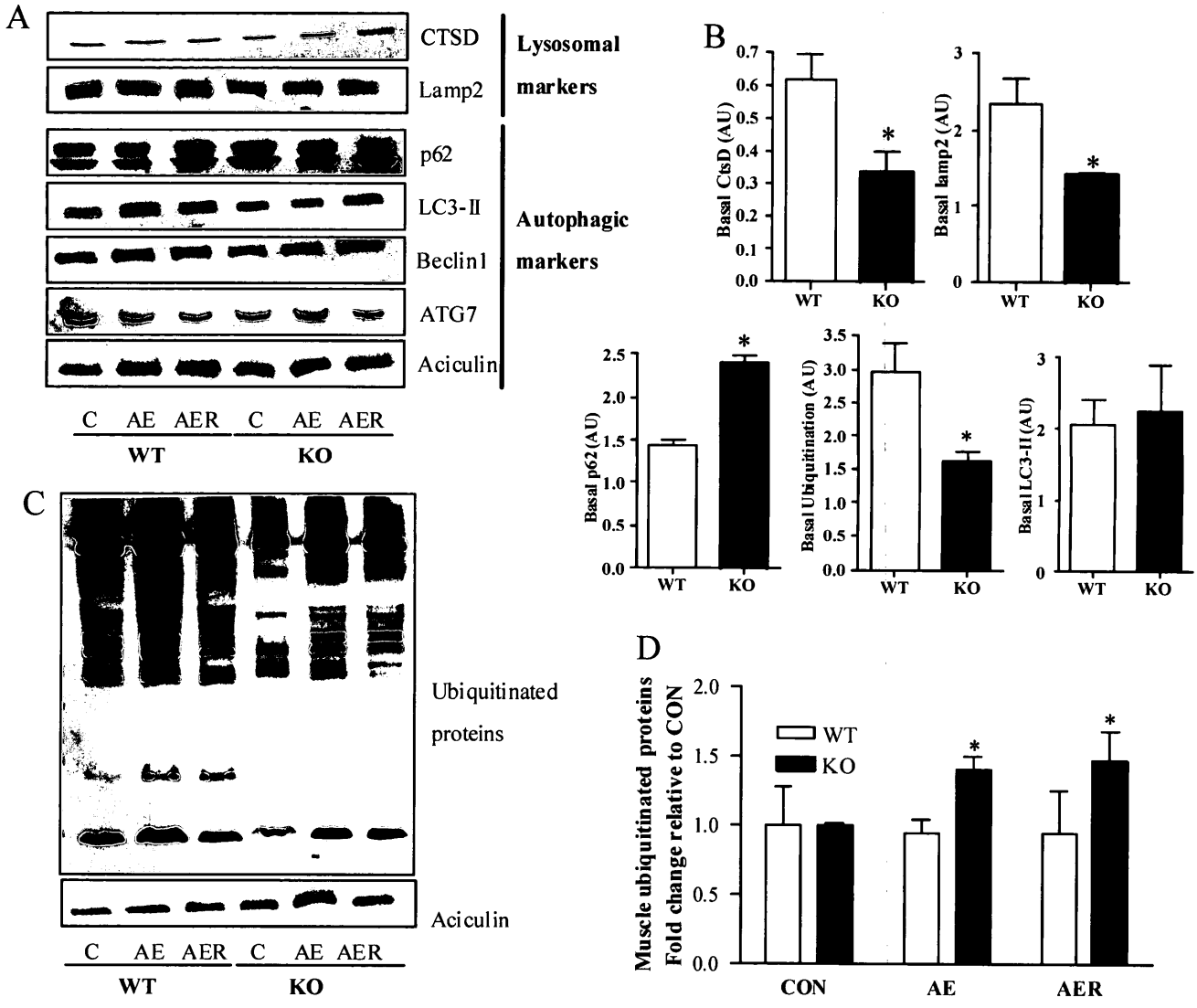
0.25
0.50
0.75
1.00
1.25
*
*
CON
AE
AER

Figure 4


A
CTSD
Lamp2
Lysosomal markers
p62
LC3-II
Beclin1
ATG7
Aciculin
Autophagic markers
C AE AER C AE AER
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B
Basal CtsD (AU)
Basal lamp2 (AU)
Basal p62 (AU)
Basal Ubiquitination (AU)
Basal LC3-II(AU)
WT KO
C
Ubiquitinated proteins
Aciculin
C AE AER C AE AER
WT KO
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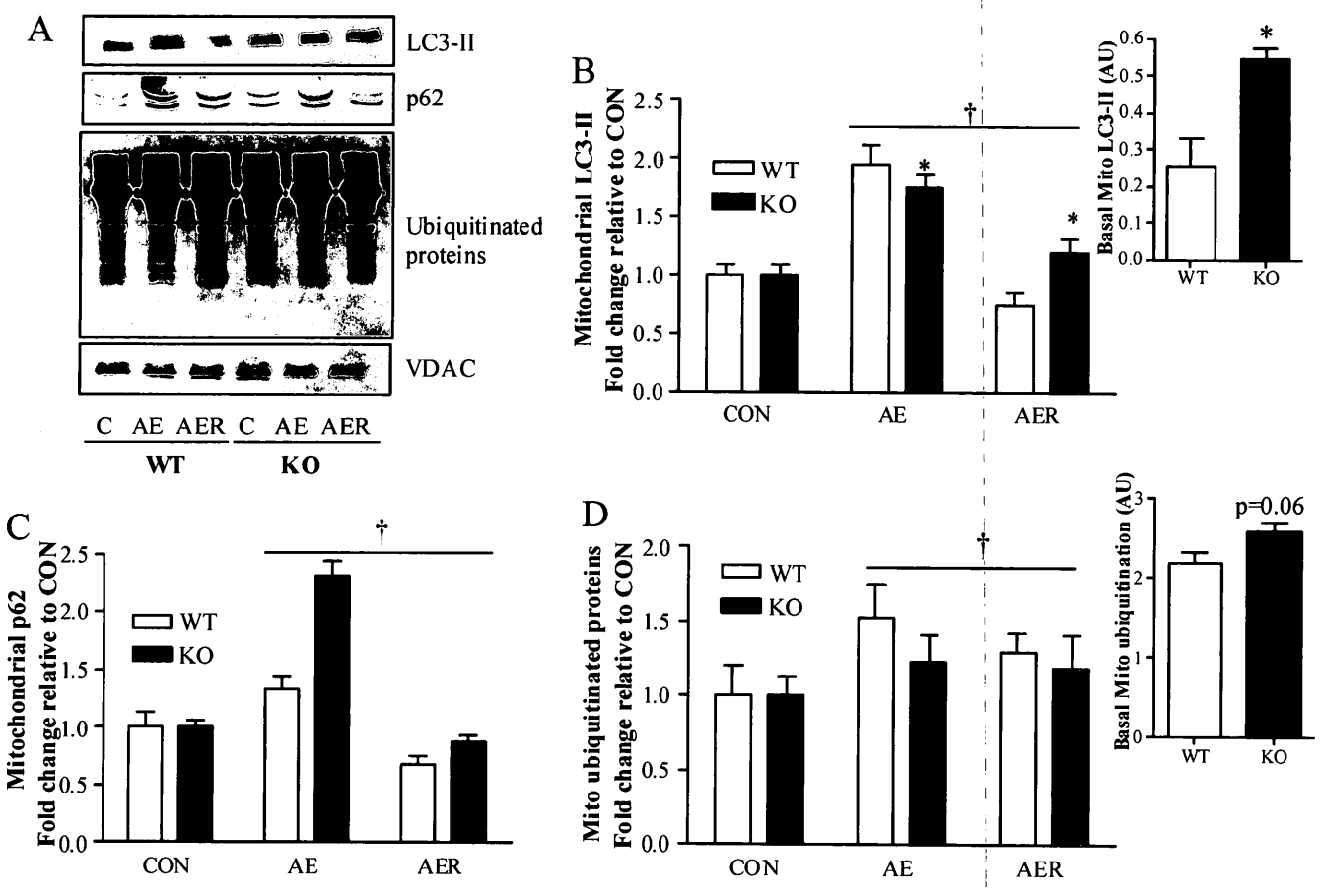
Muscle ubiquitinated proteins
Fold change relative to CON
WT
KO
CON AE AER

Figure 5


A
LC3-II
p62
Ubiquitinated proteins
VDAC
C AE AER C AE AER
WT KO
B
Mitochondrial LC3-II Fold change relative to CON
WT
KO
CON AE AER
Basal Mito LC3-II (AU)
WT KO
C
Mitochondrial p62 Fold change relative to CON
WT
KO
CON AE AER
D
Mito ubiquitinated proteins Fold change relative to CON
WT
KO
CON AE AER
Basal Mito ubiquitination (AU)
p=0.06
WT KO

CHAPTER 7:

SUMMARY AND CONCLUSION

p53 is a transcription factor that is well-known for its ability to respond to a multitude of extrinsic and intrinsic challenges to the cell including DNA damage, oxidative and UV stress, oncogene activation and hypoxia (15). It can regulate pleiotropic pathways in the cell ranging from cell cycle arrest, cell death, autophagy, senescence, metabolism, fertility, DNA repair, pro-/anti-oxidant response and angiogenesis depending on the type and severity of stress and the host tissue (3). Until about few years ago, majority of the research had investigated the role of p53 in the context of cancer cell models, understandably because of the pivotal role of p53 in halting tumorgenesis. With metabolic aberrations being recognized as a stalwart feature of cancer, and more importantly as a viable target for therapeutics, research has intensified on delineating the mechanisms by which p53 regulates mitochondrial function. There is however a dearth in literature in examining the function of p53 in skeletal muscle, a gross oversight as skeletal muscle is the largest organ system in the body, and impaired muscle mitochondrial function is linked with a variety of pathological conditions such as type 2 diabetes, obesity, cancer, and aging. Previously, we and others have shown that the absence of p53 results in a lower mitochondrial content, reduced mitochondrial respiration and poor endurance performance in animals (7; 8; 12; 13). Thus my doctoral work focused on investigating the mechanisms by which p53 induces mitochondrial biogenesis, and to attest the necessity of p53 for exercise-induced metabolic adaptations.

Objective 1: We first investigated the role of p53 in regulating mitochondrial protein import and complex IV or cytochrome *c* oxidase (COX) assembly. It has been previously demonstrated that lack of p53 results in reduced COX activity (7; 8; 12). Like many other complexes of the electron transport chain in the mitochondria, COX is made up of both mtDNA- and nuclear DNA-encoded mitochondrial proteins, which mandates the process of protein import to be important for the formation of this holoenzyme. Consequently, we hypothesized that the lower COX activity observed in p53 KO animals was a result of defective rates of protein import leading to inefficient assembly, and hence function of the complex. Additionally, p53 had been shown to be a transcriptional regulator of SCO2 (7), a factor involved in assembly of COX. Isolated mitochondria from p53 KO mice had lower expression of many important components of the protein import machinery such as Tom20, Tim23, mtHsp70, and mtHsp60. The content of cytosolic chaperone proteins in p53 KO mice was unaltered, leading us to postulate that reductions in the protein import machinery constituents would impact import kinetics. However, results from our *in vitro* import assay showed no difference between the WT and p53 KO mice. We measured COX activity and markers of ETC complexes in isolated mitochondrial sub-fractions and again noted that p53 KO samples remained unchanged from their WT counterparts. Lastly, we assessed complex IV assembly using BN-PAGE and the protein levels of Surf1, another assembly co-factor, and found both to be decreased in the absence of p53. Thus, p53 has no effect on the import of matrix-destined proteins, but lack of the protein impaired the assembly of the COX complex. This may imply that there is a compensatory increase in the COX activity of KO mice to maintain

levels comparable to WT in the face of this impairment in COX assembly. This study provides further mechanistic insight into how p53 regulates mitochondrial function in skeletal muscle.

Objective 2: Next we examined whether an acute bout of exercise activates p53. We randomly divided WT mice in to sedentary, acute exercise (AE) and acute exercise plus 3 hour recovery (AER) groups, and subjected the AE and AER groups to 90mins of treadmill running at 15m/min. We evaluated the transcript and protein content of p53, and documented that both p53 mRNA and nuclear p53 protein levels decreased upon AE compared to control conditions. A small pool of p53 has been reported to reside in the mitochondria (1), hence we measured p53 content in SS and IMF mitochondrial fractions and observed striking ~4-fold increases in p53 protein in the organelles with exercise. Furthermore, mitochondrial p53 was phosphorylated at Ser15 with exercise, indicative of improved protein stability, and an enhanced amount of p53 was immunoprecipitated with Tfam. p53 was also found in a complex with mtDNA at the D-loop region, directly or indirectly through Tfam, and this interaction increased substantially in AER conditions. We also identified putative p53 response elements in the D-loop region of mtDNA using *in silico* analysis, hence leading us to posit that p53 may be involved in co-regulating mtDNA transcription in collaboration with Tfam, the mtDNA transcription factor. Indeed the increase in mtDNA-transcribed COX subunit I with exercise and recovery was nearly abolished in p53 KO mice giving credence to our hypothesis. This study is the first to document how exercise reshuffles p53 to the mitochondria wherein it is involved in modulating mtDNA transcription and/or mtDNA integrity. This study also establishes

p53 as a bona fide transcription factor activated in response to endurance exercise in skeletal muscle.

Objective 3: Since exercise modified p53 expression and sub-cellular localization so drastically, it was intuitive to subsequently explore the necessity of p53 for exercise-mediated adaptations. Specifically, we wanted to delineate whether p53 is crucial for the post-exercise changes in cellular kinase activation, mRNA response, transcriptional regulation and autophagic/mitophagic induction. We subjected p53 WT and KO animals to a similar exercise paradigm as in objective 2, and measured downstream activation of signalling kinases. In the absence of p53, p38 MAPK phosphorylation was abolished, and CaMKII and AMPK activity was attenuated with exercise and recovery. The translocation of one of the main regulators of mitochondrial biogenesis, PGC-1α to the nucleus was impeded in the KO mice. Concomitantly, increase in the levels of mRNA transcripts commonly associated with an initiation of mitochondrial biogenesis was absent in p53 null animals. Markers of autophagy did not fluctuate with the exercise in either mice genotypes, however the induction of the mitophagic programme was evident in both WT and KO mice. This suggests that while p53 is crucial to the activation of the mitochondrial synthesis arm, and necessary for baseline levels of autophagy, it is likely not required for the exercise-mediated mitophagy/autophagy.

All together our data further cement the role of p53 as a legitimate regulator of mitochondrial biogenesis, and one that is responsive to endurance exercise, in skeletal muscle. Reversing the metabolic impediments in cancer is a viable means to treating some cancers, along with using exercise as a cancer therapy. Since p53 is involved in

regulating both oxidative capacity, and can be recruited by exercise to localize to the mitochondria, our work carries important therapeutic repercussions. Indeed, p53-mediated control of mitochondrial respiration and function may underlie the Warburg effect that is characteristic of cancer cells – an increase in glycolytic metabolism, in tandem with a decrease in oxidative phosphorylation. Coupled with the fact that p53 is one of the most often mutated proteins in tumorgenesis, focusing on treating cancer by effectively aiming to ameliorate the metabolic dysfunction in p53 mutant/KO cancers seems to be a viable therapeutic initiative that needs to be urgently addressed. More work needs to be done to fully elucidate the mechanisms by which p53 is directed to the mitochondria upon exercise, and to decipher the underpinnings of the diminished effects seen in the absence of p53 in activating mitochondrial biogenesis.

CHAPTER 8:

FUTURE DIRECTIONS

Based on our observations from the three studies summarized earlier, it is evident that p53 is implicitly involved in regulating baseline and endurance exercise-mediated mitochondrial biogenesis. Several interesting research questions arise from our observations which will form the basis of scientific studies our lab could be undertaking in future. These are detailed below:

1) Does p53 impact the rate of protein import into mitochondrial sub-compartments?

The decrease in the expression of Tom20, Tim23, and the chaperone proteins in the KO mice could be the result of a direct transcriptional regulation of these proteins by p53, or via an indirect method. While we were surprised by the unaltered matrix protein import, it is likely that import into sub-mitochondrial destinations such as the inner or outer mitochondrial membrane, or intermembrane space is impaired. Conducting further import experiments using radiolabelled precursor proteins destined for these sub-mitochondrial compartments will be valuable in answering this query.

Moreover, while we discerned no differences in cytosolic chaperones, there are instances where mitochondrial import is not different between two treatment groups, but when pre-incubated with cytosol from the respective treatment group, import kinetics were altered (16). Further work must be conducted to preclude this eventuality that may have been missed in our import study.

2) What are the upstream signalling pathways that target p53 to the mitochondria, and the subsequent post-translational modifications that the protein undergoes, in response to exercise?

Additional time course experiments need to be performed to elucidate the 'dosage' of exercise needed to re-direct p53 to the mitochondrion, and/or deplete the nuclear p53 content. Plus, it will also be of interest to subject mice to different modalities of exercise i.e., endurance vs. resistance vs. interval training. Since p53 does not have a canonical mitochondrial targeting sequence, research into investigating the molecular mechanisms (most likely post-translational modifications that dictate its mitochondrial transport) via which endurance exercise exerts its effect on p53 sub-cellular shuttling is also warranted, along with measuring protein import kinetics of p53 into the mitochondria upon exercise. Immunoprecipitating the p53 protein from different sub-fractions of the cell post-exercise, and then subjecting it to mass spectroscopy techniques will help identify the post-translational modification(s) to p53 with exercise.

We have preliminary evidence that indicates mitochondrial p53 to be phosphorylated at Ser15. The most likely candidates for this post-translational modifications are AMPK and p38 MAPK. We can elicit the contributions by the kinases by acutely administering inhibitors of these kinases (compound C for AMPK, and SB203580 for p38 MAPK) and subsequently subjecting the mice to an acute bout of acute exercise. Accumulation of p53 in the mitochondria and p53Ser15 status can then be analyzed. The next step would be to address the importance of phosphorylation at Ser15 to mitochondrial p53 translocation. Transfecting p53 KO mice with vectors expressing

p53 mutated at Ser 15, and subsequently allowing the mice to run an acute bout of treadmill exercise, would help decipher how important this post-translational modification is.

Furthermore, another intriguing question is what attracts p53 to the mitochondria upon exercise. Sablina et al. (10) once floated an interesting hypothesis that levels of ROS regulate p53 activity as the tumor suppressor protein is highly susceptible to any changes in oxidative status. It has been known for some time that exercise can briefly increase levels of oxidative stress (4; 6). It is intriguing to speculate that mitochondrial-derived ROS can serve as part of a retrograde signalling pathway that may serve to recruit p53. Treating mice with cytosolic- or mitochondrial-directed antioxidants prior to acute exercise challenge will help delineate the role of oxidative stress in directing p53 and its sub-cellular address.

3) What is the precise role of exercise-induced p53 accumulation in the mitochondria?

We believe that mitochondrial p53 may be 1) improving mtDNA integrity via its inherent base excision repair capacity, and/or by working in conjunction with mitochondrial polymerase gamma (polγ), or 2) involved in mediating mtDNA transcription. Our data support p53-mediated mtDNA transcriptional control, but it is possible that p53 is executing both of these functions. To examine this question we would need to employ a cell culture technique where primary muscle cells from p53 KO mice are differentiated and allowed to form myotubes. Recombinant p53-flagged protein can

then be transfected along with mtDNA D-loop based reporter constructs, and cells contracted *in vitro* to mimic exercise. Alternatively, since mtDNA transcription is impaired in myotubes from p53 KO mice, transient expression of p53 may rescue it. This would provide further insight into whether p53 can affect mtDNA transcription. To delineate whether p53 is playing a part in fixing mtDNA errors, p53 KO myotubes will be treated with pro-oxidants followed by mitochondrial fractions isolation. One would hypothesize that in absence of mitochondrial p53, these myotubes will accumulate more mtDNA mutations/lesions in response to pro-oxidants (such as H2O2) treatment. The isolated mitochondrial fraction from p53 KO myotubes vs, control myotubes (with and without pro-oxidant treatment for both) can be analyzed for levels of mtDNA mutations using Next Generation Sequencing. To show that p53 is involved in repairing this defect, mitochondrial fractions from these cells can be incubated with recombinant p53 followed by mtDNA sequencing to ensure that mtDNA gets repaired.

4) How does the absence of p53 impair cellular signaling in response to exercise?

First, a comprehensive assessment of the upstream regulatory components that control the activity of exercise-responsive kinases is warranted in p53 WT vs. KO muscle, including metabolites and upstream activators of AMPK and p38 MAPK such as LKB1 and p38 MAPKK. A time-course experiment needs to be conducted to ascertain whether the activity of kinases, expression of PGC-1α, and mRNA transcript levels eventually catch up to WT levels in the recovery phase post-exercise in p53 KO mice. Second, the relation between PGC-1α and p53 is a fascinating one and needs to be fully investigated.

Both proteins have been shown to exert transcriptional control over the other (2; 5; 11; 12). The decreased exercise-induced aggregation of PGC-1α to the nucleus in mice without p53 as shown in objective 3 of this dissertation could be due to inefficient post-translational modifications of PGC-1α. To further inspect this, we can subject WT and p53 KO mice to an acute bout of exercise, and immunoprecipitate nuclear and cytosolic PGC-1α protein from the respective sub-cellular components. The precipitates can thereafter be probed for the classic phosphorylation and deacetylation sites on the co-factor using immunoblotting, or subjected to mass spectroscopy, to assess the gamut of post-translational modifications of PGC-1α. Lastly, the attenuated increase in mRNA levels of genes related to mitochondrial biogenesis in the KO mice could be an indirect effect through the reduced transactivation by PGC-1α, or more directly via changes in mRNA stability. p53 can bind to RNA (9) and certainly possesses the ability to regulate the stability of its own mRNA. Also, RNPC1, an RNA-binding protein is a transcriptional target of p53 (14), which could be repressed in mice with no p53. Expression of RNPC1, and other mRNA binding proteins could be evaluated using primary myotubes derived from p53 WT and KO animals. Furthermore, using this cell culture model, we can subsequently treat the cells with actinomycin D, a transcriptional inhibitor, and RNA extracted over a time course post-treatment to gauge mRNA stability.

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APPENDIX A

ADDITIONAL DATA & GENOTYPING PROTOCOL

STUDY 1
REGULATION OF p53 mRNA EXPRESSION & STABILITY

Figure 1.

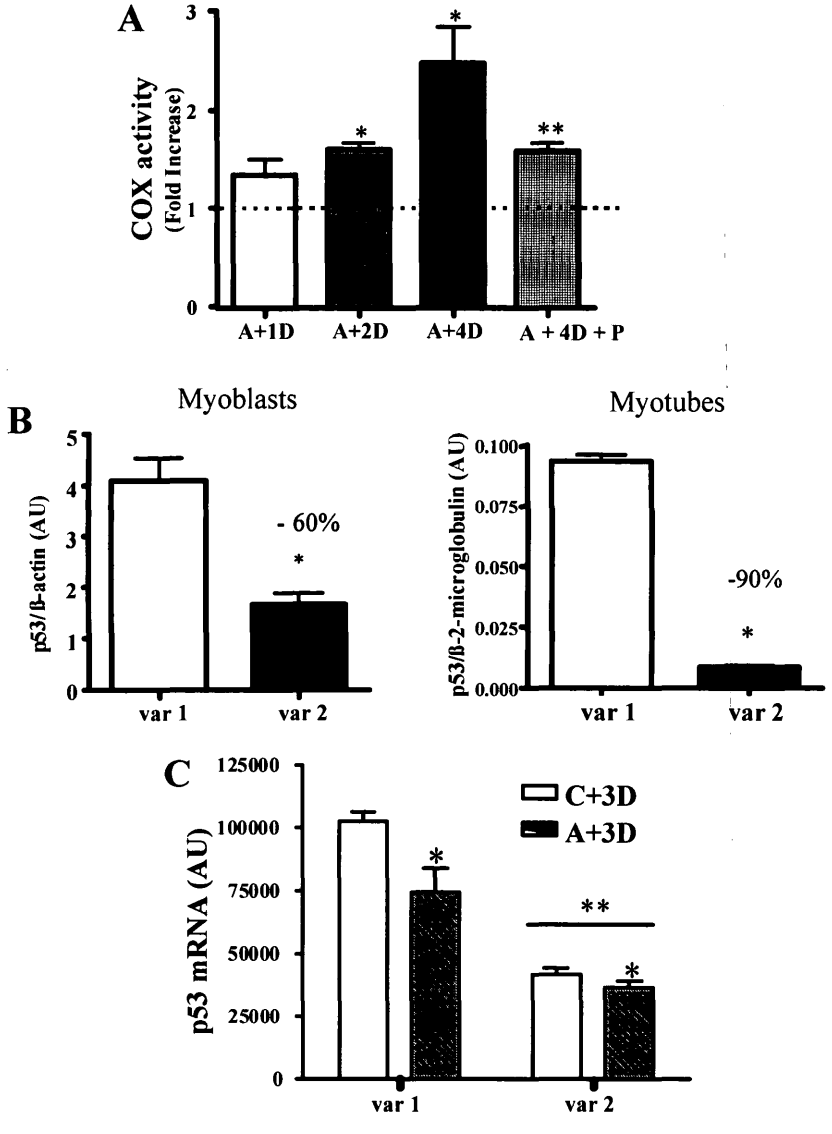


Figure 1. p53-dependent increase in COX activity and basal and AICAR-induced expression of p53 mRNA. (**A**) COX activity increased in C2C12 myoblasts with AICAR (A, 1mM) treatment for 1, 2 or 4 days (1D, 2D, 4D). With the addition of 15μM of pifithrin-α (P), a specific inhibitor of p53, the AICAR-mediated increase in COX activity was diminished. n=3-8, *p<0.05, **p<0.05 effect of pifithrin-α. (**B**) The main two isoforms of p53 mRNA in muscle. Variant 1 (var1) is expressed at 60% higher levels than variant 2 (var2) in myoblasts and at 90% higher levels in myotubes. n=3-6, *p<0.05. (**C**) mRNA expression of both p53 variants decreases in C2C12 myoblasts after 3 days of AICAR (1mM) treatment. β-actin and β-2-microglobulin were used as loading control. n=3, *p<0.05, **p<0.05, main affect of splice variant.

Figure 2

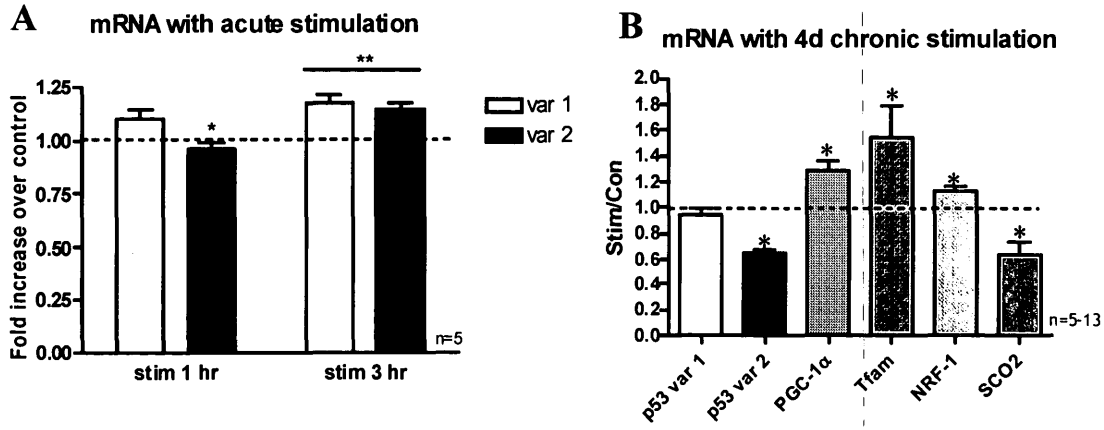
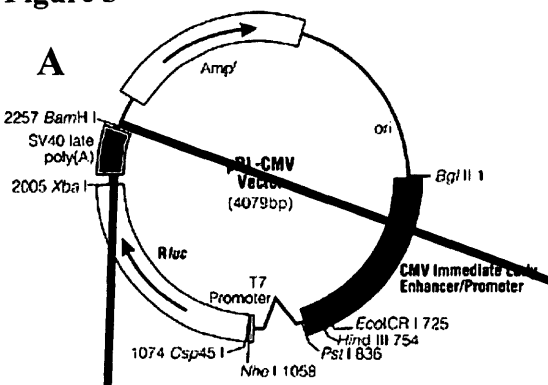


Figure 2. Acute and chronic stimulation-mediated expression of p53 mRNA variants. (**A**) 1 hour of acute stimulation at 10Hz reduced the mRNA levels of var 2, whereas 3 hours of acute stimulation (10Hz) of C2C12 myotubes resulted in a significant increase in p53 mRNA for both transcript variants. n=5, *$p<0.05$, **$p<0.05$, main effect of time. (**B**) mRNA expression of p53 decreased or remained unchanged, as opposed to other regulators of mitochondrial biogenesis such as PGC-1α, Tfam, and NRF-1 with 4 days of chronic stimulation (3hrs/day at 10Hz). SCO2 expression also decreased in tandem with p53. *$p<0.05$ vs. non-stimulated control. Values are expressed as stimulated over control. n=5-13, **$p<0.05$, main affect of splice variant.

Figure 3

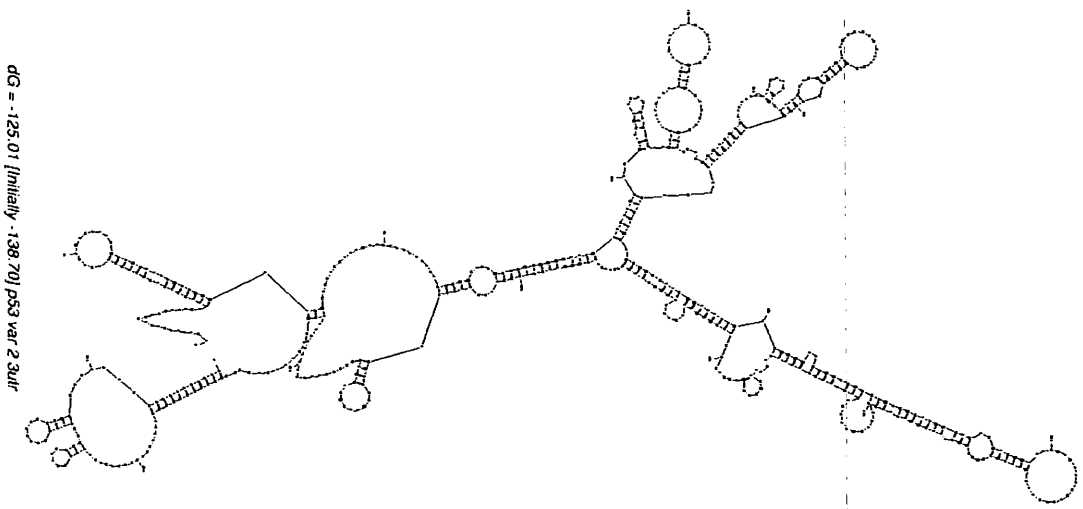
A



B

5′ctcccatcacttcatccctccccttttctgtcttcctatagctacctgaagaccaagaagggccagtctacttcccgccataaaaa
aacaatggtcaagaaagtgggacctgactcagactgactgcctctgcatcccgtccccatcaccagcctcccccctctccttgctgt
cttatgacttcagggctgagacacaatcctcccggtcccttctgctgccttttttaccttgtagctagggctcagccccctctctgag
tagtggttcctggcccaagttggggaataggttgatagttgtcaggtctctgctggcccagcgaaattctatccagccagttgttgg
accctggcacctacaatgaaatctcaccctaccccacaccctgtaagattctatcttgggccctcatagggtccatatcctccagg
gcctactttccttccattctgcaaagcctgtctgcatttatccacccccccaccctgtctccctctttttttttttttaccccttttata
tatcaatttcctatttta*caataa*aattttgttatcacttaaaaaaaaaa 3′ 564bp

C



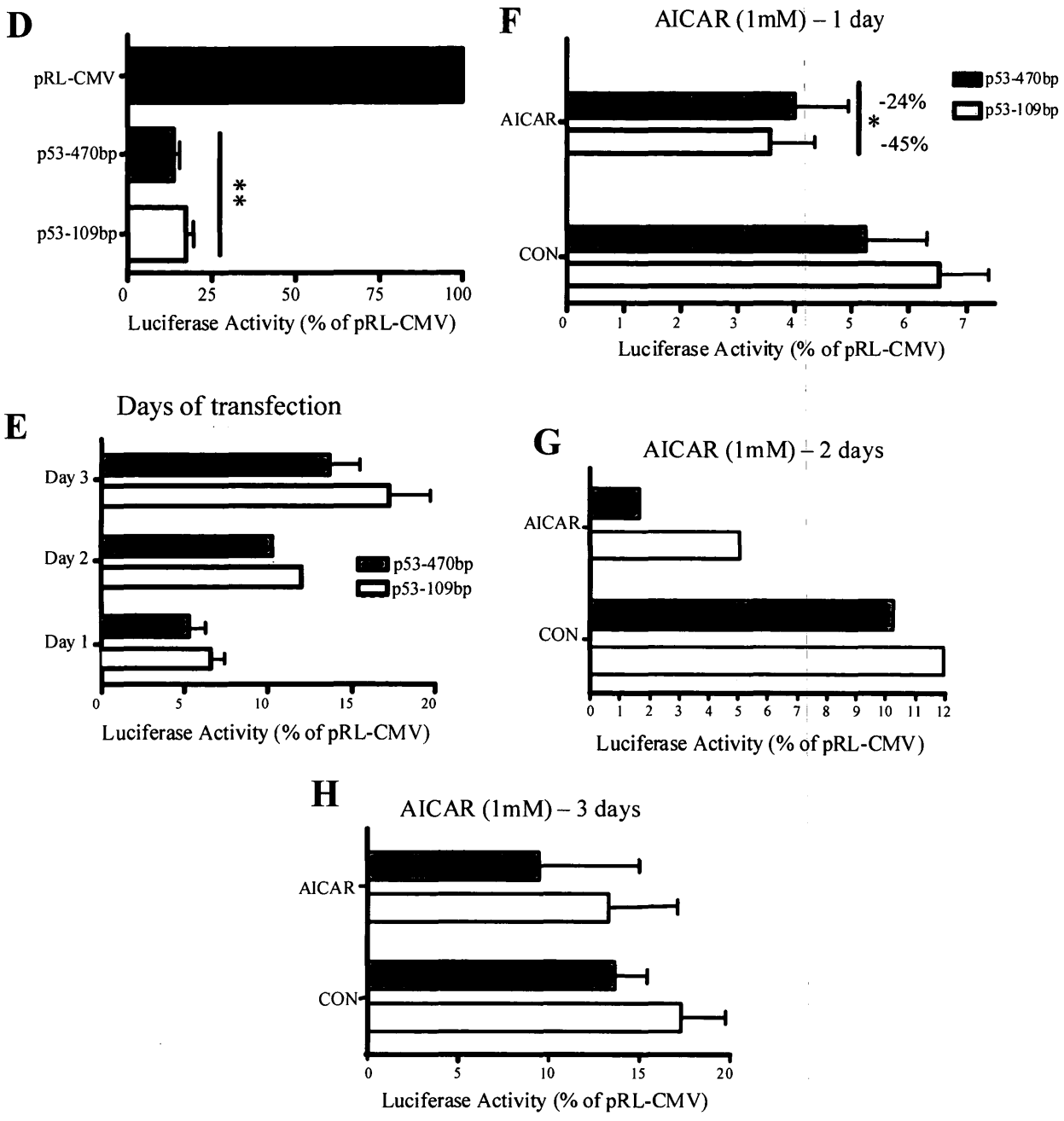

D
pRL-CMV
p53-470bp
p53-109bp
**
0
25
50
75
100
Luciferase Activity (% of pRL-CMV)
F
AICAR (1mM) – 1 day
p53-470bp
p53-109bp
AICAR
-24%
*
-45%
CON
0
1
2
3
4
5
6
7
Luciferase Activity (% of pRL-CMV)
E
Days of transfection
Day 3
Day 2
Day 1
p53-470bp
p53-109bp
0
5
10
15
20
Luciferase Activity (% of pRL-CMV)
G
AICAR (1mM) – 2 days
AICAR
CON
0
1
2
3
4
5
6
7
8
9
10
11
12
Luciferase Activity (% of pRL-CMV)
H
AICAR (1mM) – 3 days
AICAR
CON
0
5
10
15
20
Luciferase Activity (% of pRL-CMV)

Figure 3. 3′ UTR constructs of p53 var 2 and mRNA stability. (**A**) Schematic illustrating the pRL-CMV vector (Invitrogen) that has a CMV promoter driving the expression of luciferase reporter construct ligated to the 3′ UTR of the SV40 virus. (**B**) The putative full length 3′ UTR of p53 var 2 (p53-564bp). The highlighted nucleotides form the AU-rich elements (ARE) that RNA-binding proteins can bind to and affect stability of mRNA. The italicized nucleotides represent the poly-adenylation signal. The first underlined region is the smaller construct (p53-109bp). The bolded region including one ARE is the second construct (p53-474bp). We are still attempting to amplify the full length 3′UTR (p53-564bp). The other constructs were ligated into the pRL-CMV vector and transfected into C2C12 myoblasts. (**C**) The predicted secondary structure of the 3′UTR of p53 var 2 identifying the variety of stem-loop structures that offer another modicum of regulating mRNA stability, in addition to ARE regions. (**D**) Luciferase activity at 1 day post-transfection in C2C12 myoblasts in the empty vector (pRL-CMV), 3′ UTR construct (p53-470bp) and shorter 3′ UTR construct (p53-109bp), n=5, **$p<0.05$ vs. pRL-CMV. (**E**) Luciferase activity of the two constructs over 3 days of transfection, n=1-5. (**E**) Luciferase activity in myoblasts treated with 1mM AICAR for one day, n=3. *$p<0.05$, main affect of AICAR. (**F**) Luciferase activity in myoblasts treated with 1mM AICAR for two days, n=1. (**G**) Luciferase activity in myoblasts treated with 1mM AICAR for three days, n=2-5. All values are expressed as percent of control (pRL-CMV).

Figure 4

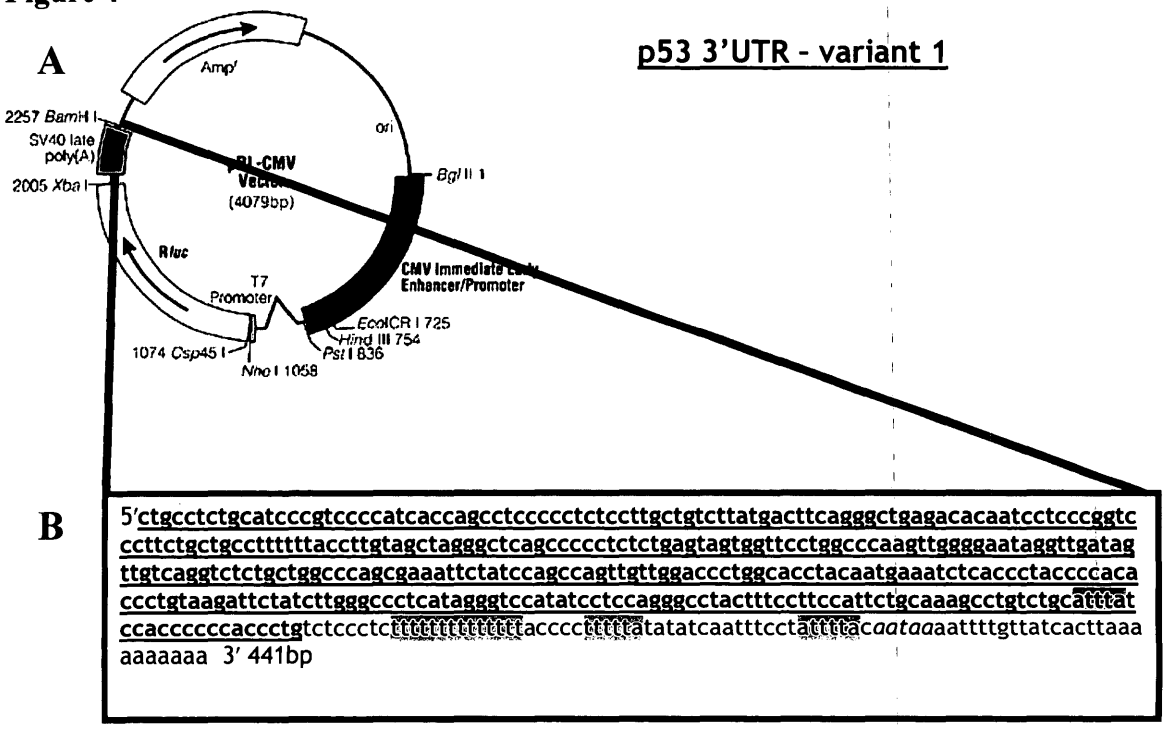

A
p53 3'UTR - variant 1
Amp'
ori
2257 BamH I
SV40 late poly(A)
2005 Xba I
RL-CMV Vector (4079bp)
Bgl II 1
Rluc
T7 Promoter
CMV Immediate Early Enhancer/Promoter
EcoICR I 725
Hind III 754
Pst I 836
1074 Csp45 I
Nhe I 1058
B
5'ctgcctctgcatcccgtccccatcaccagcctccccctctccttgctgtcttatgacttcagggctgagacacaatcctcccggtc
ccttctgctgccttttttaccttgtagctagggctcagccccctctctgagtagtggttcctggcccaagttggggaataggttgatag
ttgtcaggtctctgctggcccagcgaaattctatccagccagttgttggaccctggcacctacaatgaaatctcaccctaccccaca
ccctgtaagattctatcttgggccctcatagggtccatatcctccagggcctactttccttccattctgcaaagcctgtctgcatttat
ccacccccccaccctgtctccctctttttttttttttacccctttttatatatcaatttcctatttttacaataaaatttgttatcacttaaa
aaaaaaa 3' 441bp
C
dG = -91.02 [initially -102.30] p53 var 1 3utr
D
p53 var 1 - 366bp 3'UTR mRNA stability
Fold-change over CON
1.25
1.00
0.75
0.50
0.25
0.00
AS
AS+Rec

Figure 4. p53 variant 1 3′ UTR constructs and mRNA stability. (**A**) Schematic illustrating the pRL-CMV vector (Invitrogen) that has a CMV promoter driving the expression of luciferase reporter construct ligated to the 3′ UTR of the SV40 virus. (**B**) The putative full length 3′ UTR of p53 var 1 (p53v1-441bp). The highlighted nucleotides form the AU-rich elements (ARE) that RNA-binding proteins can bind to and affect stability of mRNA. The italicized nucleotides represent the poly-adenylation signal. The first underlined region is the smaller construct (p53v1-366bp). We are still attempting to amplify the full length 3′UTR (p53v1-441bp). The p53v1-366bp was ligated into the pRL-CMV vector and transfected into C2C12 myoblasts. These cells were subjected to 4 days of differentiation. The fully formed C2C12 myotubes were then acutely stimulated at 2hrs (AS) and AS + 2hrs recovery (**C**) The predicted secondary structure of the 3′UTR of p53 var 1 identifying the variety of stem-loop structures that offer another means of regulating mRNA stability, in addition to ARE regions. (**D**) p53v1-366bp-mediated mRNA stability does not change with AS or AS+recovery. Luciferase activity of p53v1-366bp was expressed as a ratio of pRL-CMV activity, and the resulting RFUs were presented as fold over control non-stimulated myotube values, n=6.

STUDY 2
TISSUE-SPECIFIC EFFECTS OF p53

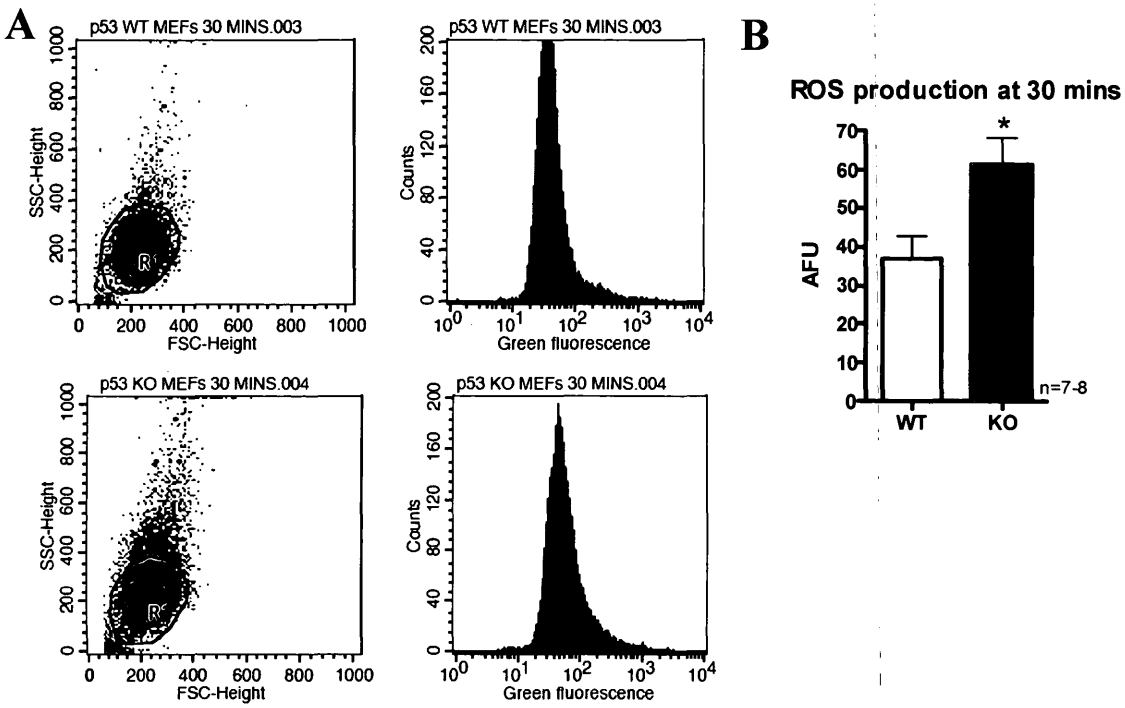


Figure 1. Basal ROS generation in mouse embryonic fibroblasts (MEFs) isolated from p53 WT and KO mice. MEFs were stained with H2DCFDA (50μM) that binds to cellular reactive oxygen species, for 30 mins. Cells were then isolated and a FACS flow cytometer was used to count the cells, and measure green fluorescence. (**A**) Representative flow cytometer read outs for p53 WT (left) and KO (right) MEFs. (**B**) quantification of the DCF staining for all experiments illustrated that p53 KO mice produce higher levels of ROS, n=7-8, *$p<0.05$.

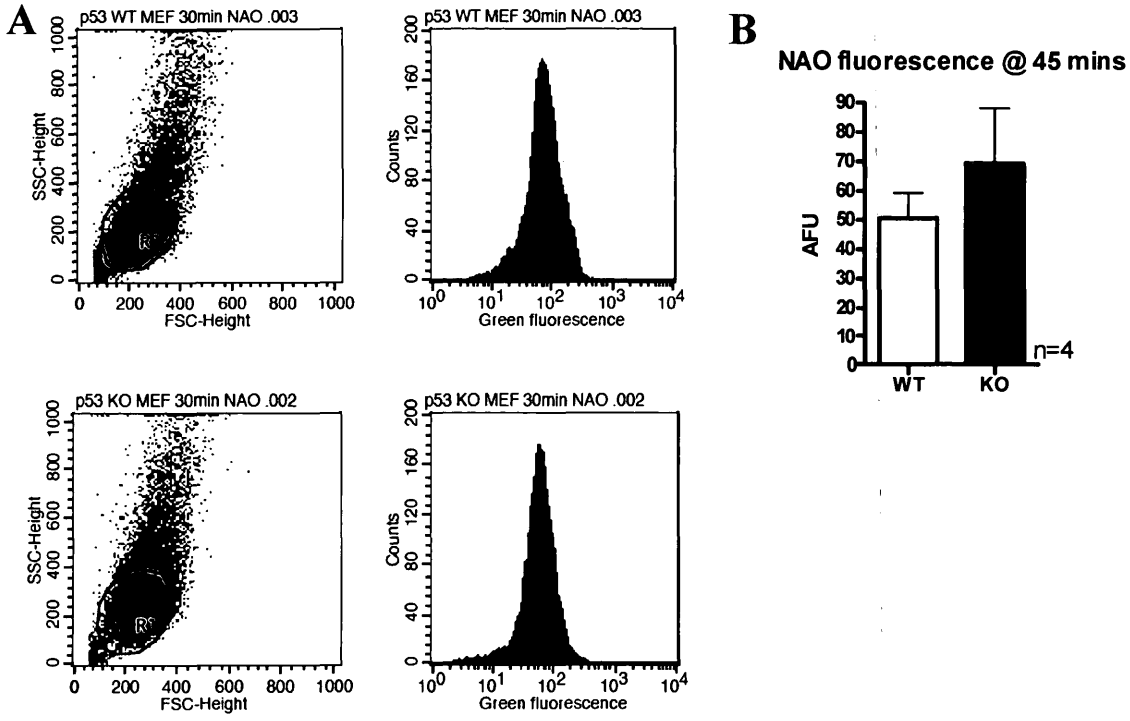


Figure 2. NAO fluorescence as a measurement of mitochondrial mass in p53 WT and KO MEFs. MEFs were stained with nonly-acridine orange (50μM) that binds to the inner mitochondrial membrane, for 45 mins. Cells were then isolated and a FACS flow cytometer was used to count the cells, and measure the fluorescence. (**A**) Representative flow cytometer read outs for p53 WT (left) and KO (right) MEFs. (**B**) quantification of the NO staining for all experiments, n=4.

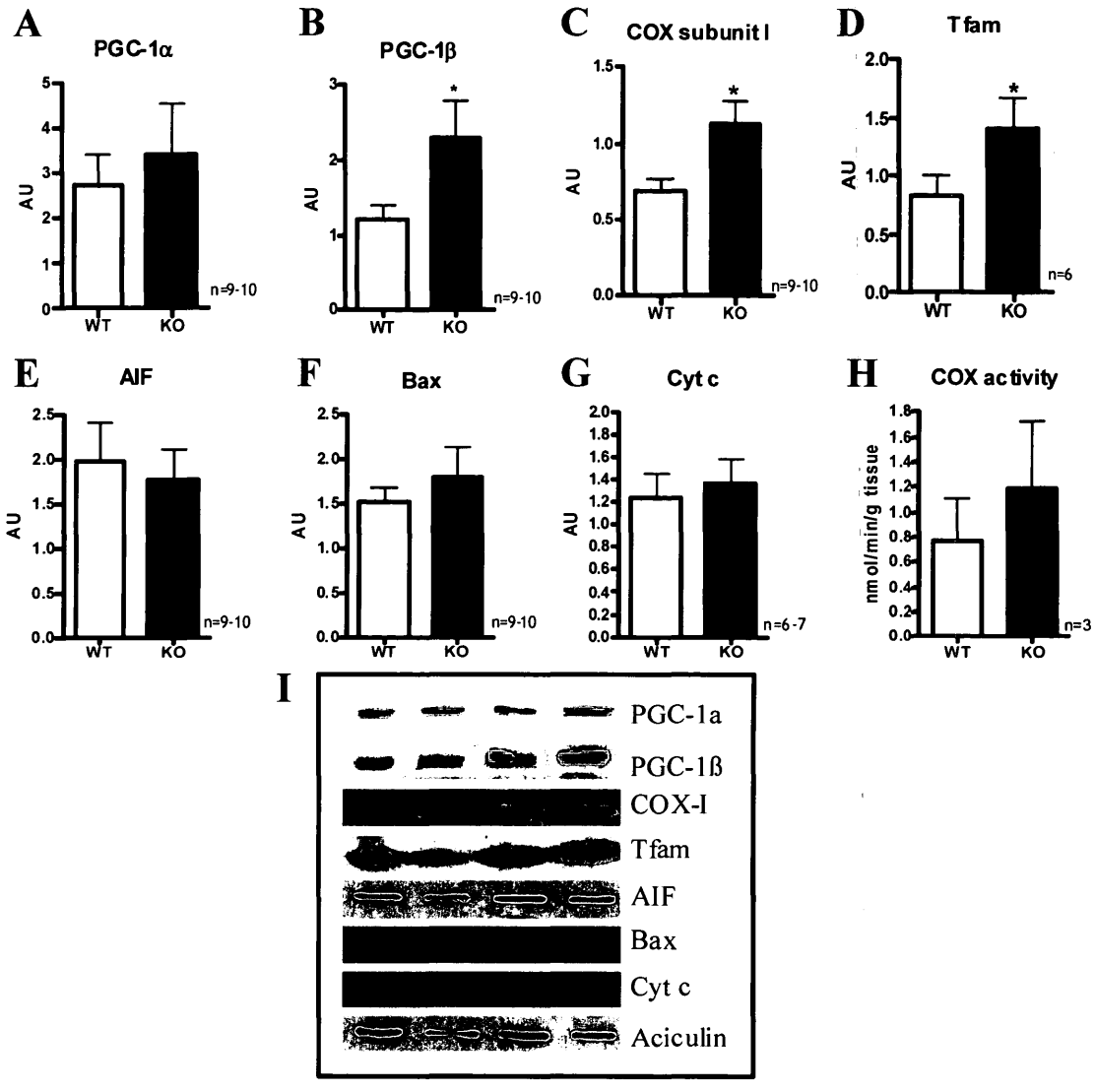


Figure 3. Protein expression of mitochondrial-related proteins, COX subunit I and COX activity in WT and p53 KO MEFs. (**A-G**) Graphical representation of PGC-1α, PGC-1β, COX subunit I, Tfam, AIF, Bax and cytochrome *c* in p53 WT and KO MEFs, (**I**) with the representative immunoblots. (**H**) COX activity in whole cell homogenates from WT and KO MEFs.

STUDY 3
ESTABLISHING PRIMARY MYOBLAST ISOLATION PROCEDURE

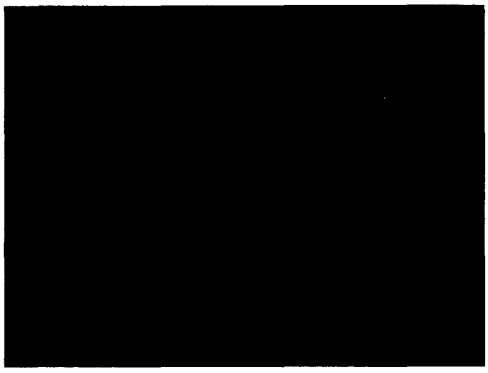
Animal Sacrifice and tissue collection:

(Use 1 week old mice – 1 mouse/well in a 6-well plate when culturing and about 6 mice with each mouse undergoing the procedure below. The myoblasts can then be pooled at the end to increase yield given the mice were all of the same genotype)

1. Euthanize mouse by cervical dislocation. Spray liberally with 70% ethanol.
2. Remove skin and excise all muscle from mouse and place in a well containing 2mls of Ham's F-10 (with 1% penicillin/streptomycin and 1% antibiotic/antimycotic). Clean the muscle of any fat, skin etc. Be quick.

Collagenase treatment:

1. In the cell culture hood, transfer muscle to a new well 1-3mls of Ham's F-10 (with peni/strep and ab/amyc) and mince thoroughly.
2. Add 1.5ml of 0.2% type II collagenase/dispase made in Ham's F-10. Use freshly prepared collagenase/dispase. Incubate at 37 °C for 30mins.
3. Triturate slurry with 25ml, add 0.5ml of collagenase/dispase solution, and incubate at 37 °C again for 30mins.
4. Triturate again with 25ml, then 10ml and 5ml pipettes.
5. Add 2ml of stop media (5% FBS in DMEM) to stop collagenase/dispase reaction.



This is what the slurry should look like before filtration.

Filtration:

1. Place a BD Falcon 70μm nylon cell strainer in a Falcon tube and wet strainer with 1ml 5% FBS in DMEM.
2. Add 1ml PBS to cell suspension and transfer all to strainer. Use 1ml PBS to rinse well and add to strainer.
3. Pipette on strainer until solution has mostly gone through.
4. No wet a 40um BD Falcon strainer with PBS and pipette the slurry again.
5. Centrifuge at 1500rpm for 15mins.
6. carefully pipette out supernate and discard. Do not disturb the pellet.
7. Add 3-4mls of Ham's F10 growth media.

Ham's F10 Growth Media (GM):
40% Ham
20% FBS
1% P/S
2% chick embryo extract (CEE)

Pre-plate, overnight plating. Initial culture (approx 2 weeks):

1. Pre-plate for 1 hour on a non-coated well.
2. Then remove supernatant and put in a collagen coated dish/well overnight.
3. Change media after 24hours
4. Pre-plate and then change media every 2 days as described above.
5. Use partial trypsinization every 2 days when plates are changed – i.e. add trypsin for 30sec and then tap once to see most of the myoblasts floating immediately whereas the fibroblasts remain stuck.. This will help purify the colony.

Growing conditions:

1. After about 2 weeks, remove CEE from growth media and add 100ng/µl bFGF (Invitrogen) fresh to 50ml aliquots of Ham's GM.
2. Stop pre-plating.
3. Use only collagen coated dishes/wells from here onwards.
4. Continue to use partial trypsinization – works very well to keep selecting myoblasts against fibroblasts.
5. Plates can be changed on a weekly basis.

Collagen coating:

1. To coat, add 2-3ml to a well or plate. Make sure it covers the surface evenly. Incubate at 37 °C for 1hr.
2. Pipette off liquid and return to stock bottle. It can be reused many times.
3. Dry the plate in the flow hood (overnight or 8 hours – do not use UV).
4. Plates can be wrapped in a bag and stored at 4 °C for many months.

Collagen:

1. Autoclave 0.2% acetic acid in ddh20. Use water NOT PBS.
2. Final solution is 0.01% collagen in 0.2% acetic acid.
3. Resuspend rat tail collagen type I (Sigma, C3867) in the bottle from the manufacturer before diluting into a larger volume. Vortex to resuspend.
4. Store collagen solution at 4 °C for many months - if kept sterile.

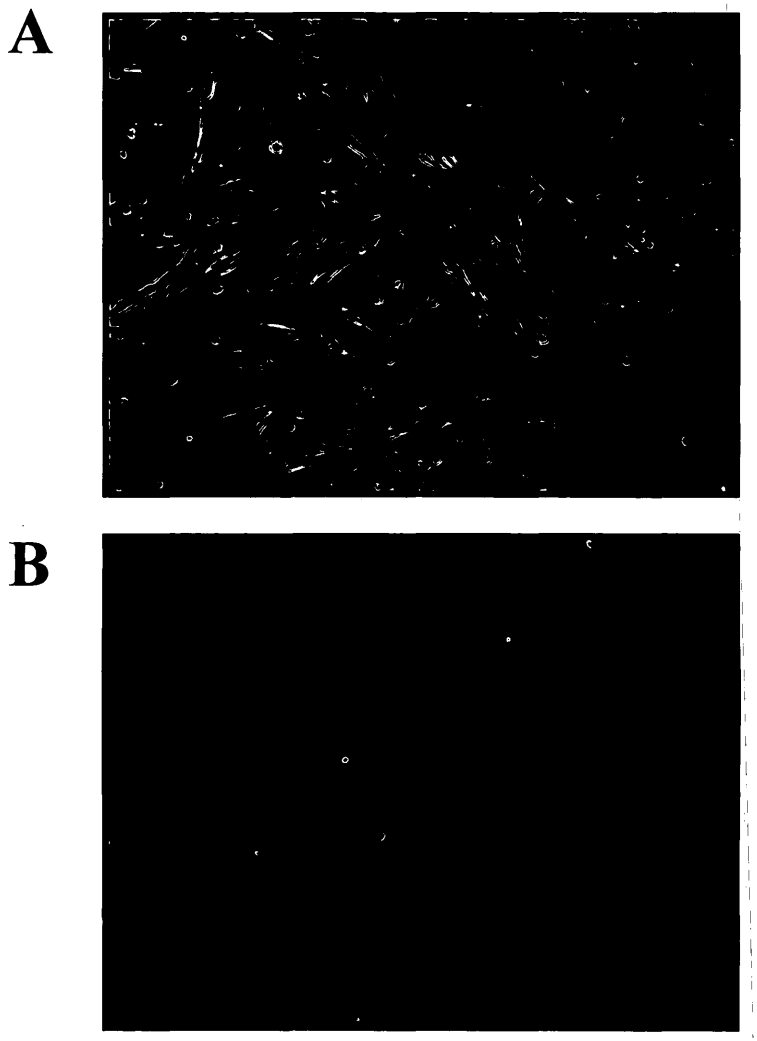
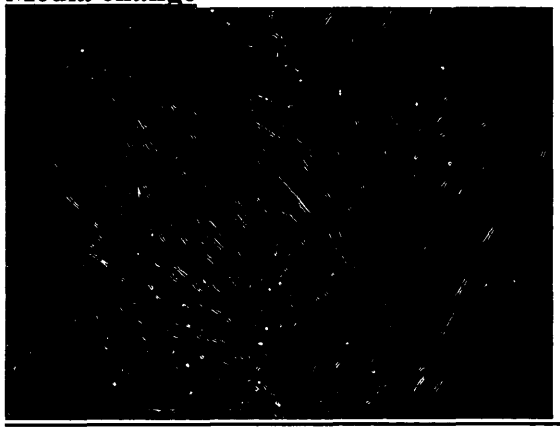


Figure 1. Proof-of-concept that the primary myoblast isolation procedure yields pure myoblast population. (**A**) Isolated myoblasts were allowed to reach 90-95% confluency and then differentiated for 5 days. The myoblasts readily formed elongated premature muscle cells or myotubes. (**B**) To further ascertain purity of isolation, myotubes were stained with skeletal muscle marker desmin, and nuclear dye DAPI. The image clearly depicts all differentiated and even the non-differentiated myoblasts in the background to be skeletal muscle cells.

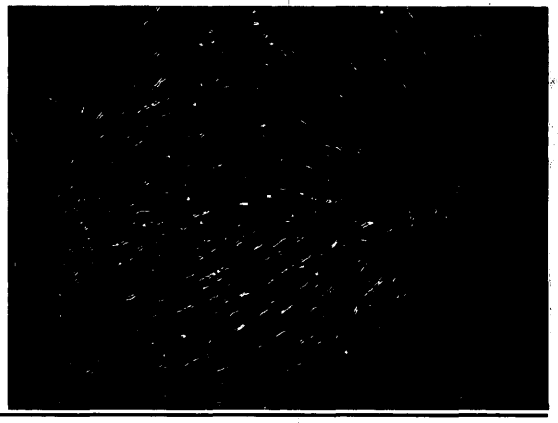
STUDY 4
EFFECT OF MEDIA CHANGE POST-STIMULATION IN C2C12 MYOTUBES

A) DAY1

Media change

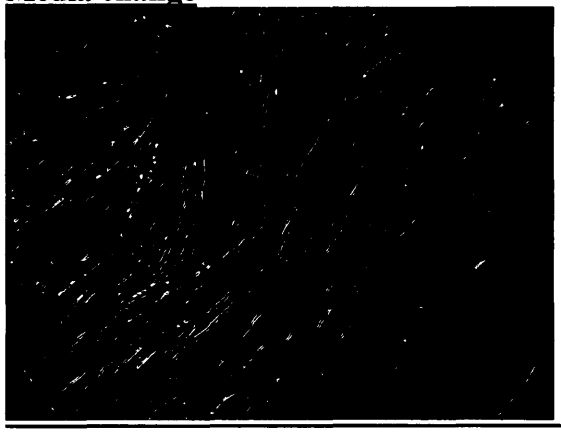


No media change



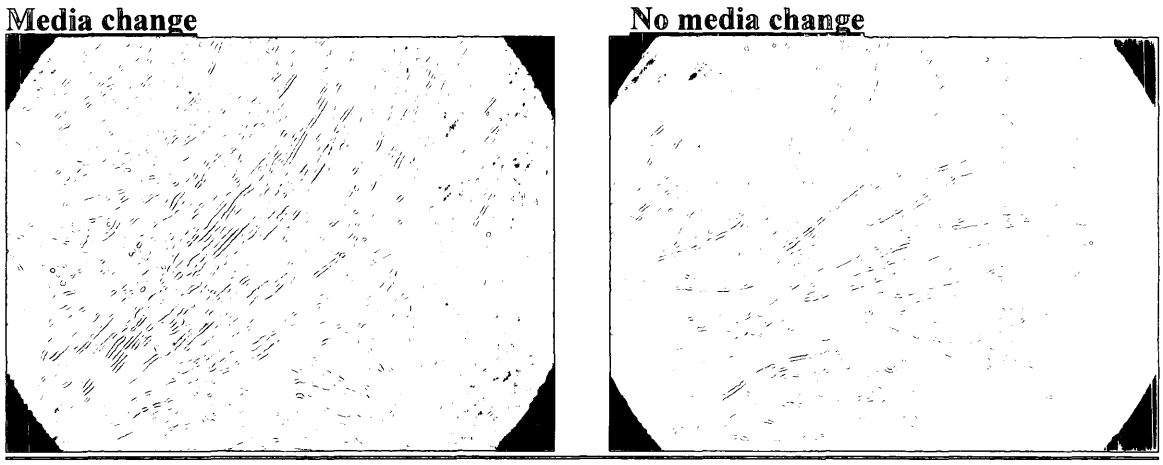
B) DAY 2

Media change



No media change

C) DAY 3



D) DAY 4

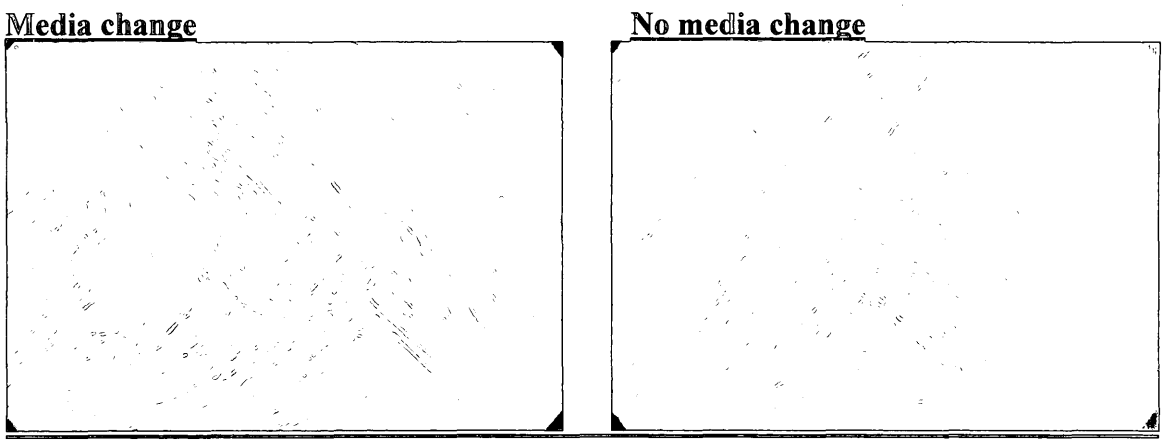


Figure 1. The effect of media change on C2C12 myotube viability after an acute bout of *in vitro* stimulation.

C2C12 myotubes were stimulated at 10Hz for 3h/day for 4 days with or without a change in media after each stimulation bout. Representative light microscopy pictures of myotubes after (A) 1 day post-stimulation, (B) 2 days of stimulation, (C) 3 days of stimulation and (D) 4 days post-stimulation. Changing the media after a bout of *in vitro* electrical stimulation improves myotube viability, especially evident during days 2 - 4.

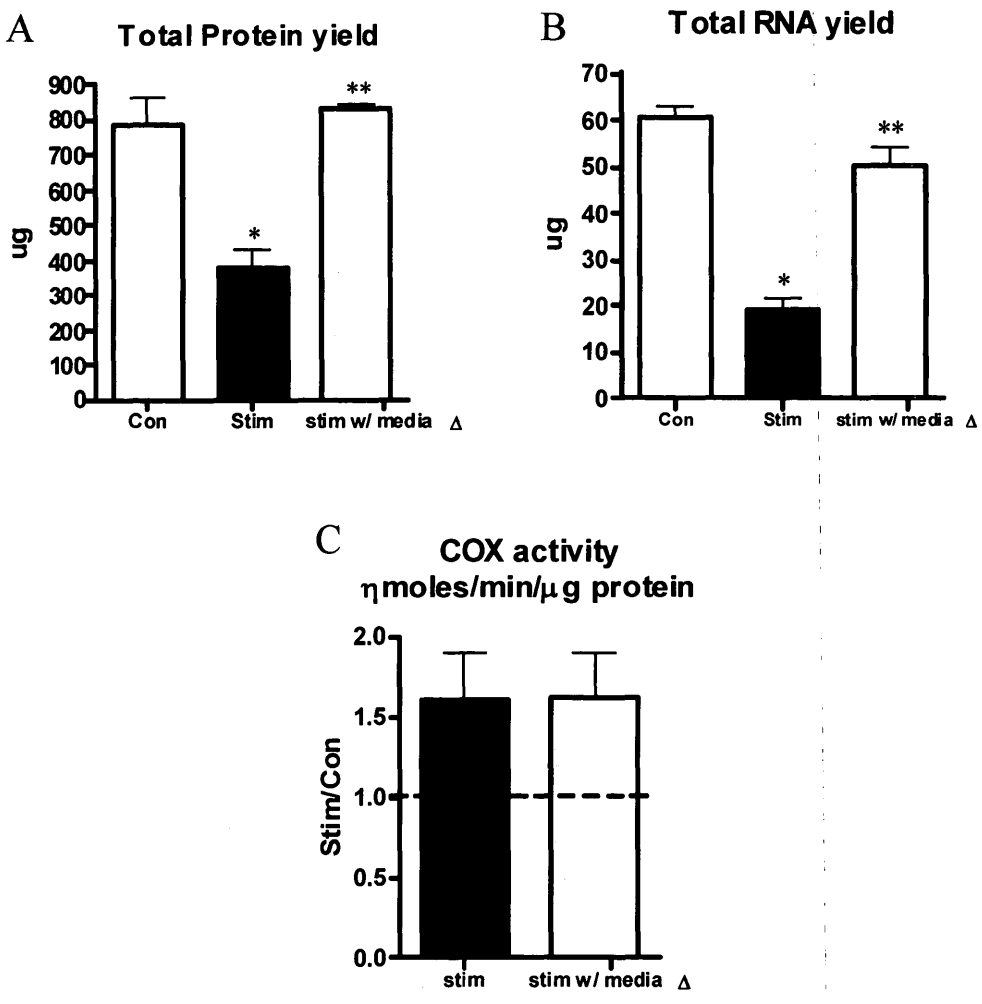


Figure 2. Total protein, RNA yield and COX activity with or without media change post-stimulation for 4 days.

C2C12 myotubes were stimulated at 10Hz for 3h/day for 4 days with or without a change in media after each stimulation bout. (**A**) Total protein yield (n=3), (**B**) total RNA yield (n=3-7) and (**C**) COX activity (n=4) from control/non-stimulated, and stimulated C2C12 myotubes with or without a change of media post-stimulation. Changing the media results in an improved protein and RNA yield, but carries no repercussions for the adaptation in mitochondrial biogenesis observed after chronic contractile activity that mimics endurance exercise training *in vitro*. *$p<0.05$, con vs. stim, **$p<0.05$, stim vs. stim with media change.

PROTOCOL
GENOTYPING P53 MICE USING PCR

***Background*:** This protocol is designed to detect sequences in the murine genome that differ between p53 wild-type and null animals using polymerase chain reaction amplification. PCR is a rapid, inexpensive and simple way of copying specific DNA fragments from minute quantities of source DNA material. There are basically 3 procedural steps involved in PCR:

1) Denaturation: DNA is heated to high temperature to separate the DNA double helix to single strands making them accessible to primers. During denaturation (94° C, 30sec), the DNA strands separate to form single strands.

2) Annealing: The reaction mixture is cooled down. Primers anneal to the complementary regions in the DNA template strands, and double strands are formed again between primers and complementary sequences. During annealing (60°C, 30sec) one primer binds to one DNA strand and another binds to the complementary strand. The annealing sites of the primers are chosen so that they will prime DNA synthesis in the region of interest during extension.

3) Extension: The DNA polymerase synthesizes a complementary strand. The enzyme reads the opposing strand sequence and extends the primers by adding nucleotides in the order in which they can pair. During extension (72°C, 45sec), DNA synthesis proceeds through the target region and for variable distances into the flanking region, giving rise to long fragments of variable lengths. The whole process is repeated over and over.

The DNA polymerase, known as Taq polymerase, is named after the hot-spring bacterium *Thermus aquaticus* from which it was originally isolated. The enzyme can withstand the high temperature needed for DNA-strand separation. The cycle of heating and cooling is repeated over and over, stimulating the primers to bind to the original sequences and to newly synthesized sequences. The enzyme will continue to extend primer sequences. This cycling of temperatures results in copying and then copying of copies, leading to an exponential increase in the number of copies of specific sequences. Because the amount of DNA placed in the tube at the beginning is very small, almost all the DNA at the end of the reaction cycles are copied sequences.

The reaction products are then separated by gel electrophoresis and visualized with the use of ethidium bromide.

Reagents

Lysis Buffer (pH=8.0)

10 mM Tris HCl (0.121g/100ml)
150 mM NaCl (0.8766g/100ml)
20 mM EDTA (0.744g/100ml)
Autoclave for 30min and store at room temperature.

Supermix

Sigma Jumpstart REDtaq Ready Mix PCR Reaction Mix (P0982)
Product contains 20 mM Tris-HCl, pH 8.3, 100mM KCl, 4 mM MgCl2, 0.002% gelatin, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), inert dye, stabilizers, 0.06 unit/µl Taq DNA polymerase, JumpStart Taq antibody.

Primers

Forward and Reverse for WT and Null Stock Concentration 500 pmol/µl
Working Concentration of Primers (10X dilution): 50 pmol/ul
To make up 50 pmol/µl: use 5 µl of 500 pmol/µl stock and add 45µl of sterile water

Proteinase K
ProK- concentration of 1mg/ml

Reagents for Agarose Gel Electrophoresis of PCR product

Agarose	50XTAE
50 X TAE	242 g TRIS
1X TAE (dilute 50X TAE with stH_2O)	500ml dH_2O
10mg/ml EtBr	100ml 0.5M EDTA (pH 8.0)
Sterile water	57.1ml Glacial Acetic Acid
Make up to 1L and autoclave	

DNA Extraction from ear clippings

1. Make **(fresh)** 10:1 mixture of lysis buffer to ProK (@concentration of 1mg/ml-**fresh**)
2. Add 20 µl of this mixture to a 1.5 ml sterile eppendorf tube.
3. Obtain ear clipping from animal, add to tube and vortex (ensure ear clipping is immersed in solution).
4. Incubate in a 55 °C water bath (no higher than 60 °C) for 30min, vortexing every 15 minutes.
5. Add 180 µl sterile distilled water.
6. Place in boiling water for 5 minutes (use hot plate) and then vortex.
7. Store at -20°C, or use immediately for PCR

PCR method

1. Make mastermix for each of the primers you will be using.
   Mastermix contains: 25 µl of Supermix sample
   1 µl of Forward Primer per sample
   1 µl of Reverse Primer per sample
   Enough sterile distilled water for a volume per sample of 50 µl.

2. For each sample use 48 µl of mastermix and 2 µl of template DNA extracted from procedure described above. Add 1 drop of mineral oil to each PCR tube to prevent evaporation of sample during cycling.

3. Cycling times:

Initial Denaturation	94°C	2min
35 cycles: Denaturation	94°C	30sec
Annealing	60°C	30sec
Extension	72°C	45sec
Final Extension	72°C	5min
Hold	4°C	

Running PCR product on gel

1. Loading buffer is already included in Supermix.

2. Preparation of a 1.2% agarose gel. ***For large gel system***: 3.6g Agarose, 6 ml 50X TAE and volumed up to 300 ml with sterile H_2O. Mix solution and note weight followed by boiling in microwave. Remove periodically to mix during boiling procedure in microwave. Upon complete dissolving of agarose and a homogenous and releatively clear agarose solution, weigh solution and replace lost amount of evaporated H_2O. Add 25µl of EtBr (10mg/ml), slightly cool solution in room temperature (5-10min), then pour into caster.
For small gel system: 1.92g Agarose, 3.2 ml 50X TAE, and 156.8ml st H_2O; follow same procedure as noted above. Only add 8 µl of EtBr (10mg/ml).
Electrophoresis Running Buffer: 1X TAE: 40 ml of 50X TAE made up to 2L with H_2O.

3. Run 10 µl of PCR product reaction on either a small or large 1.2% agarose gel for 1hr @ approximately 90V.

Example of Experimental Setup/Procedure for PCR Genotyping (15 mice)

1. Make up mastermix for both wild-type and null primers for the number of animals required for genotyping.

Mastermix(proportions)

A) WT Mastermix

25µl Supermix
1µl Forward Primer WT
1µl Reverse Primer WT
23µl Sterile Water
50µl Total

B) Null Mastermix

25µl Supermix
1µl Forward Primer Null
1µl Reverse Primer Null
23µl Sterile Water
50µl Total

X 17 reactions (over estimate, since 15 animals required)

Total Mastermix

A) WT Mastermix

425µl Supermix
17µl Forward Primer WT
17µl Reverse Primer WT
391µl Sterile Water
850µl Total

B) Null Mastermix

425µl Supermix
17µl Forward Primer Null
17µl Reverse Primer Null
391µl Sterile Water
850µl Total

1. Label PCR tubes with 1N and 1W to represent each animal and each mastermix, respectively.

2. Place 48 µl of null mastermix in PCR tubes with N designation and 48 µl of wild-type mastermix in PCR tubes with W designation.

3. Add 2µl of DNA template from animals into the appropriate PCR tubes i.e. DNA isolated from animal #1 into 1 N and 1W.

4. Include negative controls using dH_2O instead of DNA template with both Null and wild-type mastermix i.e. 2 µl of dH_2O into PCR tubes with 48 µl of WT mastermix and 2 µl of dH_2O into PCR tubes with 48 µl of Null mastermix.

5. Add 1 drop of mineral oil to each tube and place tubes into thermocycler for 35 cycles. Use file # 29 on thermocycler for the initial 2 min denaturation then STOP this program. Use file # 30 for repeated cycles. Scroll through the settings in File # 29 and change the settings to achieve:

Denaturation	94°C	30sec
Annealing	60°C	30sec
Extension	72 °C	45sec

Then link this file (#29) to file #33 which is the final extension of 72°C for 10min (protocol states 5min but 10min does not make a difference). This will be followed by a hold cycle at 4°C. Samples can be left holding overnight if necessary.

6. Prepare either large gel or small gel or both depending on number of samples. Load PCR products onto gel and run for 1-1.5 hours.

7. Visualize PCR products using UV lightbox in the molecular core facility. Null PCR product should be about 600bp whereas WT PCR product will be about 300bp. If animal #1 displays a band for both the Null and the WT, the animal is a **heterozygote**. If animal #1 exhibits only a Null band but no WT band then the animal is a **homozygous null**. If the animal displays a wild-type but no Null band then the animal is **homozygous wild-type.**

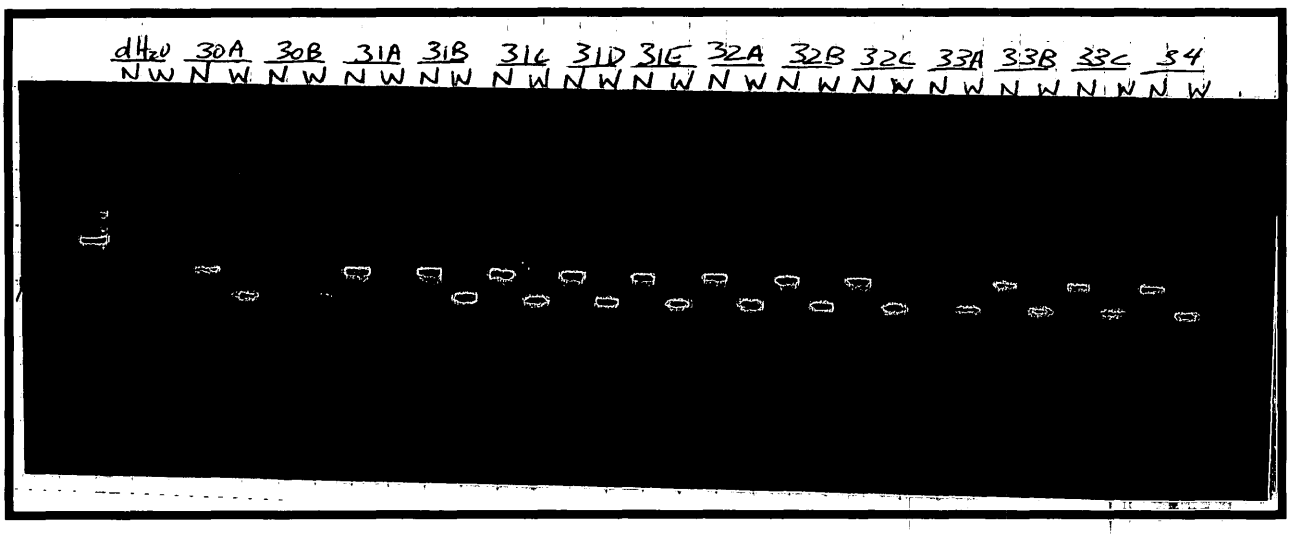


Fig.1: Typical agarose gel displaying PCR products and animal genotypes.

Genotype

30A-hetero	31E-hetero
33C-hetero	30B-hetero
32A-hetero	34-hetero
31A-null	32B-hetero
31B-hetero	32C-hetero
31C-hetero	33A-wild-type
31D-hetero	33B-hetero

APPENDIX B
OTHER CONTRIBUTIONS

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

Peer-reviewed research papers

7. Blakely K, Mero P, Arnold R, **Saleem A**, Misquitta C, Kasimer D, Kumar S, Uetrecht A, Brown KA, Datti A, Hood DA, Kim P, and Moffat J. A Lentiviral-Based Pooled Bi-Fluorescence Complementation Screen Reveals Regulators of Human Mitochondrial Function. *Proceedings of the National Academy of Scienes (PNAS).* Manuscript in revision. (Manuscript ID # 2013-07866).

6. Zhang Y, Iqbal S, O'Leary MFN, Menzies KJ, **Saleem A**, Ding S, and Hood DA. Altered mitochondrial morphology and defective protein import reveals novel roles for Bax and/or Bak in skeletal muscle. *Am J Physiol Cell Physiol.* Jun 2013 (Manuscript ID# C-00058-2013R1, in press).

5. Menzies KJ, Singh K, **Saleem A**, and Hood DA. Sirtuin 1-mediated effects of exercise and resveratrol on mitochondrial biogenesis. *J Biol Chem.* Mar 8;288(10):6968-79.

4. **Saleem A**, Heather Carter, Sobia Iqbal and Hood DA. Role of p53 Within the Regulatory Network Controlling Muscle Mitochondrial Biogenesis. *Exerc Sport Sci Rev.* 2011 Jul 14

3. **Saleem A**, and Safdar A. Exercise-induced Histone Acetylation – Playing Tag with the Genome. *J Physiol,* 2010 Mar 15;588(Pt 6):905-6.

2. Ljubicic V, Josesph AM, Adhihetty PJ, Huang JH, **Saleem A**, Uguccioni G and Hood DA. Molecular basis for an attenuated mitochondrial adaptive plasticity in aged skeletal muscle. *Aging, Vol. 1 (9)*, September 2009.

1. Ljubicic V, Joseph AM, **Saleem A**, Uguccioni G, Collu-Marchese M, Lai RY, Nguyen LM, Hood DA. Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: Effects of exercise and aging. *Biochim Biophys Acta.* 2009 Aug 12.

Book Chapters

1. Hood DA, **Saleem A**, Carter H, Vainshtein A, Ostojic O and Iqbal S. *Molecular basis of exercise training adaptations in skeletal muscle.* (2013) Textbook chapter in Exercise Physiology in Canada. MacIntosh BM (editor).

+ 16 abstracts including oral and poster presentations, and 3 non peer-reviewed publications.