

**CHARACTERIZING THE ROLE OF SULFORAPHANE IN MITOCHONDRIAL FUNCTION
AND CONTENT IN SKELETAL MUSCLE CELLS**

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ABSTRACT

A healthy mitochondrial reticulum is critical for the maintenance of skeletal muscle. Preservation of the pre-existing pool through the antioxidant response and synthesis of new mitochondria (biogenesis) ensures the quality and quantity of mitochondria is sustained. Exercise has been established an effective intervention to enhance the mitochondrial phenotype, since it elicits metabolic alterations which drive these quality control processes. To evaluate if a pharmaceutical intervention may be able to mimic or enhance exercise induced-mitochondrial adaptations, the compound sulforaphane (SFN) was examined. SFN has been reported as a potent activator of the nuclear factor erythroid 2-related factor 2 (Nrf-2) signaling cascade which enhances the expression of antioxidant and cytoprotective-related genes. Although activation of this signaling cascade has proven to be beneficial, its relevance to mitochondria has not been fully understood. To investigate this, SFN was combined with an *in vitro* model of “exercise” and mitochondrial parameters were examined. Our results suggests that SFN activates pathways that elicit exercise-induced mitochondrial adaptations.

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“Let not wisdom scoff at strange notions or isolated facts. Let them be explored. For the strange notion is a new vision and the isolated fact a new clay, possible foundations of tomorrow’s science” – Edward F. Adolph

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LIST OF ABBREVIATIONS

$\Delta\Psi_m$	Mitochondrial membrane potential
ΔpH	Potential of hydrogen
ATP	Adenosine triphosphate
ADP	Adenine diphosphate
AMP	Adenine monophosphate
ANC	Adenine nucleotide carrier
AMPK	AMP Kinase
ATF2	Activating transcription factor 2
ARE	Antioxidant response element
ATF4	Activating transcription factor 4
AhR	aryl hydrocarbon receptor
Arnt	AhR nuclear translocator
BNIP3L	BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like
COX	Cytochrome c oxidase
cGAS	cyclic GMP-AMP synthase
CAMK	Calcium/calmodulin-dependent protein kinase
CaM	Calmodulin
CnA	Calcineurin
cAMP	Cyclic adenosine monophosphate
CRE	cAMP response element
CREB	(CRE)-binding protein
CAT	Catalase
Cul3	Cullin 3
CO	Carbon monoxide
CPK	Creatine phosphokinase
c-Fos	Cellular oncogene Fos
c-Jun	Jun proto-oncogene
DAMP	Damage associated molecular pattern
ETC	Electron transport chain
e-	Electron
EPS	Electrical pulse stimulation
FAD⁺	Flavin adenine dinucleotide – oxidized
FADH₂	Flavin adenine dinucleotide – reduced
FCCP	Carbonyl cyanide-p-trifluoromethoxy
FUNDC1	Fun14 domain containing 1
Fyn	proto-oncogene tyrosine protein kinase Fyn
GCN5	General control non-repressed 5 protein
GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GR	Glutathione reductase
G6PD	Glucose-6-phosphate dehydrogenase
GSK-3β	Glycogen synthase kinase-3 β
HAT	Histone acetyl transferase
HSP90	Heat shock protein 90

HSP70	Heat shock protein 70
H⁺	Proton
H₂O₂	Hydrogen peroxide
HDAC	Histone deacetylase
HO₂•	Hydroperoxyl radical
HO-1	Heme oxygenase-1
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IMF	Intermyofibrillar
IL-6	Interleukin-6
IVR	Intervening Region
JNK	c-jun N-terminal kinase
kDa	Kilodalton
kB	Kilobase
Keap1	Kelch-like ECH-associated protein 1
LKB1	Liver kinase B1
LC3-II	Microtubule-associated protein 1A/1B-light chain 3
LDH	Lactate dehydrogenase
mtDNA	Mitochondrial DNA
MTS	Mitochondrial targeting sequence
mV	Millivolt
mPTP	Mitochondrial permeability transition pore
MEF2	Myocyte enhancer factor 2
mRNA	messenger RNA
MyoD	Myogenic determining factor
MuRF-1	Muscle ring FINGER
NuGEMPs	Nuclear genes encoding mitochondrial proteins
NAD⁺	Nicotinamide adenine dinucleotide – oxidized
NADH	Nicotinamide adenine dinucleotide – reduced
NRF-1	Nuclear respiratory factor-1
NFAT	Nuclear factor of activated T cell
Nrf-2	Nuclear factor (erythroid derived 2)-like-2
NQO1	NADPH quinone oxidoreductase
NES	Nuclear export signal
NLS	Nuclear localization signal
Neh2	Nrf-2 ECH homology
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
O₂	Oxygen
OCR	Oxygen consumption rate
OE	Over expression
OH•	Hydroxyl radical
ONOO⁻	Peroxynitrite
O₂•⁻	Superoxide
PIM	Protein import machinery

P_i	inorganic phosphate
PGC-1α	Peroxisome-proliferator-activated receptor γ co-activator 1 α
PP2	Protein phosphatase 2
P38 MAPK	p38 mitogen-activated protein kinase
P-AMPK	Phospho AMPK
P-p38	Phospho p38
P-JNK	Phospho JNK
PIC	Pre-initiation complex
PTM	Post translational modification
PLA₂	Phospholipase A ₂
cPLA₂	Cytosolic PLA ₂
iPLA₂	Calcium independent PLA ₂
sPLA₂	Calcium-dependent PLA ₂
PINK1	PTEN-induced kinase 1
rRNA	ribosomal ribonucleic acids
RyR1	Ryanodine receptor 1
ROS	Reactive Oxygen Species
Rbx1	Ring box protein 1
SR	Sarcoplasmic reticulum
SS	Subsarcolemmal
STING	Stimulator of interferon genes (STING)
Ser	Serine
SIRT1	Sirtuin 1
SP1	Specificity protein1
SOD	Superoxide dismutase
SMAD2	Mothers against decapentaplegic related protein 2
sMaf	Small Musculo-aponeurotic fibrosarcoma
TEM	Transmission electron microscopy
tRNA	transfer ribonucleic acids
TFAM	Mitochondrial transcription factor A
TOM	Translocase of the outer mitochondrial membrane
TIM	Translocase of the outer mitochondrial membrane
TF	Transcription factor
Thr	Threonine
TR	Thioredoxin reductase
Trx	Thioredoxin
TOM	Translocase of the outer mitochondrial membrane
TIM	Translocase of the inner mitochondrial membrane
UB	Ubiquinone
USF-1	Upstream stimulatory factor-1
ULK-1	Unc-51 like autophagy activating kinase-1
VO_{2Max}	Maximal oxygen consumption
vATPase	Vacuolar-type ATPase

CHAPTER 1: REVIEW OF LITERATURE

1.0 SKELETAL MUSCLE PHYSIOLOGY

Foundational research in physiology has established that human health is heavily influenced by the quality and function of skeletal muscle ¹⁻⁴. As a result, a significant amount of work has been dedicated to unravelling the structural and functional complexities of this tissue, to ultimately elucidate its role in systemic homeostasis. Among the complexities of skeletal muscle physiology, one of the most remarkable features of this tissue is its plasticity in response to stimuli, which allows it to adapt to external stressors to meet metabolic demands ^{5,6}. This adaptability is what facilitates the benefits of exercise, as well as the consequences linked to inactivity ⁷. Therefore, this highlights the importance of attaining muscle fitness and understanding the molecular mechanisms that underpin exercise-induced skeletal muscle adaptations for therapeutic purposes.

1.1 SKELETAL MUSCLE STRUCTURE

Skeletal muscle features unique morphological characteristics that distinguish it from other organs, ultimately permitting its excitability and contractility properties. At the tissue level, muscle is highly organized into elongated cylindrical fibers, known as myofibers, which run parallel to one another, giving muscle its striated appearance ⁸. Myofibers, also referred to as skeletal muscle cells, display a long, multinucleated appearance, which is a result of myogenic differentiation ⁹. During this process, myogenic progenitor cells (myoblasts) fuse to form multinucleated myotubes, which mature into myofibers ⁹.

Each muscle cell is meticulously organized, containing a series of sarcomeres which facilitate contraction. Each sarcomere is equipped with contractile apparatus, including actin and myosin filaments which physically interact in an ATP-dependent manner to enable muscle shortening ⁸. Muscle contraction is dictated by motor neurons, which innervate muscle fibers to form a motor unit. Upon motor cortex activation, motor units are activated, permitting the coordination of neural

transmission to muscle, ultimately generating force. Immediately following neural stimulation, depolarization of the muscle cell facilitates the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR) through ryanodine receptor 1 (RyR1). Ca^{2+} binds troponin C, which permits its movement and uncovering of myosin binding sites on actin. This enables contact between actin and myosin which subsequently induces cross bridge cycling ⁸.

As muscle is a post-mitotic tissue, it is reliant on resident satellite cells to permit muscle regeneration in response to injury ⁹. These quiescent myonuclei are located under the basal lamina and can contribute to the myonuclear pool to enhance muscle growth. It is important that myofibers are equipped with nuclei in succession to support the transcription and translation of target genes in a length wise manner. This maintains an optimal myonuclear domain, also known as the area a single myonucleus is responsible for, therefore supporting muscle function and adaptations ¹⁰. This requirement for protein synthesis is further highlighted when examining the composition of muscle, revealing that it is comprised of approximately 20% protein and accounts for 50-75% of the body's total protein reservoir ⁸. In addition to protein, muscle is also highly reliant on a constant influx of ATP from the mitochondria ¹¹.

1.2 SKELETAL MUSCLE MITOCHONDRIA: THE POWERHOUSES THAT KEEP US MOVING

1.2.1 MITOCHONDRIAL STRUCTURE

First discovered in 1856, Kolliker believed chondriosomes were the fundamental units of life ¹². Later renamed to “mitochondria” by Benda in 1898 ¹³, Bensley and Hoerr showed that mitochondria were distinguishable with a light microscope and could be separated from an organ with centrifugation in 1934 ¹⁴. However, it was not until the 1950s that intensive work was completed by Palade and Sjöstrand who independently examined mitochondrial structure ¹⁵. Palade established that the mitochondrion is completely enclosed within a membrane and contains internal

membrane protrusions, which he named “cristae”. He predicted that these folds bear enzymes in a linear series or “chain” for oxidative reactions. Concurrently, Sjöstrand performed more sophisticated imaging techniques (generating a different mitochondrial isolation protocol), allowing him to discover that the “cristae” are an arrangement of continuous internal double membranes, which originate from the inner bounding membrane. This seminal work on mitochondrial structure paved the way for Mitchell’s chemiosmotic theory of oxidative phosphorylation (OXPHOS) in 1961, which proposed that an electrochemical gradient is produced through the pumping of protons (H^+) across the inner mitochondrial membrane (IMM) into the intermembrane space (IMS), which can be utilized to produce ATP ¹⁶.

Today, considerable advancements in transmission electron microscopy (TEM) have provided important insight into mitochondrial organization and composition. Mitochondria contain their own genomic material, referred to as mitochondrial DNA (mtDNA) ¹¹. This characteristic is a remnant of the endosymbiotic theory, suggesting that eukaryotic mitochondria originated from the endocytosis of free-living proteobacteria over 1.5 billion years ago ¹⁷. mtDNA is a circular double stranded plasmid that is 16 kilobases (kB) long, encoding just 13 proteins out of the 1200 required for mitochondrial synthesis, or biogenesis. ¹⁸.

As postulated by Palade and Sjöstrand, mitochondria contain an outer mitochondrial membrane (OMM), which is permeable to small molecules (<10 kDa), meaning the composition of the IMS is very similar to the cytosol ¹⁹. Moving inwards, the IMM separates the mitochondrial matrix from the IMS and is much more selective than the OMM. The cristae house the OXPHOS subunits, and increases the surface area for aerobic respiration, indicating that cristae density is proportional to aerobic respiration capacity. The IMM also regulates the import of proteins into the matrix. Since over 99% of mitochondrial proteins are nuclear-encoded, nuclear genes encoding

mitochondrial proteins (NuGEMPs) must be transcribed and subsequently translated in the cytosol before entry into the mitochondria ^{11,20}. Proteins destined for the mitochondria contain a mitochondrial targeting sequence (MTS), which is recognized by cytosolic chaperones such as heat shock protein 90 (HSP90) and 70 (HSP70) ^{11,20}. These chaperones unfold and guide nuclear proteins to the mitochondrial protein import machinery (PIM), including the intricate translocase complexes TOM and TIM, found on the OMM and IMM respectively ^{11,20}. An important NuGEMP is mitochondrial transcription factor A (TFAM), which has been heavily implicated in mitochondrial biogenesis ^{21,22}.

While mitochondria are conventionally represented as singular bean-like organelles, their morphology varies dramatically based on the tissue they reside in. Within skeletal muscle, mitochondria can fuse together to form a complex interconnected network ²³. Pivotal work by Yamasaki and Brooks revealed this distinct morphology, coining this arrangement as a “mitochondrial reticulum” ^{24,25}. Morphology is largely dictated by the location of the mitochondria, generally subdividing them into two categories, subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, which exhibit different functions and adaptations to exercise due to their locations ^{23,26,27}. Phenotypically, SS mitochondria are relatively simple and circular, whereas IMF mitochondria are elongated and more reticular-like ^{23,27}. SS mitochondria are found below the sarcolemmal membrane (i.e., muscle cell membrane) and are important for nuclei and membrane function, whereas IMF mitochondria are located within the myofibrils to provide energy for contraction locally ^{23,26,27}.

1.2.2 MITOCHONDRIAL FUNCTION: ENERGY PRODUCTION AND BEYOND

1.2.2.1 RESPIRATION AND ROS

It has been established that mitochondria are important rheostats of cellular metabolism,

intimately involved in several intracellular signaling cascades. First, and most importantly, mitochondria are the site of ATP production through the process of OXPHOS as outlined by Mitchell in 1961¹⁶. This involves coupling macronutrient oxidation to ATP synthesis using ETCs that reside in the IMM. Briefly, oxidation of carbohydrates and lipids results in the reduction of co-factors flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD⁺) producing FADH₂ and NADH respectively through the Krebs' cycle²⁸. NADH is oxidized at Complex I (NADH dehydrogenase), leading to the release of 2 electrons and simultaneous pumping of 2H⁺ from the matrix into the IMS²⁸. Complex I is the largest complex in the ETC and contains a series of iron-sulfur clusters that enable rapid electron (e⁻) transfer through a sequence of redox reactions^{28,29}. These electrons are transported to Complex III (cytochrome b-c1) through the e⁻ shuttle ubiquinone (UB), therefore reducing Complex III, and oxidizing UB, allowing it to become available again to shuttle more electrons. Similarly, FADH₂ is oxidized at Complex II (succinate dehydrogenase), and 2e⁻ are moved to Complex III via UB. However, unlike Complex I, H⁺ pumping does not occur at Complex II^{28,29}. At Complex III, e⁻ movement is coupled to the translocation of H⁺, and subsequent movement of electrons down to Complex IV (COX) through cytochrome c, another mobile electron carrier. Complex IV is the last e⁻ acceptor and holds molecular oxygen (O₂) in place which is reduced to H₂O when combined with incoming electrons. The continuous translocation of H⁺ into the IMS results in the production of a protonmotive force (PMV), which encompasses two important forces, including a membrane potential ($\Delta\Psi_m$) and a difference in pH (ΔpH), which is the result of an accumulation of acidic, positively charged H⁺ across the IMM. This generates a negative mitochondrial matrix (roughly -150mV) relative to the cytoplasm (-80mV), and an electrochemical gradient that can be utilized by Complex V (ATP synthase) to synthesize ATP²⁸⁻³¹. H⁺ passes through Complex V, which triggers the physical

rotation of the rotor subunit in Complex V, and subsequent addition of inorganic phosphate (Pi) to adenine diphosphate (ADP).

Free ADP is produced as a by-product of ATPases activity, and is transported into the matrix through the adenosine nucleotide carrier (ANC), which is the rate limiting substrate for ATP production ³⁰. Hence, reductions in ATP hydrolysis reduce ADP availability and ETC function. When ADP is unavailable, H⁺ is unable to enter the matrix via Complex V, placing a backpressure on the ETC due the continuous pumping of H⁺ into the IMS. This slows e⁻ movement, and encourages their leakage from the ETC, especially at Complexes I and III ^{32,33}, with Complex III being able to release this e⁻ to both the matrix and IMS ³⁴. Once released, electrons can combine with O₂ to produce Reactive Oxygen Species (ROS) ^{32,33}. However, uncoupling proteins (UCPs) are found in the ETC, and work to dissipate the H⁺ gradient as heat to help relieve backpressure in the absence of ADP ^{29,30}.

O₂ consumption and mitochondrial ROS production provide meaningful methods to directly measure mitochondrial function. *In vitro*, O₂ consumption can be measured in living cells or isolated mitochondria with specific complex inhibitors and uncouplers. From this assay, an O₂ consumption rate (OCR) tracing is produced, permitting the measurement of respiration parameters by examining the area under the curve. Briefly, basal respiration is measured, followed by Oligomycin, a Complex V inhibitor to measure ATP linked O₂ consumption. Carbonyl cyanide-p-trifluoromethoxy phenylhydrazone (FCCP), an uncoupler that dissipates the PMF, permits the measurement of maximal OCR. Lastly, Rotenone and Antimycin A are administered to inhibit Complexes I and III respectively, demonstrating spare respiratory capacity. Other functional measurements that can be made include the assessment of $\Delta\Psi_m$, since its presence is indicative of proper ETC function ³¹.

1.2.2.2 OTHER MITOCHONDRIAL FUNCTIONS

While respiration is a primary function, mitochondria are in constant communication with the cytosol to mediate Ca^{2+} handling, cell death, propagate inflammation and maintain redox homeostasis. Mitochondria are capable of taking up cytosolic Ca^{2+} to preserve optimal intracellular Ca^{2+} levels ³⁵. While an influx of Ca^{2+} into the mitochondria has been shown to increase ETC and Krebs cycle activity ^{35–37}, much higher concentrations have been linked to mitochondrial swelling which can lead to mitochondrial-mediated cell death ^{35,37,38}. This results in the opening of the mitochondrial permeability transition pore (mPTP), permitting the release of pro-apoptotic factors such as endonucleases and cytochrome C, which can cleave DNA and propagate apoptosome formation respectively ³⁸. Opening of the mPTP can enhance the release of mtDNA, which acts as a damage associated molecular pattern (DAMP), recognized by DNA sensing pathways such as cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) ^{39,40}. This can lead to the downstream activation, synthesis and assembly of the NLRP3 inflammasome, a multi-subunit complex involved in innate immune signaling ⁴⁰. Furthermore, ROS accumulation is often associated with inflammation which can be mediated through mitochondrial dysfunction ⁴⁰.

2.0 MITOCHONDRIAL ADAPTATIONS TO EXERCISE

As mentioned earlier, skeletal muscle is a highly labile tissue that can modify its phenotype in response to the demand of exercise. This unique characteristic is what permits the beneficial adaptations observed both in locally in muscle and systemically following training, making it an important area of research for human health. Importantly, this metabolic flexibility is largely governed by the complex mitochondria that reside within it.

It is well recognized that exercise is a potent stimulus capable of activating thousands of signaling cascades that ultimately elicit robust changes in mitochondrial function and volume. Pivotal work by Holloszy demonstrated that training enhances mitochondrial biogenesis and increases aerobic respiration ⁴¹. However, these adaptations can only be achieved by training at an appropriate frequency, duration, and intensity ⁴¹, indicating that there is a time-dependent effect of training adaptations. Indeed, it has been established that significant changes in gene expression occur during the recovery period following an acute bout of exercise, which later result in protein changes and increases in mitochondrial content ¹¹. While roughly 6 weeks of endurance training is typically required to observe an increase in mitochondrial volume ¹¹, this phenomenon can be artificially modelled *in vitro* using a myotube contractile model. A more detailed description of this model is found in a later section (see section 2.4).

2.1 SIGNALING TOWARDS MITOCHONDRIAL BIOGENESIS

Exercise elicits mitochondrial biogenesis through the activation of secondary messengers such as AMP, ROS, and Ca^{2+} . Upstream signaling kinases specific to each secondary messenger sense this energetic imbalance, and collectively propagate the activation and nuclear translocation of transcription factors involved in the expression of NuGEMPs. These early signaling pathways occur rapidly in response to acute exercise to drive biogenesis, however they are blunted with training as exercise-induced adaptations are achieved.

2.1.1 AMPK

Muscle contraction is an active process that requires copious amounts of ATP. Accelerated ATP hydrolysis results in an increase in the ratio of adenosine monophosphate (AMP):ATP, which is subsequently recognized by AMP-Kinase (AMPK) ^{11,42}. AMPK is a heterotrimeric energy sensing kinase, that functions as a central regulator of cellular homeostasis ⁴². AMPK is comprised

of alpha (α), Beta (β) and gamma (γ) subunits. Mechanistically, the α -subunit contains a Ser/Thr kinase domain, which facilitates the phosphorylation of downstream targets, the γ subunit contains regulatory binding sites, and the β subunit acts as a scaffold, connecting the α and β subunits together ^{42,43}. AMP has been reported to bind directly to the γ subunit, resulting in a conformational change that is transduced to the alpha subunit, thereby enhancing kinase activity by 10-fold ^{44, 42}. While all adenine nucleotides (i.e., ATP, ADP, AMP) can competitively bind to 3 sites on the γ subunit ⁴⁵, AMP competes with a much higher affinity than ATP, therefore significantly increasing the sensitivity of AMPK to changes in AMP ⁴⁵.

This mechanism of action has been validated through the use 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMP analog which directly binds and activates AMPK in muscle ^{46,47}. AMPK activity has direct consequences on mitochondrial biogenesis, through the phosphorylation of Peroxisome-proliferator-activated receptor γ co-activator 1 α (PGC-1 α), which is commonly denoted the master regulator of mitochondrial biogenesis ⁴⁸. This leads an accumulation of PGC-1 α protein ^{46,47}, which subsequently translocates to the nucleus to enhance the expression of NuGEMPs. Specifically, PGC-1 α co-activates the transcription factor (TF) Nuclear respiratory factor-1 (NRF-1), another AMPK target ⁴⁹, which enhances TFAM expression. AMPK activity has also been linked to mitochondrial function, with muscle-specific KO of AMPK α subunits reducing mitochondrial respiration and exercise performance ⁵⁰. The role of AMPK in mitochondrial function is an active area of work, with new evidence suggesting that AMPK physically associates with the mitochondrial reticulum which may assist in maintaining energetic homeostasis during exercise ⁵¹.

In addition to allosteric activation via AMP, other modes of AMPK activation include direct phosphorylation, protection against dephosphorylation, and ROS. Indeed, binding of AMP

to AMPK significantly reduces protein phosphatase 2 (PP2)-mediated dephosphorylation (PP2) at Thr 172 ⁵², therefore sustaining pro-longed AMPK activity. The two main kinases that mediate AMPK phosphorylation in response to muscle contraction are Ca²⁺/calmodulin-dependent protein kinase (CAMK) and Liver kinase B1 (LKB1). Additionally, AMP binding has been reported to enhance LKB1-mediated AMPK phosphorylation ⁴⁴, revealing a potential positive feedback loop. Lastly, ROS have been shown to directly activate AMPK through the oxidation of cysteine residues on the α subunit, subsequently increasing kinase activity ^{53,46}. This activation is independent of AMP levels, with activation preceding changes in energy levels ⁵³. Together, this suggests that exercise-mediated ATP turnover, activation of upstream kinases, and ROS emission serve as AMPK activators which drive mitochondrial biogenesis.

2.1.2 P38 MAPK

As mentioned earlier, muscle contraction elicits an increase in ROS production. At low concentrations, ROS serve as important signaling molecules that can induce the activation of Mitogen activated protein kinase (MAPK) cascades. Importantly, this cascade can result in the activation of p38 mitogen-activated protein kinase (p38 MAPK), a stress inducible Ser/Thr kinase that can regulate PGC-1 α at the transcriptional and post-translational level. Following just 4 hours of H₂O₂ treatment in C2C12 myotubes or 3 hours of voluntary wheel running, there was a significant increase in p38 phosphorylation ^{54,55}. This resulted in the activation of activating transcription factor-2 (ATF-2), cyclic adenosine monophosphate (cAMP) response element (CRE)-binding protein (CREB), and myocyte enhancer factor 2 (MEF2), all of which bind to and enhance PGC-1 α promoter activity ⁵⁵.

p38 directly phosphorylates PGC-1 α , enhancing its stability and co-activation activity ⁵⁶. This mechanism is exemplified when examining an increase in PGC-1 α and COX IV, a nuclear-

encoded subunit of the ETC ⁵⁵, in response to p38 overexpression (OE). Conversely, p38 KO in skeletal muscle blunts exercise-induced mitochondrial biogenesis, suggesting that p38 signaling is required for maximal exercise-induced mitochondrial adaptations ⁵⁷. However, it is important to note that biogenesis is not completely ablated in response to p38 KO, suggesting that redundant signaling cascades exist to continue driving biogenesis.

2.1.3 CAMK

It is now well established that Ca^{2+} is a strong driver of mitochondrial biogenesis in muscle ¹¹. Elevations in cytosolic Ca^{2+} induce the activation of Ca^{2+} sensitive kinases and phosphatases, which ultimately transduce their signal to the nucleus to alter gene expression ¹¹. Increased cytosolic Ca^{2+} enhances mitochondrial-mediated Ca^{2+} uptake, which directly targets OXPHOS reactions. Indeed, 3 Krebs' cycle enzymes are Ca^{2+} sensitive, requiring Ca^{2+} for full activity ^{11,58}. It has also been proposed that the ANC ⁵⁹ and ATP synthase ⁶⁰ are Ca^{2+} sensitive, suggesting that Ca^{2+} may have a direct effect on ATP synthesis, persisting long after Ca^{2+} is removed ⁶¹.

There also appears to be a time and concentration-dependent increase in mitochondrial-related genes in response to Ca^{2+} in muscle cells, in part due to kinase activation upstream ⁶². Mechanistically, Ca^{2+} binds to the protein calmodulin (CaM), thereby inducing a global conformational change that exposes CaM binding domains ⁶³. CaM then binds and activates CaMKs, a family of Ser/Thr kinases, that can phosphorylate a plethora of downstream substrates. Similarly, protein phosphatase calcineurin A (CnA), a Ca^{2+} -sensitive phosphatase, is activated upon binding to Ca^{2+} /CaM ⁶³, and is involved in propagating PGC-1 α transcriptional activity (this will be further discussed in section 2.1.4.1).

Substantial work has corroborated that Ca^{2+} -mediated PGC-1 α activity directly enhances mitochondrial biogenesis, evident through an increase mitochondrially-encoded genes, mtDNA replication and ETC subunits ^{62,64–67}. Independent of PGC-1 α transcription, Ca^{2+} enhances PGC-1 α activity through CAMK-mediated AMPK phosphorylation ⁶⁸, together suggesting that Ca^{2+} is a strong driver of biogenesis.

2.1.4 PGC-1 α

Though TFs bind directly to DNA, they often lack the ability to remodel chromatin and recruit RNA polymerase for gene expression ⁶⁹. Hence, transcriptional co-activators are required, forming a multiprotein complex with the target TF to enhance transcription ⁶⁹. One of the best studied group of co-activators which has emerged as regulators of cellular function is PGC-1. Although PGC-1 does not contain its own histone acetylase transferase (HAT) activity, it binds several proteins with HAT activity which subsequently remodel chromatin and recruit components of the pre-initiation complex (PIC) ^{70,71}. This action displaces repressors such as histone deacetylases (HDACs) to induce gene activation ⁷⁰.

This small family of transcriptional co-activators is made up of PGC-1 β , PGC-1 α , and PGC-1-related coactivator (PRC), all of which co-activate TFs involved in OXPHOS gene expression. From this group, PGC-1 α is the best characterized and has reported as highly inducible upon both acute and chronic exercise within skeletal muscle ⁷². Indeed, several gain of function studies have revealed that PGC-1 α plays an important role in exercise-stimulated mitochondrial biogenesis. Early work by Spiegelman demonstrated that PGC-1 α OE enhanced mitochondrial biogenesis, volume, and respiration in C2C12 myotubes ⁷³ and skeletal muscle ⁷⁴, accompanied by the formation of slow-twitch fibers which are rich in mitochondrial content ⁷⁴. Conversely, PGC-1 α ablation mildly reduced mitochondrial gene expression and respiration, indicating PGC-1 α is

somewhat required for mitochondrial maintenance basally, however other family members may be able to compensate for this loss ^{71,75,76}.

While PGC-1 α is clearly an integral responder to exercise, with several signaling kinases converging onto this coactivator via phosphorylation, it may not be required to achieve mitochondrial adaptations following exercise. Whilst untrained PGC-1 α KO mice exhibit lower mitochondrial proteins, training induced a similar fold increase in mitochondrial-related proteins, suggesting that PGC-1 α is not mandatory for training-induced adaptation, despite its requirement to some degree basally ^{76,77}. It is however required in aged muscle ⁷⁸, where mitochondrial adaptations cannot occur following training without PGC-1 α ⁷⁹.

2.1.4.1 TRANSCRIPTIONAL REGULATION OF PGC-1 α

In response to exercise-induced signaling, PGC-1 α transcription is enhanced by up to 10-fold in muscle, peaking approximately 2hrs post exercise ⁸⁰. As previously mentioned, this increase in mRNA is mediated by upstream signaling kinases, such as CaMK, which phosphorylate CREB, permitting its nuclear translocation ⁶³. Characterization of the PGC-1 α promoter has revealed CRE binding sites, indicating CREB may be an important regulator of PGC-1 α transcription ⁴⁶. Furthermore, while OE of CaMK enhanced PGC-1 α mRNA by 3-fold, this effect was completely ablated when combined with CREB KD, demonstrating CREB is a downstream target and major effector of CaMK-mediated PGC-1 α promoter activity ⁶⁴.

CnA also appears to be important for PGC-1 α transcription, since CaMK OE coupled with CnA OE enhanced PGC-1 α promoter activity by 7-fold ⁶⁴. This additive effect arose due to nuclear factor of activated T cell (NFAT) and MEF2, which are downstream targets of CnA. Upon dephosphorylation, these TFs enter the nucleus and bind to the promoter region of PGC-1 α ⁶⁴. While on its own, MEF2 did not affect PGC-1 α transcription, when combined with PGC-1 α ,

promoter activity was enhanced by 4-fold, suggesting that PGC-1 α co-activates MEF2 to enhance its own transcription through an autoregulatory loop ⁶⁴.

AMPK further increases PGC-1 α transcription by enhancing the binding of Upstream stimulatory factor-1 (USF-1) to enhancer box (E-box) elements found in the promoter region of PGC-1 α . This subsequently drives promoter activity, leading to an increase in PGC-1 α mRNA ⁴⁶. Interestingly, this action was not accompanied by an increase in USF-1 protein, indicating the increased binding may be due to enhanced recruitment and not USF-1 transcription. USF-1 OE also enhances PGC-1 α promoter activity, suggesting that USF-1 regulates PGC-1 α transcription independent of AMPK ⁴⁶.

P38 and JNK enhance the phosphorylation of ATF-2, another CRE binding TF which has been shown to coordinate the expression of PGC-1 α by directly binding the promoter region ^{57,81}. ATF2 KO reduced PGC-1 α expression by 40%, suggesting that ATF-2 maintains PGC-1 α transcription basally ⁸². GC rich areas in the promoter region of PGC-1 α have also been identified as a binding site for Specificity protein 1 (SP1) ⁴⁶. Indeed, this protein is upregulated following contractile activity ⁸³, and directly binds to the promoter region of PGC-1 α , enhancing PGC-1 α transcription ⁸⁴, as well as other mitochondrially encoded targets, such as cytochrome C ⁸³, signifying its importance for exercise-induced mitochondrial biogenesis.

Tumour suppressor protein p53 has also been established as a potent TF that drives transcriptional events observed with exercise. P53 is a stress response factor, famously known for inducing senescence and apoptosis, however it is now established that p53 is involved in mitochondrial biogenesis and maintenance ⁸⁵. Both AMPK and P38 MAPK have been shown to phosphorylate and activate p53, implicating it in exercise-induced signaling cascades. Following an acute bout of exercise, p53 has been reported to translocate to the nucleus and mitochondria to

induce its effects. In the nucleus, p53 binds to the promoter region of PGC-1 α in response to mild oxidative stress⁸⁶. However, it is important to note that PGC-1 α upregulation was not accompanied by mitochondrial biogenesis, instead there was an increase in antioxidant signaling mediated by the TF nuclear factor (erythroid derived 2)-like-2 (Nrf-2), suggesting that a PGC-1 α -Nrf-2 axis may exist to mitigate oxidative stress. While oxidative stress is a hallmark of acute exercise, p53 exhibits a significantly different effect following an acute bout of exercise. Indeed, p53 translocates to the mitochondria to form a complex with TFAM subsequently enhancing mtDNA replication⁸⁷. This mechanism is further illuminated when examining p53 KO mice, which were unable to display an increase in COX I, a mtDNA-encoded protein, following exercise⁸⁷, suggesting that the subcellular localization of p53 is highly dependent on the entire cellular milieu.

2.1.4.2 PROTEIN STABILITY OF PGC-1 α

Like other proteins, PGC-1 α is regulated by post-translational modifications (PTMs) which confer its stability and subsequent nuclear action. These modifications are particularly important for PGC-1 α , since it has been deemed an intrinsically disordered protein that is “degraded by default” by the 20S proteasome⁸⁸. However, NADPH quinone oxidoreductase (NQO1), a downstream target of Nrf-2 has been shown to bind to PGC-1 α and protect from its 20S-proteasome-mediated degradation, indicating that activation of Nrf-2 may help propagate PGC-1 α action⁸⁸. PGC-1 α protein has an extremely short half-life (around 30 minutes), quickly returning back to pre-exercise levels when physical activity stops⁸⁰. In aged muscle this half-life is reduced even further, indicating that these PTMs may become increasingly important with age to support biogenesis⁷⁸. Trained muscle exhibits a persistent elevation of PGC-1 α between bouts, demonstrating the adaptability of this protein to chronic exercise⁸⁹. The most prominent PTMs which helps impart this stability is its phosphorylation and acetylation status. AMPK

phosphorylates PGC-1 α at Thr177 and Ser538 ⁴⁸, while P38 MAPK phosphorylates PGC-1 α on Thr262, Thr298 and Ser265, resulting in its stabilization and transcriptional co-activation activity ⁵⁶. Deacetylation by Sirtuin 1 (SIRT1) has also been shown to enhance PGC-1 α activation ⁹⁰, as PGC-1 α is heavily acetylated by the acetyltransferase, general control non-repressed 5 protein (GCN5) ⁹¹. SIRT1 is a NAD⁺-dependent deacetylase, indicating SIRT1 activity is regulated by metabolic status ⁸¹. SIRT1 OE has been shown to increase PGC-1 α transcription by positively regulating myogenic determining factor (MyoD)-mediated PGC-1 α transcription, suggesting that energy status is a major driver of PGC-1 α transcription and activation ⁹².

2.2 REACTIVE OXYGEN SPECIES

It was first reported almost 50 years ago that pro-longed endurance exercise is associated with oxidative damage ⁹³, however it was not until 4 years after that discovery, work by Lester Packer's group revealed that contracting muscle produce ROS ⁹⁴ thereby promoting oxidative stress ⁹⁵. "Oxidative stress" was first defined by Helmut Sies 3 years later, describing this phenomenon as a disturbance in the pro and anti-oxidants, favouring pro-oxidation ⁹⁵. However, this definition has since been criticized due to its simplicity and was later revised by Sies and Dean Jones, further elaborating on how oxidative stress disrupts redox signaling which can permit cellular damage ⁹⁶. While this definition will undoubtedly be modified as the field of redox biology advances, it has been generally accepted and adopted by muscle physiologists when describing oxidative stress ⁹⁷.

2.2.1 SOURCES AND SITES OF ROS PRODUCTION

ROS production occurs at many sites in muscle, including the mitochondria, SR, transverse tubules, and sarcolemma ⁹⁷. However, the specific sources of ROS which have been investigated include the ETC, NADPH Oxidase (NOX) enzymes, and phospholipase A2 (PLA₂) ⁹⁷. While

earlier studies often recognize mitochondria as the largest contributor to ROS production during exercise, newer studies have revealed that mitochondria produce more ROS basally (state 4) in comparison to ADP stimulated respiration (state 3), suggesting that other sources may be responsible for the elevation in ROS observed during contraction ^{98,99}. NOX enzymes have since been identified as the prominent contributor of superoxide ($O_2^{\bullet-}$) production during both exercise and rest ^{100,101}. NOX are membrane bound enzymes, which are found in the SR, transverse tubules, mitochondria, and sarcolemma ⁹⁷. Skeletal muscle expresses 2 isoforms (NOX2, NOX4), with NOX2 being found in the sarcolemma, and T-tubules ¹⁰², and NOX4 within the SR ¹⁰³ and mitochondria, due to the presence of a MTS found within the N-terminus ¹⁰⁴. While NOX4 is constitutively active, thereby contributing to basal ROS production ^{105,106}, NOX2 is inducible and is activated in response to mechanical force and stretching ¹⁰⁷. This subsequently facilitates the assembly of NOX regulatory subunits and translocation to the corresponding membrane, thereby permitting its activation ¹⁰⁷. Lastly, the PLA₂ pathway is another mechanism implicated in $O_2^{\bullet-}$ production ¹⁰⁸. PLA₂ catalyzes the cleavage of the ester bond linking glycerol to fatty acid chains in phospholipids, resulting in the production of arachidonic acid ¹⁰⁸. This fatty acid is subsequently utilized by several enzymes, including lipoxygenases (LOX) and cyclooxygenases (COX), which produce $O_2^{\bullet-}$ as a by-product of their activity ¹⁰⁹. PLA₂ exists as both Ca^{2+} -dependent (sPLA₂) and independent (iPLA₂) isoforms, which contribute to ROS production basally ¹¹⁰ and upon Ca^{2+} release ^{107,111}. PLA₂ is found in the cytosol and mitochondria, and has been shown to regulate both cellular and mitochondrial ROS production ¹¹². Upon Ca^{2+} uptake, mitochondrial PLA₂-dependent processes promote lipid peroxidation in the ETC, enhancing Ca^{2+} -mediated mitochondrial ROS emission ¹¹¹. The addition of arachidonic acid to the ETC stimulates ROS production, whereas the inhibition of PLA₂ blunts Ca^{2+} -induced ROS emission ^{111,113}. Furthermore, PLA₂ has been reported

to stimulate NOX assembly ¹¹⁴. Inhibition of cytosolic PLA₂ diminished the translocation of NOX regulatory subunits to membrane rich fractions, suggesting that PLA₂ may regulate NOX activity by enhancing the association of NOX subunits ¹¹⁴. Taken together, these findings suggest that cellular redox status is regulated by several key players which may work interdependently to mediate ROS production (Fig. 1).

2.2.2 ACUTE AND CHRONIC EXERCISE

Though still a relatively new topic, it is now widely accepted that exercise-induced oxidative stress damages tissue and increases muscle fatigability ^{97,115}. Naturally, it has become a booming area of research to determine how ROS operate to mediate tissue damage during exercise. At low amounts, ROS propagate signal transduction and gene regulation, yet at high levels, ROS can damage macromolecules, induce senescence, and even cell death ¹¹⁶. The intensity and duration of exercise have also emerged as major determinants of ROS production. Short duration, low intensity exercise [i.e., <1 minute, 30% maximal O₂ consumption (VO_{2max})] does not increase oxidative stress ¹¹⁷, whereas an acute bout of sustained, high intensity exercise enhances markers of oxidative stress ¹¹⁸. In terms of chronic muscle use, endurance training has been shown to mitigate contraction-induced oxidative stress observed after a single bout, through the upregulation antioxidant defense pathways. Interestingly, this protective effect is observed after both short (5 days) ¹¹⁹ and prolonged endurance training, suggesting that antioxidant proteins are quickly mobilized to reduce muscle damage.

2.2.3 MAJOR REACTIVE OXYGEN SPECIES

ROS is a broad term used to describe O₂ centered radicals or non-radical derivatives. While all ROS share this characteristic, they differ greatly in their half-life, oxidizing potential, and action. O₂•⁻ is the most abundant O₂ centered radical within cells ¹²⁰, and can be generated through

the incomplete reduction of O_2 in the ETC or as a by-product of enzymatic reactions. The negative charge of $O_2^{\bullet-}$ renders it impermeable to membranes, which helps contain its action, but $O_2^{\bullet-}$ has a reasonably long half-life (in comparison to other radicals) which can increase the number of substrates it can target ¹²⁰. While $O_2^{\bullet-}$ is relatively unreactive, it is highly unstable and can become protonated to produce hydroperoxyl radical ($HO_2^{\bullet}$) which may permit its movement across membranes ¹²¹. However, if this action occurs at physiological pH remains debated, suggesting this may be possible only during exercise when H^+ is abundant.

Hydrogen peroxide (H_2O_2) is highly stable, has a long half-life and is permeable to plasma membranes ¹²⁰. While these properties deem H_2O_2 destructive, H_2O_2 has a relatively low oxidizing potential which diminishes the amount of damage it can cause ¹²⁰. H_2O_2 is unable to oxidize DNA, proteins, and lipids, however the hydroxyl radical ($OH^{\bullet}$), a derivative of H_2O_2 , has robust oxidizing potential ^{97,120}. $OH^{\bullet}$ are also the most damaging ROS, and are not membrane permeable, thereby oxidizing nearby material due to their high reactivity ¹²⁰.

2.2.4 CELLULAR ANTIOXIDANT DEFENSE SYSTEMS

Skeletal muscle cells are equipped with various antioxidant strategies to regulate ROS in a well-organized manner to dampen metabolic disturbance and mitigate tissue damage. These antioxidants exist as both enzymes and non-enzymatic molecules that are cleverly placed in various localizations to locally reduce ROS.

2.2.4.1 ANTIOXIDANT ENZYMES

The 3 major antioxidant enzymes involved in the reduction of ROS are superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) ¹²². SOD exists as 3 isoforms, found in various cellular compartments to reduce $O_2^{\bullet-}$ into H_2O_2 ¹²². SOD1 resides within the cytosol and IMS, SOD2 within the mitochondrial matrix, and SOD3 within the extracellular space

¹²². SOD2 distribution within the mitochondria is particularly important, since removing $O_2^{\bullet-}$ at the site of its production helps protect mtDNA and proteins that are in proximity ¹²². Indeed, mtDNA is not protected by histones and undergoes less efficient DNA repair upon damage, highlighting the importance of antioxidant systems for mitochondrial preservation ¹²³. Though $O_2^{\bullet-}$ is a relatively poor oxidizer, its removal ensures other more reactive derivatives (e.g., $OH^{\bullet}$, $ONOO^-$) are not produced ^{120,122}. SOD activity is highest within oxidative muscle (rich in type 1 and 2a fibers), though it may also be modified in response to exercise ^{124,125}. Many groups have shown that both SOD1 and SOD2 activity is enhanced in endurance trained muscle ^{124-128,133}, however it is important to note others have reported no change ¹²⁹⁻¹³¹. SOD activity also appears to be intensity and duration-dependent, with longer and more intensely trained muscle exhibiting higher activity ^{124,132}.

GPXs are a family of enzymes that mediate the reduction of H_2O_2 into H_2O ⁹⁷. To perform this action, GPX requires glutathione (GSH) in its reduced state to donate $2H^+$ which consequently oxidizes GSH into glutathione disulfide (GSSG). GSSG is reduced back to GSH by glutathione reductase (GR), an NADPH-dependent enzyme that facilitates GSH regeneration ⁹⁷. NADPH is produced primarily through glucose 6 phosphate dehydrogenase (G6PD), the terminal enzyme in the pentose phosphate pathway. NADPH is an important co-factor for other antioxidant enzymes as well ¹³⁸, namely, thioredoxin reductase (TR), an enzyme that reduces thioredoxin (Trx), a thiol specific antioxidant which reduces oxidized protein ⁹⁷. Similar to SOD, GPX activity is highest in oxidative fibers ^{124,128,131} and is enhanced following endurance training in an intensity and duration-dependent manner ^{124,125,127,128,131}. This is accompanied by an increase in GR ^{129,134}, suggesting that training induces a higher capacity for GPX-mediated ROS removal.

Like GPX, CAT is another enzyme that reduces H_2O_2 and can be found in various cellular compartments, including the cytosol and mitochondria ¹³³. However, GPX and CAT vary considerably in terms of substrate specificity and mechanism of action ¹³³. For example, CAT has a much lower affinity for H_2O_2 at low concentrations, suggesting that GPX may be the principal mediator of H_2O_2 removal under physiological conditions ^{95,133}. Furthermore, CAT does bind NADPH, which confers it protection from radicals ¹³⁵, however it is not required to perform its reductive role ¹³⁶. CAT exclusively reduces H_2O_2 , while GPX can reduce H_2O_2 derivatives (e.g., hydroperoxide), demonstrating the selectivity for each enzyme ¹³³. CAT activity is also highest in oxidative fibers, consistent with other antioxidants ^{124,128,131}, however its expression in response to training has been quite controversial. Indeed, some groups have shown CAT activity is increased ^{119,137}, unchanged ¹²⁴ or reduced ^{124,131,138} following endurance training (Fig.1).

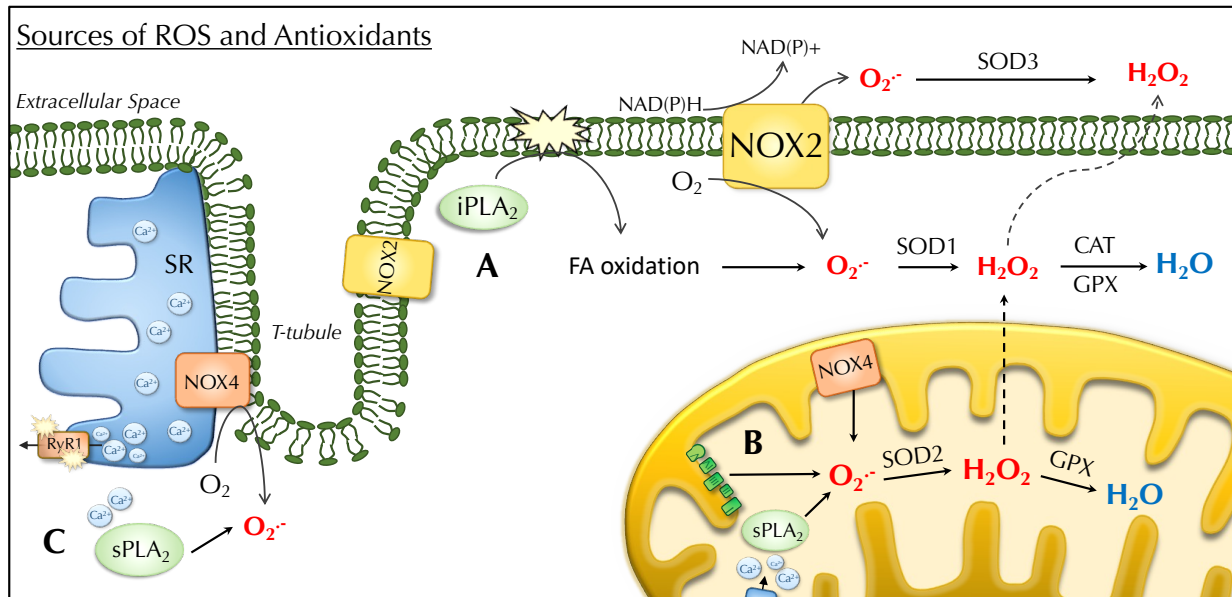


Figure 1: Sources of reactive oxygen species (ROS) and the antioxidants that reduce them. A) The NADPH oxidase (NOX) enzyme NOX2 is embedded within the plasma membrane and T-tubules, where it mediates contraction-induced superoxide ($O_2^{\bullet-}$) formation. Phospholipase A₂ (PLA₂), exists as both Ca^{2+} -dependent (sPLA₂) and independent (iPLA₂) isoforms, which contribute to cellular $O_2^{\bullet-}$ production. $O_2^{\bullet-}$ can be reduced into H_2O_2 by superoxide dismutase (SOD) and eventually H_2O by Glutathione peroxidase (GPX) or catalase (CAT). **B)** Mitochondria contain NOX4, which contributes to mitochondrial $O_2^{\bullet-}$ production basally. In addition to $O_2^{\bullet-}$ emission from the electron transport chain (ETC), sPLA₂ is found within the mitochondrial matrix and contributes to $O_2^{\bullet-}$ production upon Ca^{2+} influx. **C)** sPLA₂-mediated ROS emission enhances oxidation of ryanodine receptor 1 (RyR1) within the sarcoplasmic reticulum (SR), which permits Ca^{2+} leakage and further activation of cytosolic sPLA₂'s.

2.2.4.2 NON-ENZYMATIC ANTIOXIDANTS

The effects of endurance training on non-enzymatic antioxidants in skeletal muscle have been significantly underexplored relative to antioxidant enzymes. However, it is important to appreciate the complexity and intersectionality between both classes of antioxidants to combat oxidative stress. Important non-enzymatic antioxidants that will be discussed include GSH, Vitamin E and C, and coenzyme Q₁₀ (ubiquinone).

The antioxidant which has garnered the most attention within this category is GSH. While GSH can be produced in muscle, it is primarily synthesized within the liver and disseminated to periphery tissue through circulation¹³⁹. GSH content is higher in more oxidative fiber types^{128,140},

and has been reported to increase with endurance training ^{128,138,141}. This may be due to an increase in vascularization, facilitating greater uptake of circulating GSH, coupled with enhanced synthesis of GSH locally ¹⁴¹. While GSH serves as an e- donor for GPX activity, GSH can also directly reduce radicals (e.g., OH•) and Vitamin E by donating a H⁺, highlighting its ubiquitous in redox homeostasis ¹²⁰.

Dietary antioxidants Vitamin E (α -tocopherol) and Vitamin C (ascorbate) may further enhance protection from radicals and other forms of ROS. Vitamin E is a lipid soluble antioxidant, found in the membrane of the mitochondria, SR, and plasma membrane ¹³⁹. Vitamin E is an effective radical scavenger, consequently stabilizing and protecting membranes from oxidative damage ¹³⁹. This is particularly important with age, where membrane fluidity is increased, resulting in enhanced permeability and poor stabilization ¹⁴². Unlike Vitamin E, Vitamin C is a hydrophilic antioxidant, widely distributed within the cell to reduce superoxide's and Vitamin E back to its reduced state upon radical-mediated oxidation ¹⁴³.

Lastly, ubiquinone (coenzyme Q₁₀) is a noteworthy antioxidant to mention. Primarily known for its role as an electron carrier in the ETC, it possesses antioxidant effects by inhibiting lipid peroxidation due to its association with membranes ¹⁴⁴. Much of this antioxidant capacity is owing to its phenol ring, which provides a plethora of H⁺ to reduce ROS ¹⁴⁵. While almost 50% of ubiquinone exists within mitochondria, ubiquinone is also found in the nucleus, endoplasmic reticulum, and cytosol where it confers cellular protection ¹⁴⁶.

2.2.4.3 MEASURING CELLULAR REDOX STATUS

To evaluate the pro-oxidant environment within a cell, there are generally 4 broad categories that can be evaluated, which include the measurement of oxidants, antioxidants, oxidation products, and the balance between antioxidants and pro-oxidation ¹²⁰. Firstly, measuring

oxidants is the most direct method to conclude that ROS production is enhanced, however this can be difficult to assess due to the short half-life and instability of ROS. To address this limitation, specific dyes have been developed to interact with oxidants and produce a quantifiable fluorescent product with a relatively long half-life. Limitations to these dyes involve a disruption in normal function and cellular toxicity ¹²⁰. Antioxidants can also be evaluated to corroborates changes in oxidation. For example, GSH, vitamin C, and vitamin E are depleted upon oxidative stress ¹³⁹, whereas certain antioxidant enzymes are enhanced, such as GPX1 ¹⁴⁷. The product of oxidants can also be evaluated to draw conclusions about the substrates ROS are targeting. Protein oxidation can be assessed by measuring protein carbonylation ¹²⁰, while lipid peroxidation can be evaluated by measuring 4-hydroxyl-2-nonenol (4-HNE) and Malondialdehyde (MDA), which are both by-products of lipid peroxidation ¹⁴⁸. Finally, DNA damage can be assessed by evaluating oxidation of the guanine base, in the form of 8-hydroxy-2'-deoxyguanosine (8-OH-dG).

The last method is to assess the redox balance with the cell. This is most frequently completed by examining the ratio of oxidized and reduced glutathione ¹²⁰, however, the ratio of cysteines oxidation can also be measured, as cysteine residues in proteins are common substrates for ROS to attack. Upon oxidation, the unique thiol group (-SH) on cysteine residues form a disulfide bond (S-S) with a neighbouring cysteine, which can have consequences on protein structure and function ¹²⁰. Overall, there are several methods that can be employed to provide a robust understanding of cellular redox status.

2.2.4.4 ROS-MEDIATED SIGNALING: JNK

In response to acute exercise-mediated ROS production, upstream MAPK signaling cascades can activate Jun proto-oncogene (c-jun) N-terminal kinase (JNK), a downstream stress inducible kinase ¹⁴⁹. Activated as early as 15 minutes of exercise ¹⁴⁹, JNK can be stimulated by a

variety of stressors, such as the unfolded protein response (UPR) or pro-inflammatory cytokines, both which occur with an acute bout of exercise ⁶⁷. Interestingly, the downstream effects of JNK appear to be different depending on the training modality employed. In response to resistance exercise-induced mechanical stress, JNK signaling, which normally mediates atrophy related gene expression, is blunted thus supporting muscle hypertrophy ¹⁴⁹. Indeed, JNK phosphorylated the TF small body size and Mothers against decapentaplegic-related protein 2 (SMAD2), inhibiting its nuclear translocation and transcription of E3 ubiquitin ligases muscle ring FINGER (MuRF-1) and Atrogin-1 ¹⁵¹. Whereas with endurance training, JNK activation is diminished, thereby inhibiting muscle hypertrophy ¹⁴⁹. Together this suggests that JNK mediates distinct remodelling programs that alter muscle phenotypes in response to different exercise modalities.

JNK activation has also been implicated in the antioxidant response. JNK1 directly phosphorylates the transcription factors ATF-2 ¹⁵² and c-jun ¹⁵³ which heterodimerize to form the activator protein 1 (AP-1) complex. This in turn can bind to CRE and TRE sites found upstream of target genes GPX-1 and CAT, which both reduce cytosolic H₂O₂ ¹⁵⁴

2.3 MITOCHONDRIAL DYNAMICS

Mitochondria are frequently depicted as round structures, confined to a single subcellular compartment. Yet this classic illustration is generally an oversimplified and incomplete view of mitochondrial morphology in muscle. These organelles exist in a reticular formation, encompassing a large network of mitochondria that are physically bound and electrically coupled through a continuous membrane. EM imaging by Brian Glancy's group has shown that neighbouring mitochondria are connected by nanotunnels, which permit the formation of a reticulum and sharing of metabolites and substrates ^{23,155}. The linkage between mitochondria is

extremely dynamic, capable of changing within seconds ¹⁵⁶, hence the study of the everchanging mitochondrial morphology is referred to as mitochondrial dynamics.

2.3.1 FUSION

Mitochondria can change their morphology in response to an alteration in their environment by either fusing with adjacent mitochondria or becoming fragmented. Upon contractile activity-induced mitochondrial biogenesis, fusion processes occur to expand the pre-existing reticulum, thereby enabling more efficient substrate oxidation and O₂ consumption. A reticular formation is advantageous since neighbouring mitochondria can compensate for deficits by sharing resources and reducing diffusion time for O₂. This intermixing of mitochondrial material following endurance training was first observed by George Brooks, where he revealed enhanced fusion following training ¹⁵⁷. Fusion of the OMM and IMM are catalyzed by two major proteins, including two mitofusin isoforms (MFN1/2) and optic atrophy 1 (OPA1) respectively. MFN1 and MFN2 function in a GTPase-dependent manner, working to tether adjacent mitochondria and bring them into proximity. While structurally similar, these proteins facilitate distinctive functions. MFN1 is the major mediator of mitochondrial tethering whereas MFN2 may play more of a regulatory role ¹⁵⁸. Fusion of the inner membrane via OPA1 requires MFN1 but not MFN2, highlighting the divergent role of these two isoforms in fusion processes ¹⁵⁹. Lastly, OPA1 OE enhances tubular morphology, while ablation enhances fragmentation ¹⁵⁹.

2.3.2 FISSION AND MITOPHAGY

There are certainly advantages to a reticular morphology, however this can be a disadvantage when a portion of the network becomes damaged or is not functioning optimally. To mitigate any harm to the entire network, mitochondria can be physically segregated through fission processes and subsequently degraded. Indeed, TEM images have revealed intermitochondrial

junctions form to facilitate this separation ¹⁶⁰. Receptor proteins, such as fission protein 1 (Fis1), reside on the OMM and recruit dynamin related protein 1 (Drp1). Drp1 wraps around the mitochondrion and pinches off the organelle for subsequent removal through mitophagy, which is the selective removal of mitochondria through the autophagy machinery. Upon signaling towards mitophagy, PTEN-induced kinase 1 (PINK1) accumulates on the OMM and undergoes dimerization and autophosphorylation ¹⁶¹. While normally, translocated into the mitochondria and cleaved upon entry, PINK1 import is impaired upon mitochondrial stress, such as loss of $\Delta\Psi_m$, allowing it to serve as an extremely sensitive protein in mitochondrial status ¹⁶¹. PINK1 phosphorylates ubiquitin on the OMM ¹⁶², recruiting the E3 ubiquitin ligase Parkin ¹⁶³. PINK1 phosphorylates and activates Parkin, which subsequently ubiquitinates the OMM ¹⁶³. These ubiquitin chains are recognized by adaptor proteins such as p62, which bind to Microtubule-associated protein 1A/1B-light chain 3 (LC3-II), a protein which helps regulate cargo capture within the autophagosome ^{164,165}. When the mitochondrion is completely engulfed within the autophagosome, it travels to the lysosome fusing to form an autophagolysosome. Lysosomes contain hydrolases, including cathepsin B and D, which work to break down cellular cargo. These hydrolases require an acidic environment to function, which is established through the vacuolar-type ATPase (vATPase), utilizing ATP to pump H^+ against their gradient ¹⁶⁶. Ubiquitin independent mitophagy pathways also exist and can be employed for mitochondrion removal. Receptors such as Fun14 domain containing 1 (FUNDC1) ¹⁶⁷ and BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like (BNIP3L) ¹⁶⁸ have been reported to bind directly to LC3-II to facilitate mitochondrial degradation (Fig. 2).

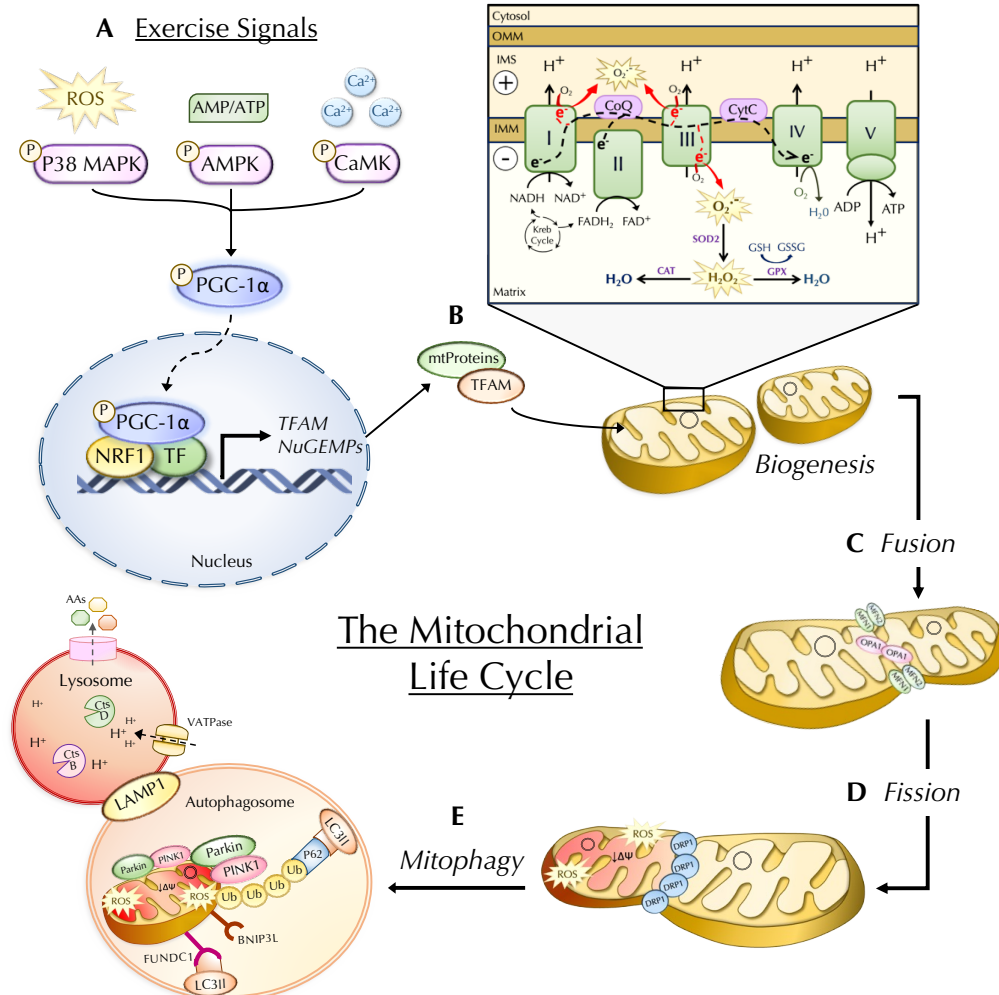


Figure 2: The mitochondrial life cycle and quality control processes during exercise. **A)** Contractile activity results in an increase in reactive oxygen species (ROS), AMP, and Ca^{2+} release, which subsequently activate upstream kinases. These kinases phosphorylate PGC-1 α , which co-activates transcription factors that drive the expression of nuclear genes encoding mitochondrial proteins (NuGEMPs), including nuclear respiratory factor-1 (NRF-1) which activates the transcription of mitochondrial transcription factor A (TFAM). **B).** TFAM binds mitochondrial DNA (mtDNA) and facilitates replication, whereas mitochondrial proteins are incorporated into the reticulum to facilitate expansion. Electron leakage may occur at Complexes I and III, which can combine with O_2 to form superoxide ($\text{O}_2^{\bullet-}$). $\text{O}_2^{\bullet-}$ can be reduced into hydrogen peroxide (H_2O_2) by superoxide dismutase 2 (SOD2), and finally H_2O by catalase (CAT) or glutathione peroxidase (GPX). **C)** New mitochondria fuse to the pre-existing network through the help of mitofusin 1/2 (MFN1/2) and optic atrophy 1 (OPA1). **D)** Dysfunctional mitochondria are pinched off through fission processes to target the organelle towards the lysosome for degradation. **E)** The mitochondrion is tagged and engulfed by an autophagosome before fusing to the lysosome to be recycled into its cellular constituents, such as amino acids (AA). Mitochondria can be tagged with ubiquitin (Ub) via PTEN-induced kinase 1 (PINK1)-Parkin-mediated mechanisms, or by binding directly to LC3-II through mitochondrial receptors BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like (BNIP3L) or Fun14 domain containing 1 (FUNDC1).

2.4 MYOTUBE CONTRACTILE MODEL

Regular exercise is one of the best interventions currently available to enhance overall quality of life and prevent disease. However, it is often difficult to delineate the pathways that are activated in response to contractile activity due to whole-body involvement. Therefore, an *in vitro* model of exercise using C2C12 myotubes can be employed to study conserved biological processes within muscle exclusively. Electrical pulse stimulation (EPS) can be applied to model motor neuron activity, thereby permitting muscle contraction which can be readily observed under a light microscope. EPS is an established model of exercise for *in vitro* studies due to the many advantages to this system^{83,169–179}. Importantly, this model generates a highly controlled closed system which allows the experimenter to modify environmental aspects, including O₂, temperature, fed state, exercise duration, and recovery period. This is particularly useful when the experimenter is interested in examining the effect of a certain drug during different stages of exercise. Another advantage is that both immortalized cell lines^{83,170,172–174,177} and primary cells retrieved from subjects can be used in this system¹⁷⁸. This may provide a valuable mode to study exercise when the subject is exercise intolerant^{180,181} or when an exercise study is implemented later, since cell stocks can be stored. Lastly, the electrical pulse generators that propagate the electrical current can transmit a wide range of electrical potential (volts), frequency (Hz), and intensity (amps). Due to this versatility, EPS has been used to model both resistance^{169,179,182} and endurance^{83,171,183} exercise by adjusting these parameters, making it a very flexible system to study different exercise modalities. For the purposes of this study, a model of endurance training was employed which has been previously verified to model endurance training adaptations including no hypertrophy, an increase in mitochondrial content, and activation of conserved pathways consistent with acute exercise^{47,83,183–185}. However, a major limitation to this model which must be acknowledged is that

it may not be the most physiologically relevant model. This is due to the lack of whole-body factors (i.e., hormones, cytokines,), simplified environmental model (i.e., one cell type), and no account for changes in O₂ delivery via vascularization in muscle.

3.0 THE NRF-2 KEAP1 PATHWAY

ROS are important molecules have been implicated in the progression of various conditions, including musculoskeletal diseases, aging, and diabetes. Therefore, it is imperative that protective mechanisms are in place to reduce cellular susceptibility to oxidative damage and blunt muscle atrophy. While many redox sensitive antioxidant pathways exist to mitigate oxidative stress, this review will focus on the Nrf-2 pathway, and its regulator kelch-like ECH-associated protein 1 (Keap1). The Nrf-2-Keap1 axis is a stress inducible pathway that mediates the expression of over 1000 cytoprotective genes ¹⁸⁶. This axis was first thoroughly described in 1997, which demonstrated that Nrf-2 is a TF that binds to *cis* elements, denoted Antioxidant response elements (AREs) found upstream of target genes ¹⁸⁷. Further examination of the structural domains of Nrf-2 revealed that the Nrf-2 ECH homology (Neh)-2 domain interacts with the cytoplasmic protein Keap1, which facilitates its negative regulation ¹⁸⁸. Keap1 OE repressed Nrf-2 accumulation in a concentration-dependent manner, while ablation of the Neh2 domain enhanced Nrf-2 accumulation, demonstrating Nrf-2 is strictly regulated by Keap1 through its interaction on the Neh2 domain ¹⁸⁸ (Fig. 3).

Keap1 negatively regulates Nrf-2 in two ways, which include facilitating its degradation through the 26S proteasome and inhibiting its nuclear translocation. Firstly, the C-terminal Kelch domain of Keap1 binds to the DLG and ETGE motifs which flank the Neh2 domain within Nrf-2, supporting a tight interaction. Other regions of Keap1, including the N-terminal BTB domain, permit Keap1 homodimerization and its interaction with Cullin 3 (Cul3) and ring box protein 1

(Rbx1), which together form an E3 ubiquitin ligase complex ¹⁸⁹. The intervening region (IVR) domain of Keap1 further supports Cul3-Rbx1 complex assembly ¹⁹⁰, thereby enhancing the polyubiquitination and degradation of Nrf-2 protein ¹⁹¹. Treatment of fibroblasts with MG132, a well-documented proteasome inhibitor, increased Nrf-2 protein demonstrating its degradation is indeed mediated by proteasome action ¹⁹².

Keap1 contains a half-life of approximately 12.7 hours ¹⁹³, while Nrf-2 is only 13 minutes ¹⁹⁴, indicating that Keap1-mediated Nrf-2 turnover maintains very low amounts of Nrf-2 protein basally. Interestingly, Keap1 expression is enhanced with age due to demethylation of the Keap1 promoter region, suggesting that perturbations to the Nrf-2-Keap1 may occur during the aging process ¹⁹⁵. The other way Keap1 inhibits Nrf-2 action is by physically segregating it within the cytosol. Keap1 is a cytoskeleton binding protein, thereby sequestering Nrf-2 by tethering the complex to the actin cytoskeleton ^{188,196}. However, other groups have shown that sequestration may not be due to actin tethering, but instead due to the nuclear export signal (NES) found in the IVR of Keap1, thereby keeping Nrf-2 cytosolic ¹⁹⁷ (Fig. 3A, B).

Keap1 is a cysteine rich protein, which means upon exposure to electrophiles such as ROS, these sensitive residues are oxidized, thereby permitting a conformational change within Keap1. This action results in the release of Keap1 from the DLG motif of Nrf-2, while remaining attached to the ETGE motif of Nrf-2. This is because the ETG region binds with a much greater affinity than DLG, resulting in an opening which has been termed the “hinge-latch” model ¹⁹⁸. This conformational change ensures Nrf-2 is no longer in the correct orientation to become ubiquitinated, thus permitting its accumulation ¹⁹⁹. However, it is important to note that the Nrf-2-Keap1 axis can be disrupted independent of cellular redox state, specifically by the selective removal of Keap1 through autophagy. Phosphorylation of p62 (Ser351) via mTOR, a protein

kinase complex which associates with downstream kinase Unc-51 like autophagy activating kinase-1 (ULK-1) to regulate starvation-induced autophagy ²⁰⁰, enhances the binding affinity of p62 for Keap1. P62 competes with Nrf-2 to bind to Keap1, resulting the removal of Keap1 via autophagy ^{201,202}. This can occur since p62 contains a DPSTGE motif, which is very similar to the ETGE motif found on Nrf-2. OE of p62 reduces Keap1 and enhances Nrf-2 protein, demonstrating it is an important regulator of this axis ²⁰³. P62 is a downstream target of Nrf-2, consequently creating a positive feedback loop since Nrf-2 indirectly drives its own activity (Fig. 3C) ²⁰⁴.

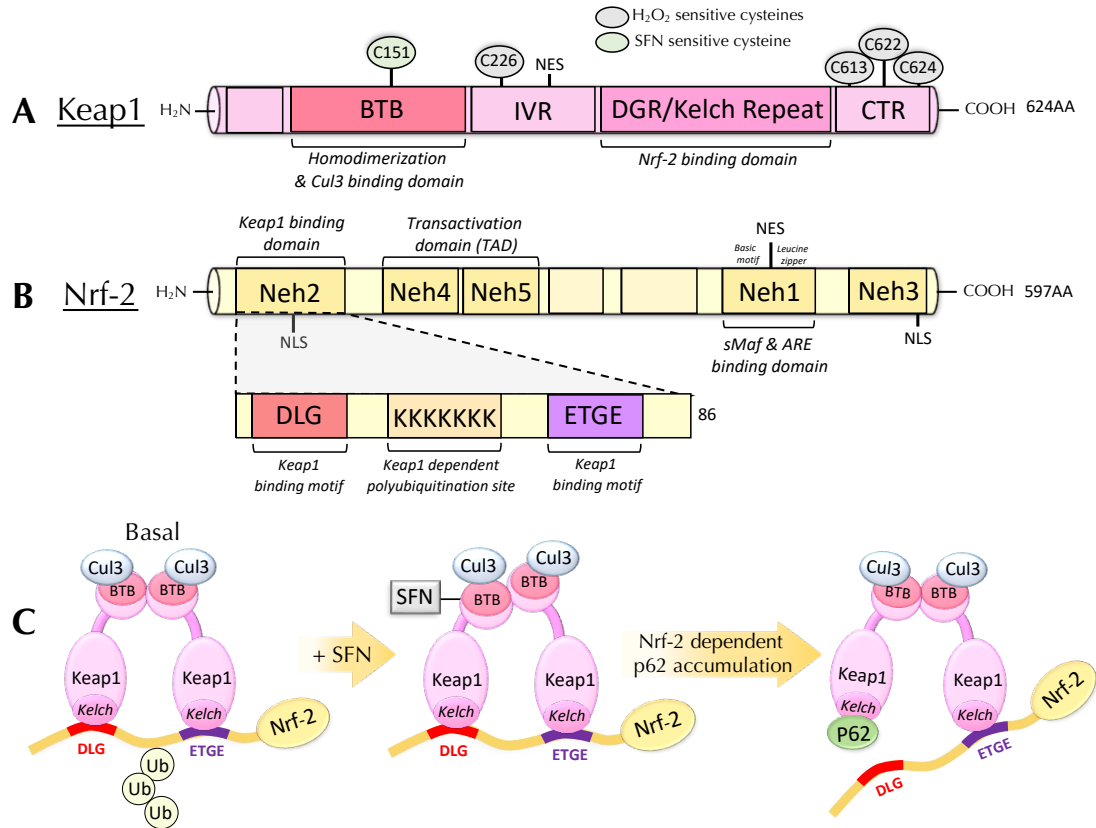


Figure 3: Structure of Keap1 and Nrf-2 protein, depicting their protein-protein interaction in response to Sulforaphane **A)** Keap1 consists of a BTB domain, which facilitates the interaction between another Keap1 and Cullin 3 (Cul3). The central intervening region (IVR) contains redox sensitive cysteine residues and a nuclear export signal (NES) which functions to sequesters Nrf-2 in the cytosol. The Kelch domain binds Nrf-2, while the C terminal region (CTR) also houses multiple redox sensitive cysteine residues that are responsive to hydrogen peroxide (H_2O_2). **B)** Nrf-2 binds Keap1 through the Nrf-2-ECH homology (Neh)-2 domain, specifically the DLG and ETGE motif. The Neh2 domain features lysine (K) residues, which are a target for ubiquitination. Neh3-5 enhance the recruitment of proteins involved in transcription initiation, while Neh1 mediates dimerization with small musculoaponeurotic fibrosarcoma (sMaf) proteins and DNA binding to antioxidant response elements (AREs). Neh1 also contains a nuclear export signal (NES). **C)** Under basal conditions, Nrf-2 is bound to Keap1, facilitating its ubiquitination and subsequent degradation. Sulforaphane interacts with Cys151 of Keap1, which disrupts the ubiquitination of Nrf-2. Nrf-2 enhances p62 accumulation, which binds Keap1 and disrupts the DLG-Keap1 interaction, permitting the open conformation of the “hinge-latch” model, thereby enhancing Nrf-2 accumulation.

3.1 NRF-2

Nrf-2 is a transcription factor that belongs to the cap n collar (CNC) family, which are members characterized by their basic region leucine zipper (bZIP) domain. This affords them DNA binding abilities through their basic domain and dimerization abilities through their leucine zipper. Nrf-2 must heterodimerize with a member of the small Musculo-aponeurotic fibrosarcoma (sMaf) family to permit DNA binding to AREs found within the promoter region of antioxidant target genes ²⁰⁵. Indeed, KO of sMaf proteins significantly blunts electrophilic-mediated induction of Nrf-2 genes ²⁰⁵. However, the absence of complete abolishment may be due to other Nrf-2 dimerizing partners which have recently been identified, including Activating transcription factor 4 (ATF4) ²⁰⁶ and c-jun ²⁰⁷.

As mentioned earlier, electrophilic compounds such as ROS can enhance Nrf-2 signaling, indicating acute exercise-induced ROS production enhances this axis. This has been previously reported *in vitro*, whereby acute exercise enhanced Nrf-2 accumulation and downstream antioxidants during recovery ¹⁷³. This has been corroborated *in vivo* by various groups, demonstrating Nrf-2 accumulates, translocates to nucleus, and binds DNA following acute exercise ^{208–210}. Activation of the Nrf-2 axis appears to be dependent on exercise intensity and duration, with more intense and longer exercise protocols eliciting more robust increases in Nrf-2 signaling. *In vitro* models indicate that a minimum of 3hrs of EPS at a high intensity is required ¹⁷³, while *in vivo* studies indicate 90 minutes is sufficient to see protein changes within skeletal muscle ²¹¹, suggesting that an *in vitro* model of Nrf-2 signaling may underscore physiological conditions. In contrast, chronic endurance exercise has been shown to increase Nrf-2 signaling ²¹², with trained individuals showing a greater ratio of Nrf-2:Keap1 ²¹³. This is particularly important since exercise training has been shown to restore perturbations to Nrf-2 signaling observed with

age. While aging has been associated with reduced Nrf-2 protein and downstream signaling, training restores the activation of this axis, suggesting that exercise may help mitigate age-induced oxidative stress through modulation of redox homeostasis, however more work needs to be done to substantiate this ^{209,212,213}.

Nrf-2 activity has also been implicated in mitochondrial biogenesis, with the most convincing evidence being that Nrf-2 enhances the transcription of NRF-1 due to the presence of 4 ARE sites found upstream ²¹⁴. As NRF-1 directly enhances TFAM, and subsequently mtDNA replication, this finding provided substantial evidence in Nrf-2 driving biogenesis. Furthermore, H₂O₂ has been shown to increase NRF-1 and TFAM mRNA, however KO of Nrf-2 blunts this expression ²¹⁴. Similarly, Nrf-2 KO resulted in a marked reduction in NRF-1, PGC-1 α , ²¹⁵ TFAM, mtDNA and mitochondrial content in response to sepsis ²¹⁶, suggesting that Nrf-2 may be required for stress-induced mitochondrial biogenesis. Interestingly, a complex relationship seems to exist between Nrf-2 and PGC-1 α , which has not been fully elucidated. Upon oxidative stress (i.e., GSH depletion) p53 interacts with PGC-1 α to enhance Nrf-2 transcription. KD of either PGC-1 α or p53 resulted in a marked reduction in Nrf-2 protein and rendered Nrf-2 unresponsive to oxidative stress, suggesting that the p53-PGC-1 α axis may play a role in the Nrf-2-mediated antioxidant response. Other groups have also observed that PGC-1 α KD reduces Nrf-2-mediated expression of SOD2 ²¹⁷ and Nrf-2-dependent mitochondrial biogenesis ²¹⁸, further indicating the presence of this axis. While Nrf-2 is not required for maintenance of mitochondrial content basally, whole body KO of Nrf-2 reduces respiration and increases ROS production ²¹⁰. Nrf-2 KO blunts ²¹⁰ or completely eliminates ²⁰⁸ exercise-induced mitochondrial biogenesis, suggesting that Nrf-2 may be required under stress conditions such as exercise and oxidative stress.

3.1.1 TRANSCRIPTIONAL REGULATION

Encoded by the *Nfe2l2* gene, Nrf-2 is regulated by a small number of transcription factors as another mode of regulation. The Nrf-2 protomer contains a Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) binding site, allowing inducible Nrf-2 activity in response to inflammatory stimuli. Lipopolysaccharide (LPS), a strong inducer of the inflammatory response, elicited NF- κ B binding to the Nrf-2 promoter, resulting in an increase in Nrf-2 mRNA and protein ²¹⁹. Furthermore, the high Nrf-2 activity observed in acute myeloid leukemia has been credited to increased NF- κ B expression and binding to the Nrf-2 promoter ²²⁰. Another well documented regulator of Nrf-2 transcription is aryl hydrocarbon receptor (AhR), which binds as a heterodimer with AhR nuclear translocator (Arnt) to the Nrf-2 promoter in response to xenobiotics (i.e., foreign compounds, not naturally occurring within organisms), thereby increasing Nrf-2 mRNA ²²¹. For example, administration of butylated hydroxytoluene (BHT), a lab formulated food preservative, significantly enhanced Nrf-2 mRNA. However, BHT significantly reduced survivability when coupled with Nrf-2 KO, suggesting that Nrf-2 is a key mediator of toxicological responses ²²². Importantly Nrf-2 has also been reported to bind to and enhance its own promoter activity through ARE like sequences. Nrf-2 OE coupled with a truncated Nrf-2 promoter (lacking ARE like sequences) results in a marked reduction in Nrf-2 promoter activity, suggesting that Nrf-2 is a major driver of its own activation ²²³. Due to this ARE-like sequence, Nrf-2 can heterodimerize with other bZIP proteins to modulate its own transcription. Dimerization with c-jun enhances Nrf-2-induced transcription ²²⁴, whereas dimerization with cellular oncogene Fos (c-fos) diminishes it ²²⁵, demonstrating the importance of dimerization partners in Nrf-2 regulation. Nrf-2 also dimerizes with ATF4 to enhance Heme oxygenase-1 (HO-1) expression, a downstream target of Nrf-2, indicating sMaf proteins may not always be obligatory dimerization partners for activation of the Nrf-2-ARE axis ²⁰⁶.

3.1.2 PROTEIN REGULATION

Keap1 is the major mediator of Nrf-2 protein stability, however other cellular regulators have been reported to modulate Nrf-2. Interestingly, although Keap1-mediated degradation of Nrf-2 is blunted upon oxidative stress, Nrf-2 remains physically bound to Keap1 and sequestered within the cytosol due to the NES found on Keap1. Therefore, it has been suggested that Nrf-2 accumulation may be due to both the liberation of Nrf-2 from Keap1 via phosphorylation, as well as its de novo synthesis, which allows newly synthesized Nrf-2 to bypass Keap1. Nevertheless, the exact mechanism of Nrf-2 regulation remains unknown and is an active area of research. Evidence delineating the role of stabilization versus transcription has not been established.

Nrf-2 can be modified by cytosolic kinases to modulate its translocation. Most notably, AMPK has been reported to enhance Nrf-2 activation. Following AICAR treatment, AMPK enhanced phosphorylation of Nrf-2 at Ser550, which resulted in a marked increase in nuclear Nrf-2 phosphorylated at Ser550. Phosphorylation at Ser550 occurs in the NES found in the Neh1 domain, suggesting that AMPK mediates the nuclear retention of Nrf-2 (Fig. 3B). Indeed, substitution of Serine to Alanine at Ser550 blunted AICAR-mediated Nrf-2 translocation, further suggesting that Nrf-2 is a downstream target of AMPK ²²⁶. AMPK activation is also coupled with the inhibition of Glycogen synthase kinase-3 β (GSK-3 β), a constitutively active kinase that enhances proto-oncogene tyrosine protein kinase Fyn (Fyn)-mediated phosphorylation of Nrf-2 at Tyr568. Upon phosphorylation, Fyn translocates to the nucleus and places an inhibitory phosphate on Nrf-2, enhancing its nuclear export. Furthermore, OE of GSK-3 β significantly reduced both cytosolic and nuclear Nrf-2, suggesting that Nrf-2 is exported out, ubiquitinated, and degraded ²²⁶.

PKC directly phosphorylates Nrf-2 at Ser40 which enhances its dissociation from Keap1 by 50% ²²⁷. Since PKC requires Ca²⁺ for its activation, it may be possible that exercise-induced

Ca²⁺ release supports PKC-mediated Nrf-2 nuclear translocation, however this hypothesis has not been explored. Lastly, MAPK signaling has been implicated in Nrf-2 regulation, providing differential results. P38 MAPK has been shown to promote the interaction between Keap1 and Nrf-2, which subsequently reduces its nuclear translocation²²⁸, whereas ERK2 and JNK1 directly phosphorylate Nrf-2, which permits its release from Keap1 and nuclear translocation²²⁹.

3.2 NQO1

NQO1 is a direct downstream target of Nrf-2 activation, with KO of Nrf-2 resulting in a marked reduction in NQO1²¹⁰. The main function of NQO1 is to catalyze the 2e⁻ reduction of quinones (e.g., ubiquinone, vitamin E) into hydroquinones using the electron donor NADPH. This complete reduction ensures harmful electrophilic intermediates (i.e., semiquinones) are not produced, therefore further conferring another layer of cellular protection. In addition to its enzymatic capacity as an antioxidant, NQO1 has non metabolic functions which have heavily implicated it in mitochondrial biogenesis. NQO1 physically interacts with a variety of proteins and protects them from proteasome-mediated degradation. Ground-breaking work completed by Spiegelman's group showed that NQO1 binds and protects PGC-1 α from the 20S proteasome, enhancing its accumulation. KD of NQO1 resulted in reduced of PGC-1 α protein, suggesting NQO1 that may be required for the accumulation of PGC-1 α basally within hepatocytes⁸⁸. NQO1 also binds and protects p53 and Sirt1 which have been reported to enhance PGC-1 α transcription and activation respectively^{230,231}, suggesting that NQO1 may play multiple roles in biogenesis.

3.3 HO-1

HO-1 is significantly upregulated following oxidative stress, mainly functioning to catalyze the degradation of free heme, which is released into the plasma upon microtrauma to muscle during exercise²³². Free heme is considered cytotoxic, since it acts as a DAMP that can

activate the NLRP3 inflammasome, thereby classifying it as a proinflammatory molecule ²³³. Luckily, HO-1 scavenges and metabolizes heme to avoid cytotoxicity and consequently produce biliverdin, iron and carbon monoxide. Biliverdin reductase quickly converts biliverdin into bilirubin, a potent antioxidant which has been reported to inhibit NOX4 generated superoxide ²³⁴. Furthermore, CO has been reported to have anti-inflammatory effects, reducing pro-inflammatory cytokines TNF- α and IL-1 β , suggesting that HO-1's anti-inflammatory role occurs in a dual manner ²³⁵. Indeed, HO-1 KO animals exhibited greater elevations in lipid peroxidation, muscle atrophy and fatigability ²³². HO-1 has also been reported to enhance mitochondrial biogenesis and maintenance, with OE increasing NRF-1, TFAM and PGC-1 α protein ²³⁶ while KO significantly reduced mitochondrial content and respiration ²³².

3.4 SULFORAPHANE

It is certainly not a new concept that vegetables are an important component of a healthy diet. Epidemiological studies have identified the positive impact of broccoli and other cruciferous vegetables on human health. A cohort study of over 12,000 participants revealed that a dose-response relationship exists between the consumption of broccoli and the onset of cardiovascular disease and tumor mortality, demonstrating that broccoli ingestion was inversely associated with the risk of mortality ²³⁷. While the exact mechanism for these benefits is undoubtedly multifaceted, one reason may be due to sulforaphane (SFN), an antioxidant compound found within broccoli.

SFN [$\text{CH}_3\text{S}(\text{O})(\text{CH}_2)_4\text{-N}=\text{C}=\text{S}$] is a small organosulfur molecule that has been described as one of the most potent activators of the Nrf-2-Keap1 pathway ²³⁸. SFN stimulates this pathway by acting as a mild prooxidant, utilizing its electrophilic terminal carbon [$\text{-N}=\text{C}=\text{S}$] to react with the thiol group [R-S-H] of Cys151 of the BTB domain of Keap1 ²³⁹. This results in the formation of SFN-Keap1 adducts, which subsequently enhances Nrf-2 accumulation (Fig. 3C). While the

exact mechanism of SFN action remains up for debate, several groups have proposed compelling arguments. Nuclear magnetic resonance (NMR) spectroscopy experiments have definitively shown that 1 Keap1 homodimer, bound to 2 Cul3 monomers, bind a single Nrf-2 protein to regulate its degradation, however it remains uncertain if it is the activity or the composition of the Keap1-Cul3 complex that is responsible for SFN-mediated Nrf-2 accumulation ¹⁹². Indeed, some groups have shown that SFN reduced the Keap1-Cul3 interaction ²⁴⁰, whereas other groups demonstrated no change in their association upon treatment ¹⁹². However, it should be noted that dissociation occurred at a higher concentration (20μM), relative to the other study that saw no change (10μM), suggesting that the disruption of this complex may be concentration dependent.

Nevertheless, SFN has been shown to enhance Nrf-2 accumulation without utilizing the “hinge-latch” model. Nrf-2 remains tightly bound to Keap1, promoting the formation of the closed state of the “hinge-latch” model, thereby inhibiting its nuclear translocation. While this mechanism may seem contradictory, it suggests that newly synthesized Nrf-2 is required for the induction of antioxidant gene expression, and not necessarily release from Keap1. Inhibition of translation via cycloheximide completely blunted Nrf-2 accumulation in response to SFN, further suggesting that newly synthesized Nrf-2 is the major mechanism of SFN-mediated Nrf-2 accumulation ²⁴¹. Liam Baird further elaborated on these findings, suggesting that Keap1 remains saturated with old Nrf-2, permitting newly translated protein to bypass Keap1 and translocate to the nucleus ²⁴², however definitive evidence is still required to corroborate this hypothesis (Fig. 4).

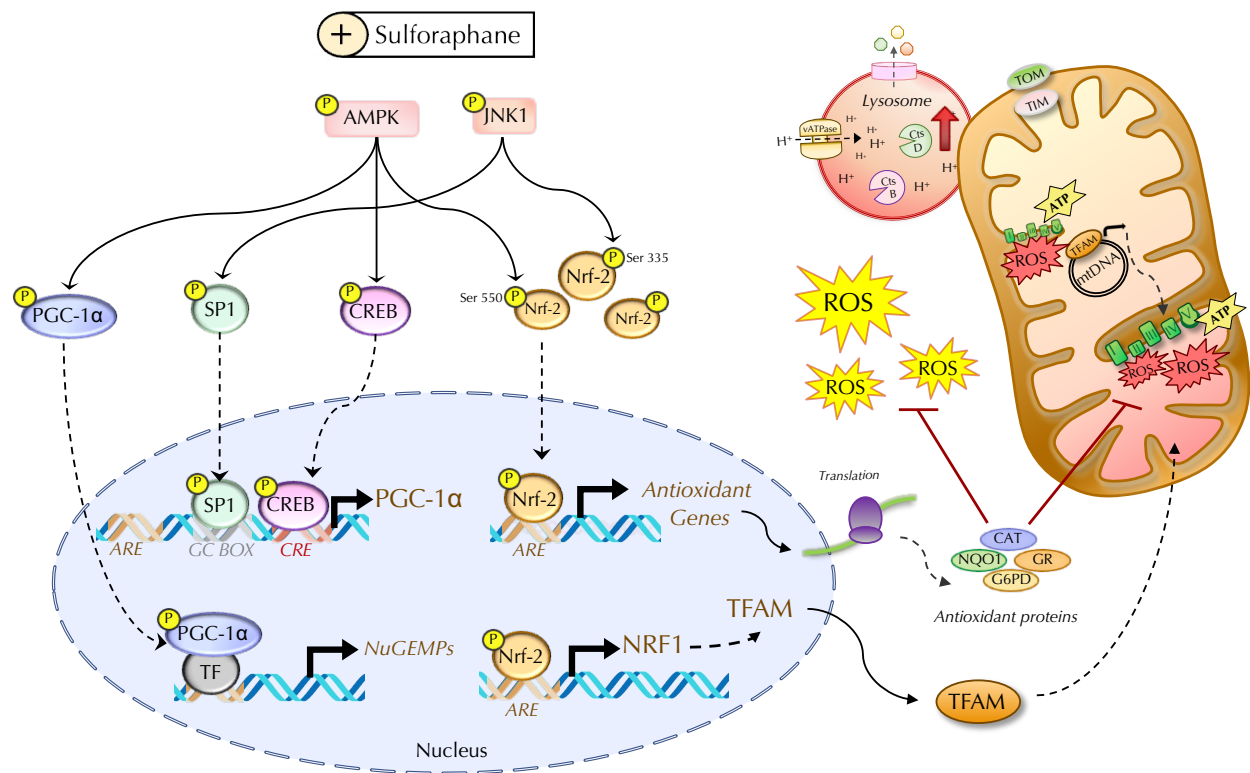


Figure 4: Proposed mechanism of action for Sulforaphane. Upon sulforaphane (SFN) treatment, Keap1-induced degradation of Nrf-2 is disrupted, permitting Nrf-2 accumulation and nuclear translocation. Nrf-2 binds antioxidant response elements (ARE), enhancing the expression of antioxidant genes which blunt cellular and mitochondrial reactive oxygen species (ROS). SFN-mediated Nrf-2 accumulation enhances TFAM expression which consequently drives mtDNA replication. SFN increases the activation of AMPK and JNK1 which may mediate Nrf-2 and CREB activation. CREB and SP1 translocate to the nucleus and enhance PGC-1 α promoter activity. PGC-1 α translocates to the nucleus to increase the expression of NuGEMPs. Lastly, SFN treatment increases markers of lysosomal content and degradation capacity.

3.4.1 TRANSLATIONAL RELEVANCE

As mentioned previously SFN is commonly found in cruciferous vegetables, however a very little amount of free SFN actually exists within plants. Instead, a majority of this compound is present as its inactive precursor glucoraphanin. Derived from the amino acid methionine, glucoraphanin is stable and biologically inert pending plant injury (i.e., cutting, chewing). Upon damage, the enzyme myrosinase, which is normally sequestered within the plant, encounters glucoraphanin and cleaves the precursor into SFN. Hence, this indicates the formation of SFN begins in the mouth upon chewing, later being absorbed at the intestine and peaking around 2 hours post-consumption within the plasma, reflecting the excellent bioavailability of this compound ²⁴³. The half-life of SFN is around 2.2 hours, and is subsequently excreted through the urinary tract, conjugated to glutathione ²⁴⁴. While mammalian cells do not produce myrosinase, bacteria found in the gut microbiome do, which enable the conversion into SFN. Indeed, subjects who consume glucoraphanin without myrosinase, produced 5 times less SFN, evident through the excretion of SFN metabolites ²⁴⁵, therefore highlighting the importance of gastrointestinal health for attaining maximal SFN benefits.

SFN has been deemed a clinically-relevant nutraceutical in the treatment of chronic diseases that are characterized by oxidative stress, with evidence both *in vitro* and *in vivo*. Most famously known for its anticancer properties, SFN has been reported to inhibit the migration and invasiveness of lung tumor cells. When administered both before and after tumor inoculation, SFN inhibited tumor development and increased the survivability of tumor-bearing mice ²⁴⁶. Pre-treatment appeared to be more effective than post-tumor inoculation, suggesting that SFN may have cancer preventative properties ^{246,247}. SFN has also been reported to ameliorate symptoms of neurodegenerative diseases such as Alzheimer's, blunting cognitive deficits and amyloid plaque

in the brain, a hallmark of Alzheimer's ²⁴⁸. When considering muscle health, SFN has had significant positive outcomes, positioning it as a robust metabolic modulator. SFN enhanced grip strength, muscle mass, and reduced pro-inflammatory proteins in type two diabetic mice, suggesting that SFN may protect against skeletal muscle deterioration in diabetic models ²⁴⁹. SFN has also been implicated in muscle growth by enhancing AMPK signaling towards protein synthesis ²⁵⁰, as well as blunting oxidative stress-induced atrophy of C2C12 myotubes ²⁵¹, together suggesting that SFN may be a powerful modifier of muscle mass. SFN has also been investigated in relation to exercise since exhaustive acute exercise is known to induce oxidative stress and muscle damage. Indeed, SFN pre-treatment blunted endurance exercise-induced muscle damage, apparent through a marked reduction in plasma creatine phosphokinase (CPK) and Lactate Dehydrogenase (LDH) relative to non-treated exercised rats ²⁵². Similar results have been observed following high-intensity resistance exercise, where 4 weeks of SFN treatment significantly reduced CPK and the pro-inflammatory cytokine interleukin-6 (IL-6) in human participants following exercise, indicating SFN may be a useful therapeutic to prevent muscle damage observed with exercise ²⁵³. While the protective effects of SFN have proven to be multifaceted, its effects seem to be highly specific to its activation of the Nrf-2-Keap1 axis, suggesting that targeting this pathway may be a fruitful avenue to enhance muscle health.

RESEARCH OBJECTIVES

1. Investigate the ability of Sulforaphane to enhance the activation of the Nrf-2-Keap1 pathway and ameliorate rotenone-induced mitochondrial dysfunction through the induction of downstream antioxidant proteins.
2. Assess if Sulforaphane is able to modulate PGC-1 α -mediated mitochondrial biogenesis, total mitochondrial content, and respiration.
3. Evaluate the effect of Sulforaphane when combined with chronic contractile activity (CCA) *in vitro* on mitochondrial content and antioxidant enzymes.

HYPOTHESES

1. Sulforaphane will increase Nrf-2 protein which will subsequently enhance the expression of antioxidant enzymes and reduce Reactive Oxygen Species (ROS) when challenged with a mitochondrial stressor.
2. Sulforaphane will enhance mitochondrial biogenesis and respiration.
3. Sulforaphane treatment will work synergistically with CCA to increase mitochondrial content, surpassing untreated “exercised” cells.

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CHAPTER 2: MANUSCRIPT

MANUSCRIPT AUTHOR CONTRIBUTIONS:

Sabrina Champs: Performed all cell culture procedures and drug treatments; whole-cell, nuclear, and cytosolic cell extraction; western blotting; confocal microscopy; flow cytometry experiments; Seahorse XF assays; luciferase assays; C2C12 chronic contractile activity experiments; data analysis and interpretation; wrote the manuscript.

Dr. David A. Hood: Supervised this project and is the Principal Investigator.

**CHARACTERIZING THE ROLE OF SULFORAPHANE IN MITOCHONDRIAL FUNCTION
AND CONTENT IN SKELETAL MUSCLE CELLS**

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ABSTRACT

Mitochondria are metabolic hubs that mediate signaling cascades that govern skeletal muscle health. While exercise has been established as a powerful inducer of quality control processes that ultimately enhance the mitochondrial phenotype, there are currently limited pharmaceutical interventions available that emulate or enhance exercise-induced mitochondrial adaptations. To investigate a novel nutraceutical candidate for this role, we examined Sulforaphane (SFN) a naturally occurring compound found in cruciferous vegetables. SFN has been documented as a potent antioxidant inducer through its activation of the Nrf-2-ARE pathway. However, its effects on muscle health have been underexplored. To investigate the interplay between chronic exercise and SFN, C2C12 myotubes were electrically stimulated to model chronic contractile activity (CCA) and were treated with SFN for various timepoints. Following treatment, SFN enhanced mitochondrial respiration and upregulated key antioxidant proteins including NQO1, CAT and GR. These adaptations were accompanied by a reduction in cellular and mitochondrial ROS emission. Signaling towards biogenesis was enhanced, demonstrated by an increase in TFAM protein and PGC-1 α nuclear translocation. Nuclear fractions revealed an increase in transcription factors SP1 and CREB, previously shown to enhance PGC-1 α transcription. This was accompanied by an increase in PGC-1 α promoter activity, mitochondrial content, and branching, suggesting a larger more interconnected mitochondrial pool. Lysosomal proteins TFE3 and Cathepsin B were also significantly upregulated. Importantly, there was no difference in mitochondrial and antioxidant-related proteins between CCA and non-CCA SFN-treated cells. Our data suggests that SFN activates signaling cascades that are common to those produced by regular exercise, suggesting that SFN-centered therapeutic strategies may improve the mitochondrial phenotype in skeletal muscle.

INTRODUCTION

Skeletal muscle health is heavily influenced by the quantity and quality of the mitochondria that reside within it. Loss of mitochondrial content and excessive mitochondrial ROS emission have both been linked to muscle atrophy, indicating that the mitochondrial phenotype is tightly linked to muscle health ¹. Mitochondria are exceedingly adaptable, capable of modulating their structure and molecular response in accordance with the tissue's energy demand. This plasticity is exemplified during exercise, where several intracellular signaling cascades are activated which ultimately drive mitochondrial quality control processes which mediate favourable mitochondrial adaptations ².

During muscle contraction, metabolic alterations enhance cytosolic calcium (Ca^{2+}), reactive oxygen species (ROS) and elevate AMP relative to ATP. These secondary messengers activate sensitive upstream signaling kinases, including Ca^{2+} /calmodulin-dependent protein kinase (CaMK) ³, P38 mitogen-activated protein kinase (MAPK) ⁴ and AMP-kinase (AMPK) respectively ⁵. These kinases phosphorylate and activate Peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 α , a protein involved in the co-activation of transcription factors (TFs) which enhance the expression of nuclear genes encoding mitochondrial proteins (NuGEMPs). These post-translational modifications (PTMs) permit PGC-1 α nuclear translocation, resulting in an increase in mitochondrial biogenesis. Most notably, PGC-1 α co-activates nuclear respiratory factor 1 (NRF-1), a TF involved in the expression of mitochondrial transcription factor A (TFAM), a mediator of mitochondrial DNA (mtDNA) replication ⁶.

Chronic contractile activity also enhances the capacity for mitochondrial removal through lysosomal biogenesis, thereby enhancing mitochondrial turnover through lysosomal breakdown ⁷. Transcription factor E3 (TFE3) and transcription factor EB (TFEB) homo or heterodimerize,

enabling them to enhance lysosomal and autophagy-related genes. Furthermore, training reduces ROS through the upregulation of antioxidant enzymes, including glutathione peroxidase-1 (GPX-1), glutathione reductase (GR), and superoxide dismutase (SOD) ⁸. These target genes can be upregulated by the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf-2) ⁹.

Under basal conditions, Nrf-2 is sequestered within the cytosol by its negative regulator kelch-like ECH-associated protein 1 (Keap1). Keap1 acts as an adaptor for the E3 ubiquitin ligase complex cullin 3 (Cul3), permitting the ubiquitination and subsequent proteasome mediated degradation of Nrf-2, thereby keeping protein levels very low ¹⁰. During exercise, ROS modify sensitive cysteine residues on Keap1, resulting in a reduction in Nrf-2 ubiquitination. Nrf-2 is then able to accumulate, translocate to the nucleus and heterodimerize with members of the small Musculo-aponeurotic fibrosarcoma (sMaf) protein family, permitting its binding to antioxidant response elements (ARE) found upstream of target genes ¹¹. While this signaling cascade has been reported to occur during exercise ¹² it can be chemically induced by the nutraceutical sulforaphane (SFN). SFN has proven to be an extremely strong inducer of the Nrf-2-Keap1 axis, and therefore has been employed in several studies to examine the activation and downstream effectors of this pathway.

SFN is a naturally occurring compound found in cruciferous vegetables, such as broccoli and cauliflower ¹³. While previously work has positioned SFN as a chemopreventive agent ¹⁴, recent work has revealed it may play an important role in skeletal muscle homeostasis. Since SFN has been described as among one of the most potent Nrf-2 activators available, we chose to investigate the effects of SFN in C2C12 myotubes to elucidate if 1) Nrf-2 may be able to preserve or enhance the mitochondrial content and function and if 2) sustained Nrf-2 activation may

augment mitochondrial adaptations in response to exercise using an *in vitro* model of chronic contractile activity (CCA).

METHODS

Growth, maintenance, and differentiation of C2C12 murine skeletal muscle cells. C2C12 myoblasts (ATCC; CRL-1772) were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (P/S) [growth medium (GM)]. Cells were incubated at 37°C and 5% CO₂ and passaged upon 60% confluency to maintain their myoblast form. For subsequent treatment and analysis of SFN on myotubes, C2C12 myoblasts are seeded in 6-well cell culture plates supplemented with GM. After reaching 80-90% confluency, GM was removed and cells were washed with PBS before incubation with differentiation media (DM), containing 5% heat-inactivated horse serum and 1% P/S to promote differentiation. DM was replaced daily to promote cell health and differentiation. After six days of differentiation, myotube morphology was confirmed using a phase contrast microscope before performing subsequent treatment and analysis.

Drug treatments. SFN (MedChemExpress) was prepared using DMSO as the diluent and was added to DM to produce a final working concentration of 10 μ M. Vehicle treatment was prepared by adding the same volume of DMSO to DM. The proteasome inhibitor N-carbobenzoxyl-Leu-Leu-leucinal (MG132; Sigma; M8699-1MG) was prepared as a 2mM intermediate stock and was added to SFN or vehicle-treated media to produce a final working concentration of 10 μ M. Cells were co-treated for 6 hours in phenol-free media prior to imaging. Positive and negative controls for flow cytometry were prepared fresh and performed every time a new vial of dye was used. The antioxidant N-acetylcysteine (NAC; Sigma; A8199) was resuspended in DM at a final concentration of 20mM, a concentration previously optimized in C2C12 myotubes ⁵, for 24 hours.

Menadione (Sigma; M5625), a ROS inducer¹⁵, was used at a working concentration of 100 μ M for 1 hour as per the manufacturer's instructions. The mitochondrial uncoupler Carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma; 215911) was applied for 30 minutes at a final working concentration of 25 μ M¹⁶.

Whole-cell isolation and protein extraction. Following treatment, media was aspirated, and cells were rinsed twice with warm PBS. Cells were then trypsinized for five minutes at 37°C to permit cell detachment before being scraped, collected, and spun down at 1400xg for 4 minutes. Following centrifugation, the supernatant was aspirated, and the pellet was resuspended in ice-cold PBS. Cells were spun down again at 1400xg for 4 minutes and PBS was aspirated. The pellet was placed on ice and resuspended in cell extraction buffer containing 1X lysis buffer (Promega), phosphatase inhibitor cocktails (sigma), and a protease inhibitor (sigma). Four freeze-thaw cycles were performed by flash-freezing cells with liquid nitrogen then thawing them in a 37°C water bath. This procedure damages the cell membrane and promotes cell lysis. Finally, cell lysates were centrifuged at 2500xg for 10 minutes at 4°C and the supernatant fraction was collected and stored in -80°C for future use.

Nuclear-cytoplasmic fractionation. C2C12 myoblasts were seeded and differentiated in 15cm² cell culture plates before treatment with SFN or vehicle. Whole cells were collected in cytoplasmic buffer, containing phosphatase inhibitor cocktails (sigma), and a protease inhibitor (sigma). Samples were placed on ice for five minutes before being rocked on the orbital shaker for five minutes at 4°C, then centrifuged at 2500xg for 4 minutes. The supernatant fraction was collected and stored as the cytoplasmic fraction at -80°C for future use. The remaining nuclear fraction was resuspended with cytoplasmic buffer, rocked for three minutes at 4°C, and then centrifuged at

3200xg for 4 minutes before discarding the supernatant fraction. This workflow was repeated 10-12 times to ensure the purity of the nuclear fraction. After the final cycle, 0.15 unit/ μ L of benzonase was added to the nuclear fraction and incubated on ice for 20 minutes before storing the samples at -80°C for subsequent western blotting.

Western blotting. The protein concentration of each sample was determined using the Bradford assay. Equal amounts of protein, ranging from 15-30 μ g depending on the protein targets, were prepared, and loaded into 8-15% Sodium Dodecyl Sulfate (SDS) polyacrylamide gels and separated using electrophoresis. Following separation, proteins were transferred onto nitrocellulose membranes and washed with Ponceau S stain to confirm that transferring was successful. Membranes were labelled and cut at the appropriate molecular weight for each target protein. Ponceau S stain was washed off the membranes with 1x TBS-T for five minutes before blocking for one hour in 5% skim milk at room temperature with light agitation. Afterwards, primary antibodies (see Table 1) were applied to membranes overnight at 4°C . The following day, membranes were incubated with the corresponding secondary antibody for one hour at room temperature. Equal portions of Clarity Max Enhanced Chemiluminescence (ECL) substrates were combined and applied to membranes for four minutes before being imaged using the iBright Imaging System (ThermoFisher). Optical density was quantified using ImageJ software (ver. 1.53) for subsequent quantification using GraphPad Prism Software (ver. 8.2.1).

Flow cytometry. Myoblasts were proliferated and differentiated in a 6-well cell culture plate, before treatment with SFN and vehicle. Cells were stained with 100nM MitoTracker Green (MTG), 100nM MitoTracker Red (MTR) 5mM CellROX Green, or 15 μ M MitoSOX Red in phenol-free media for 30 minutes at 37°C . Following incubation, myotubes were washed with

warm PBS, trypsinized for 5 minutes at 37°C, then scraped for collection and centrifugation at 1400xg for 4 minutes. Pellets were washed then resuspended in ice-cold PBS before being analyzed on the Flow Cytometer (Beckman, CytoFlex). MTG and CellROX Green were evaluated using the Fluorescein isothiocyanate (FITC) filter, while MTR and MitoSOX Red were assessed using the phycoerythrin (PE) filter, both utilizing the 488nm laser. Unstained, single stains, and double-stained samples were produced for each experiment to generate accurate gating for dot plots. Compensation was performed when applying more than one dye to correct for spectral overlap, thereby providing a more accurate fluorescence intensity for each dye (Fig. S1).

Assessment of mitochondrial content, morphology, and PGC-1 α via confocal microscopy.

Following either SFN alone, MG132 treatment, or CCA, C2C12 myotubes were stained as described above with 10 μ M Hoechst, and either MTG or MTR in phenol-free DMEM immediately prior to imaging. Myotubes were imaged with the confocal microscope using a Nikon Eclipse TE2000-U Fluorescence microscope coupled to a Nikon C2 microscope. All image analysis was performed using ImageJ (ver. 1.53), ensuring fully visible myotubes were traced (i.e., minimum 2 nuclei per cell). To measure dye intensity, images were processed using “unsharp mask” (radius = 1.0-pixel, mask = 0.60) and “median” (radius = 2.0 pixels) filter tools. Images were made binary and skeletonized before measuring the integrated density (fluorescence intensity) of each channel. To assess the colocalization of MTG and MTR or Hoechst and GFP, the plugin JACoP (https://github.com/fabricecordelieres/IJ-Plugin_JACoP) was employed¹⁷. Costes’ automatic threshold was utilized to determine Mander’s Coefficient for each overlapping fraction. To assess mitochondrial morphology, the Mitochondria Analyzer Toolset, available as a plugin on ImageJ (<https://github.com/AhsenChaudhry/Mitochondria-Analyzer>), was used according to the provided user manual. This permitted the evaluation of mitochondrial branching parameters, including

branch length, and the number of branch junctions per mitochondria. Branch length refers to how long the mitochondria are, whereas branch junction refers to points in the reticulum where 3 or more branches intersect. Both length and intersectionality are parameters indicative of mitochondrial connectivity.

Mitochondrial respiration. Mitochondrial respirometry was assessed using the Seahorse XF96 Cell Mito Stress Test Kit (Agilent). Myoblasts were seeded at a density of 2500 cells per well and subsequently differentiated in a Seahorse cell microculture plate (Agilent) before treatment with SFN and vehicle for 24hrs and 48hrs. Each condition was plated in 23 replicate wells across the microplate and averaged for each condition (n=1). The experiment was completed 5 times on separate plates (n=5). The Seahorse XF96 Extracellular Flux Cartridge is used to measure the Oxygen Consumption Rate (OCR) of each well after adding Oligomycin, FCCP, and Rotenone + Antimycin A. After the assay was completed, the cells were washed with phenol-free DMEM (Wisent; 319-065-CL) supplemented with glucose and glutamine, then incubated with 10 μ M Hoechst (Thermo; 33342) and 100nM MitoTracker Green (Thermo) for 30 minutes at 37°C to stain the nuclei and mitochondria respectively. Following incubation, fluorescence was measured on the plate reader (BioTek Instruments Inc). MitoTracker Green was normalized to Hoechst to obtain a measurement of mitochondria per cell. These values were used to normalize the OCR obtained per well using the XF Wave Software (Agilent), therefore providing mitochondrial respiration per cell.

Chronic Contractile Activity (CCA). Following differentiation and confirmation of appropriate morphology, myotubes were electrically stimulated as previously described^{18–20}. Briefly, the plastic lids corresponding to 6-well cell plates were retrofitted with 2 platinum electrodes which

span the length of the well. Before stimulation, cells were washed and supplemented with 4.5mL of fresh media per well, ensuring each electrode was fully submerged. Myotubes were subjected to electrical pulse stimulation (EPS) at 5Hz and 18V for 3hrs, followed by a 21-hour recovery period where cells were supplemented with either vehicle or sulforaphane (SFN). Visual confirmation of myotube contraction was confirmed for every bout and monitored periodically to ensure that sustained contraction was achieved. Stimulation was repeated for four days, and cells were collected for analysis after the last recovery period (Fig. 7A).

Acute Contractile Activity (ACA). Myotubes were subjected to the same contractile model as described above, however only underwent a single bout of EPS which lasted 3hrs, in order to model acute “exercise”. Cells were collected immediately following stimulation and were treated with either SFN or vehicle during the bout. Unstimulated cells were treated for 3hrs and collected at the same time as “exercised cells”.

Promoter-reporter transient transfections. A custom PGC-1 α promoter-firefly-luciferase-reporter construct was purchased from Applied Biological Materials Inc (Richmond, BC Canada), containing 4-Kb of the mouse PGC-1 α promoter region. For DNA amplification, the plasmid was transformed into competent *E. coli* cells, which were incubated on ice for 30 minutes. Following incubation, cells were heat-shocked for 30 seconds and then placed back on ice for 5 minutes before adding 475 μ L of outgrowth media and being placed on a shaker plate at 37°C and 250 RPM for 1 hour. Cells were inoculated on agar plates supplemented with the corresponding antibiotic (Kanamycin or Ampicillin). Following overnight incubation, single colonies were selected and amplified in LB media overnight on the shaker (250 RPM), followed by plasmid isolation using the HiSpeed Plasmid Maxi Prep Kit (Qiagen; 12663). The PGC-1 α plasmid was

co-transfected with a CMV-promoter-pRL3-reporter vector (Promega; E2261), expressing renilla luciferase, which served as a loading control for transfection efficiency. An empty vector (EV; Promega) was co-transfected with the pRL3 vector to provide a baseline of luciferase activity, accounting for any non-specific activity which contributes to the background signal. At 40% confluency, cells were transfected with the plasmids and Lipofectamine2000 (Fisher) in serum-free DMEM. Following 17hrs of transfection, myoblasts were washed with PBS, proliferated, differentiated, and treated with SFN or vehicle for 24hrs. Following treatment, whole cells were collected, and luciferase activity was determined using the Steady-Glo Luciferase Assay System (Promega; 72530).

Similarly, cells were transfected with a CMV-promoter-GFP-PGC-1 α -reporter plasmid using the same transfection procedure as described above. This is a constitutively active plasmid, expressing PGC-1 α bound to GFP, permitting the observation of PGC-1 α localization.

Statistical analysis. All graphs were produced, and statistics were analyzed using the GraphPad Prism software (version 8) and represented as means $\pm$ SEM. For comparisons between vehicle and SFN in respects to nuclear-cytosolic fractions, confocal imaging, and certain whole cell analyses as specified within figures, a two-tailed unpaired T-test was performed. A one-way analysis of variance (ANOVA) was completed followed by a Tukey's post hoc test for the analysis of oxygen consumption, PGC-1 α promoter activity, reactive oxygen species measurements, MG132 treatment, and protein analysis following chronic contractile activity. Data were considered significant when $p < 0.05$.

Table 1: List of antibodies used for western blotting.

Antibody	Manufacturer	Catalog No.	Molecular Weight (kDa)	[1°Ab]	[2°Ab]
Nrf-2	Cell Signaling	12721S	100	1:500	1:1000
P62 (SQSTM1)	Cell Signaling	5114S	62	1:1000	1:1000
Keap1	ProteinTech	10503-2-AP	74	1:1000	1:2000
H2B	Cell Signaling	2934S	15	1:1000	1:1000
α -tubulin	Millipore Sigma	CP06- 100UG	50	1:1000	1:1000
GAPDH	Abcam	ab9485	37	1:100000	1:5000
TFAM	Abcam	ab176558	24	1:500	1:1000
COX I	Abcam	ab14705	39	1:500	1:1000
MFN2	Abcam	ab50843	86	1:1000	1:1000
Glutathione Reductase	Santa Cruz	sc-133245	55	1:2000	1:1000
G6PD	Cell Signaling	8866S	58	1:1000	1:1000
NQO1	Abcam	ab34173	31	1:1000	1:1000
Catalase	Cell Signaling	14097S	60	1:2000	1:1000
GPX-1	Abcam	Ab22604	22-24	1:1000	1:1000
SP1	Santa Cruz	SC-420	92	1:1250	1:1000
CREB1	Santa Cruz	SC-058	43	1:1000	1:1000
ATF-2	Cell Signaling	20F1	65-75	1:1250	1:1000
USF-1	Santa Cruz	sc-8983	44	1:1000	1:1000
TFE3	Sigma	HPA023881	73	1:500	1:1000
UQCRC2	Abcam	ab14745	48	1:1000	1:1000
VDAC	Abcam	ab14734	33	1:500	1:1000
vATPase	Santa Cruz	sc-55544	50	1:1000	1:1000
Cathepsin B	Cell Signaling	31718S	24-44	1:1000	1:1000
HO-1	Abcam	ab13248	32	1:1000	1:1000
Drp-1	BD BioSciences	611738	27	1:1000	1:1000
P-AMPK (Thr172)	Cell Signaling	2535S	64	1:1000	1:1000
AMPK	Cell Signaling	2532S	64	1:1000	1:1000
P-JNK (Thr183, Tyr185)	Cell Signaling	4668S	37-54	1:1000	1:1000
T-JNK	Cell Signaling	9252T	37-54	1:1000	1:1000
P-P38 MAPK (Thr180/Tyr182)	Cell Signaling	9211S	43	1:1000	1:1000
P38 MAPK	Cell Signaling	9212S	40	1:1000	1:1000
P-CAMKII- α (Thr286)	Cell Signaling	3361S	50	1:1000	1:1000
T-CAMKII- α	Cell Signaling	3362S	50-60	1:1000	1:1000
P-GSK-3B	Cell Signaling	9322S	46	1:1000	1:1000
T-GSK-3B	Cell Signaling	9832S	46	1:1000	1:1000
Anti-Mouse HRP- linked 2°	Cell Signaling	7076S	-	-	-
Anti-Rabbit HRP- linked 2°	Cell Signaling	7074S	-	-	-

RESULTS

Sulforaphane increases Nrf-2 accumulation and nuclear localization. SFN has been well documented as a potent inducer of the Nrf-2-ARE axis by directly modifying Keap1, the negative regulator of Nrf-2. To examine the upstream activation of this pathway, myotubes were treated with SFN for 24 hrs and collected. Whole-cell lysates revealed a 6-fold increase in Nrf-2 protein (**** $p < 0.0001$) relative to vehicle-treated cells, indicating Nrf-2 accumulation (Fig. 1A, C). To determine if this transcription factor was translocating to the nucleus to enhance the upregulation of target genes, nuclear-cytosolic fractions were prepared, which demonstrated a dramatic increase in nuclear Nrf-2 (Fig. 1B, C; **** $p < 0.0001$). This was accompanied by a reduction in Keap1 (* $p < 0.05$) and an increase in p62 (** $p < 0.01$), an autophagy receptor which facilitates the removal of cellular cargo (Fig. 1A, C).

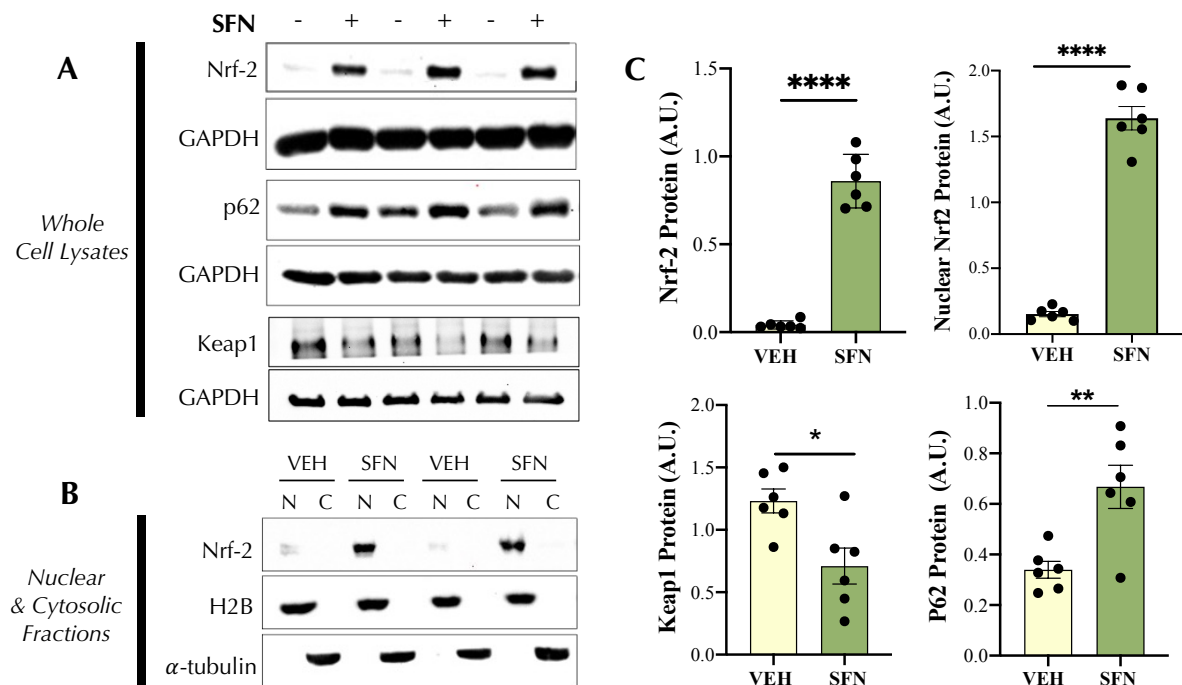


Figure 1: Expression of upstream proteins involved in the Nrf-2-ARE antioxidant axis following 24hr SFN treatment. **A, C)** Representative western blots and quantifications of Nrf-2 (**** $p < 0.0001$), p62 (** $p < 0.01$) and Keap1 (* $p < 0.05$), normalized to the corresponding GAPDH in whole cell lysates. Negative (-) indicates cells were treated with vehicle. **B, C)** Representative nuclear (N)-cytosolic (C) western blot of Nrf-2 (**** $p < 0.0001$) and corresponding quantification. Nrf-2 was normalized to H2B for the nuclear fraction, and purity was evaluated with α -tubulin. An unpaired two-tailed T-test was performed for each graph ($n=6$), and all values are represented as $\pm$ SEM. VEH=Vehicle, SFN=Sulforaphane.

Sulforaphane enhances mitochondrial content and respiration. Nrf-2 has been reported to increase mitochondrial biogenesis through its own action, as well as its downstream effectors, including NQO1 and HO-1. To evaluate if SFN may be able to augment Nrf-2-mediated biogenesis within skeletal muscle cells, western blotting and flow cytometry experiments were performed to detect changes in mitochondrial content following treatment. After 24_hrs, there was a significant increase in TFAM (**p<0.01), an important mediator of mtDNA replication. This was accompanied by a marked elevation in COX I (*p<0.05), a subunit of Complex IV of the electron transport chain (ETC), and MFN2 (*p<0.05), a key protein involved in mitochondrial fusion, by 48_hrs. At the same time point, flow cytometry revealed an increase in mitochondrial mass and membrane potential intact using MitoTracker Green (**P<0.01) and MitoTracker Red (**P<0.01) respectively (Fig. 2). Confocal imaging revealed a significant increase in mitochondria with their membrane potential intact, evident through enhanced colocalization of MTG and MTR (Fig. 3B, C). Assessment of mitochondrial respiration parameters, including basal respiration (****SFN<0.0001), ATP production (****SFN<0.0001), maximal respiration (***P<0.001), and spare respiration (***P<0.001) demonstrated a main effect of treatment. No effect of time was observed, demonstrating the same enhancement in respiration at both 24hrs and 48hrs (Fig. 3A).

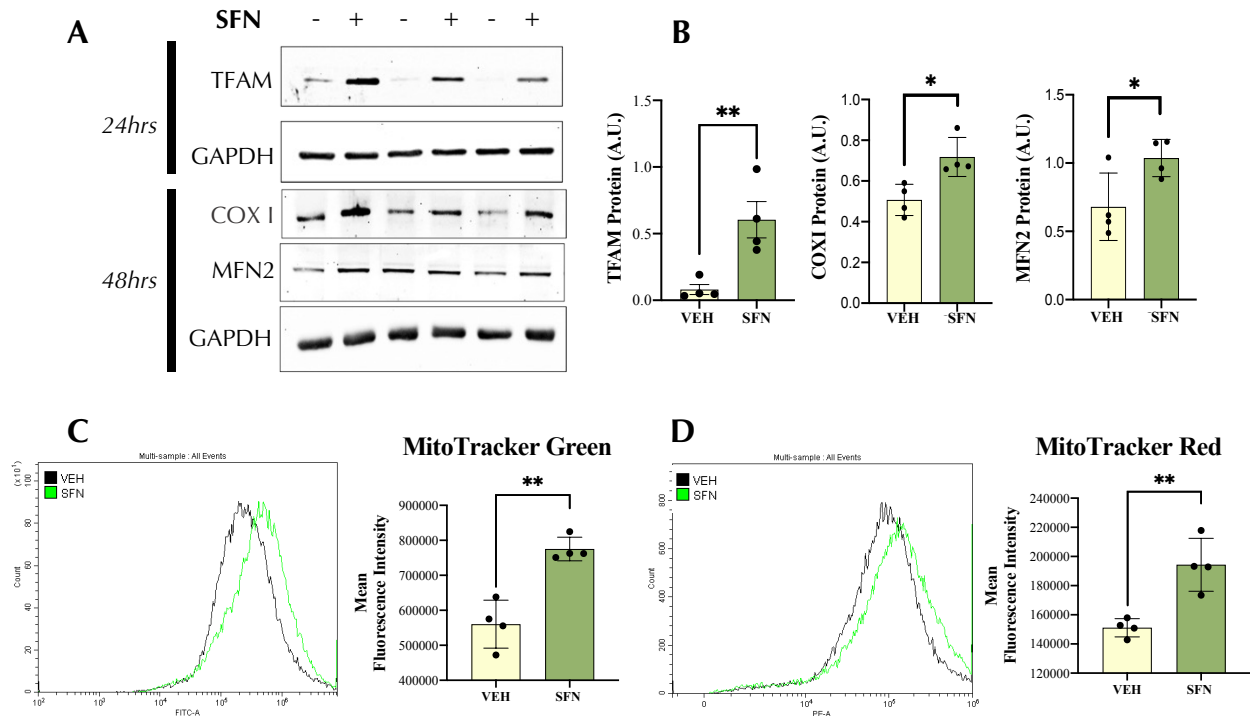


Figure 2: Mitochondrial content following SFN. **A)** Representative western blots of TFAM following 24hr SFN, as well as mitochondrially encoded COX I, and the fusion-related protein MFN2 following 48hrs SFN, re-supplemented at 24hrs. **B)** Western blot quantifications of TFAM (n=4, **P<0.01), COX I (n=4, *P<0.05), and MFN2 (n=4, *P<0.05). All targets were normalized to GAPDH. **C, D)** Representative histogram and corresponding quantification of MitoTracker Green, for a measure of total mitochondrial content (n=4, **P<0.01) and MitoTracker Red, as a functional measurement, as this stain only accumulates in mitochondria with their membrane potential intact. An unpaired two-tailed T-test was performed for each graph. VEH=Vehicle, SFN=Sulforaphane. All values are represented as $\pm$ SEM.

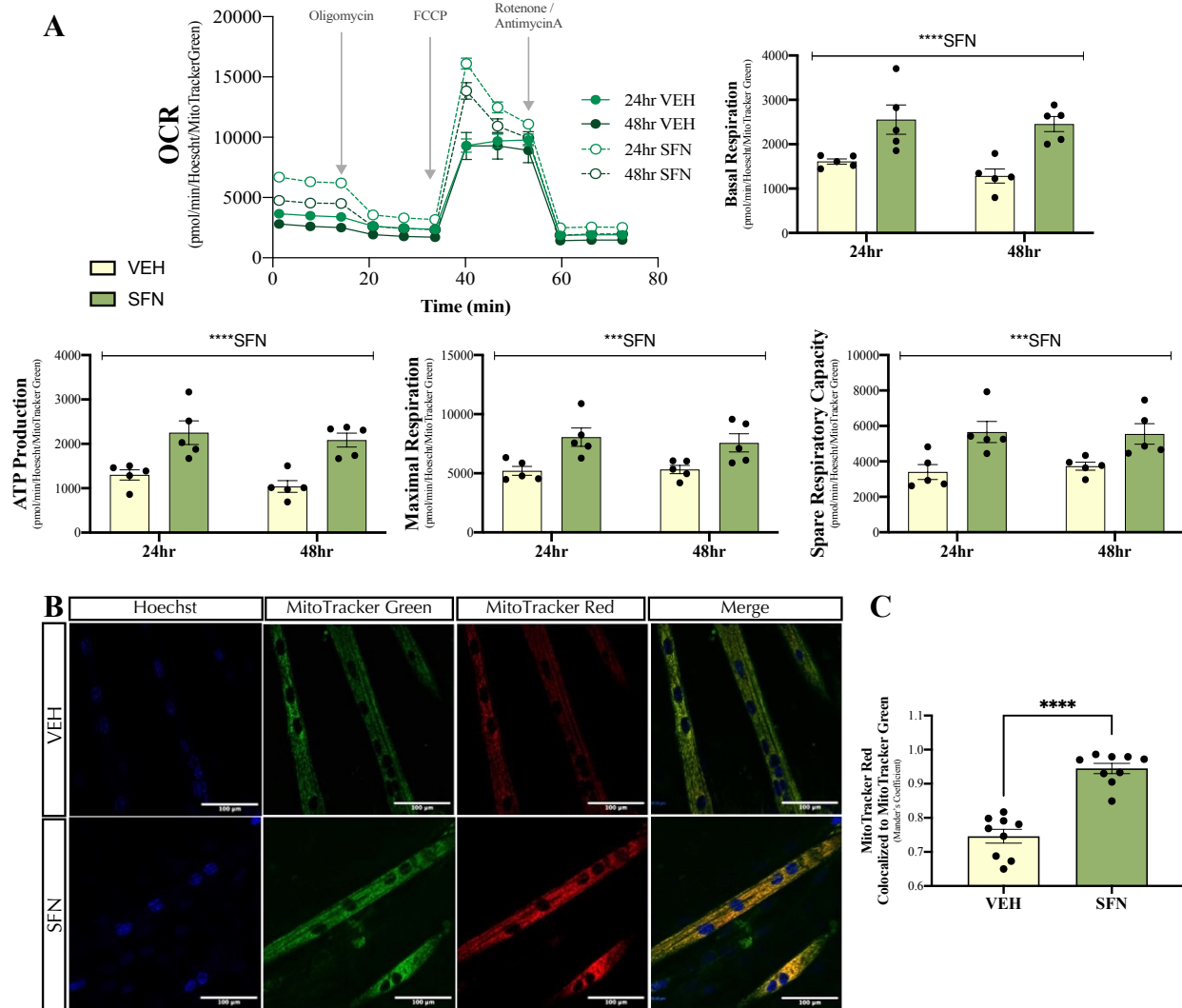


Figure 3: Mitochondrial respiration and functional parameters following SFN treatment. A) Representative Seahorse Oxygen Consumption Rate (OCR) tracing following the addition of substrates as shown, and the corresponding graphical quantifications following 24hr and 48hr SFN treatment. Cells were re-supplemented with treatment at 24hrs. ATP production, basal respiration, spare respiratory capacity, and maximal respiration capacity were normalized to mitochondrial content and nuclei to obtain respiration per cell. A two-way ANOVA was performed for each graph to determine the effect of SFN and time ($n=5$; *** $P<0.001$, **** $P<0.0001$). “SFN” indicates a main effect of SFN; * Denotes the P value of SFN. **B)** Representative confocal microscopy images of MitoTracker Green and Red following 48hr SFN or vehicle treatment (60X magnification). Co-localization was determined using Mander’s overlap coefficient (**** $p<0.0001$), evaluating the fraction of MitoTracker red localized to MitoTracker Green ($n=9$; 2-3 myotubes per image). A two-tailed unpaired T-test was performed. All values are represented as $\pm$ SEM.

Sulforaphane pre-treatment blunts rotenone-induced oxidative stress through the activation of antioxidant enzymes related to the Nrf-2-ARE axis. After observing a robust accumulation of nuclear Nrf-2, we sought to evaluate if SFN may be able to enhance the activation of downstream antioxidant proteins related to the Nrf-2-ARE axis and confer cellular and mitochondrial protection against oxidative stress. Cells were pre-treated with SFN for 24hrs, followed by a 24hr treatment with rotenone, an inhibitor of Complex I of the ETC (Fig. 4A). Previous reports have shown that rotenone is a powerful inducer of mitochondrial-mediated oxidative stress, leading to cellular dysfunction ²¹. Following treatment, glutathione reductase (GR; ***SFN<0.001), glucose-6-phosphate-dehydrogenase (G6PD; ***SFN<0.001), NAD(P)H Quinone Oxidoreductase (NQO1; ****SFN<0.0001), catalase (CAT; ****SFN<0.0001) and glutathione peroxidase-1 (GPX-1; *SFN<0.05) all demonstrated a main effect of SFN. CAT (**ROT<0.01) and GPX-1 (*ROT<0.05) showed a main effect of rotenone, along with an interaction effect for CAT (*SFNxROT<0.5) and GPX-1 (**SFNxROT<0.01).

To determine if alterations in antioxidant proteins may be able to provide a rescue effect in terms of reactive oxygen species (ROS), cellular and mitochondrial ROS were detected with CellROX Green and MitoSOX Red respectively, along with MitoTracker Red (MTR) to evaluate membrane potential. Basally, SFN treatment significantly reduced mitochondrial ROS (***SFN<0.001; Fig. 4E) and enhanced membrane potential (***SFN<0.001; Fig. 4F). Rotenone induced cellular (*ROT<0.05; Fig. 4D) and mitochondrial (**ROT<0.01; Fig. 4E) ROS emission, along with a reduction in membrane potential (*ROT<0.05). This oxidative stress was blunted with SFN pre-treatment, evident through a reduction in cellular (*SFNxROT<0.05) and mitochondrial ROS (****SFNxROT<0.0001), as indicated through an interaction effect.

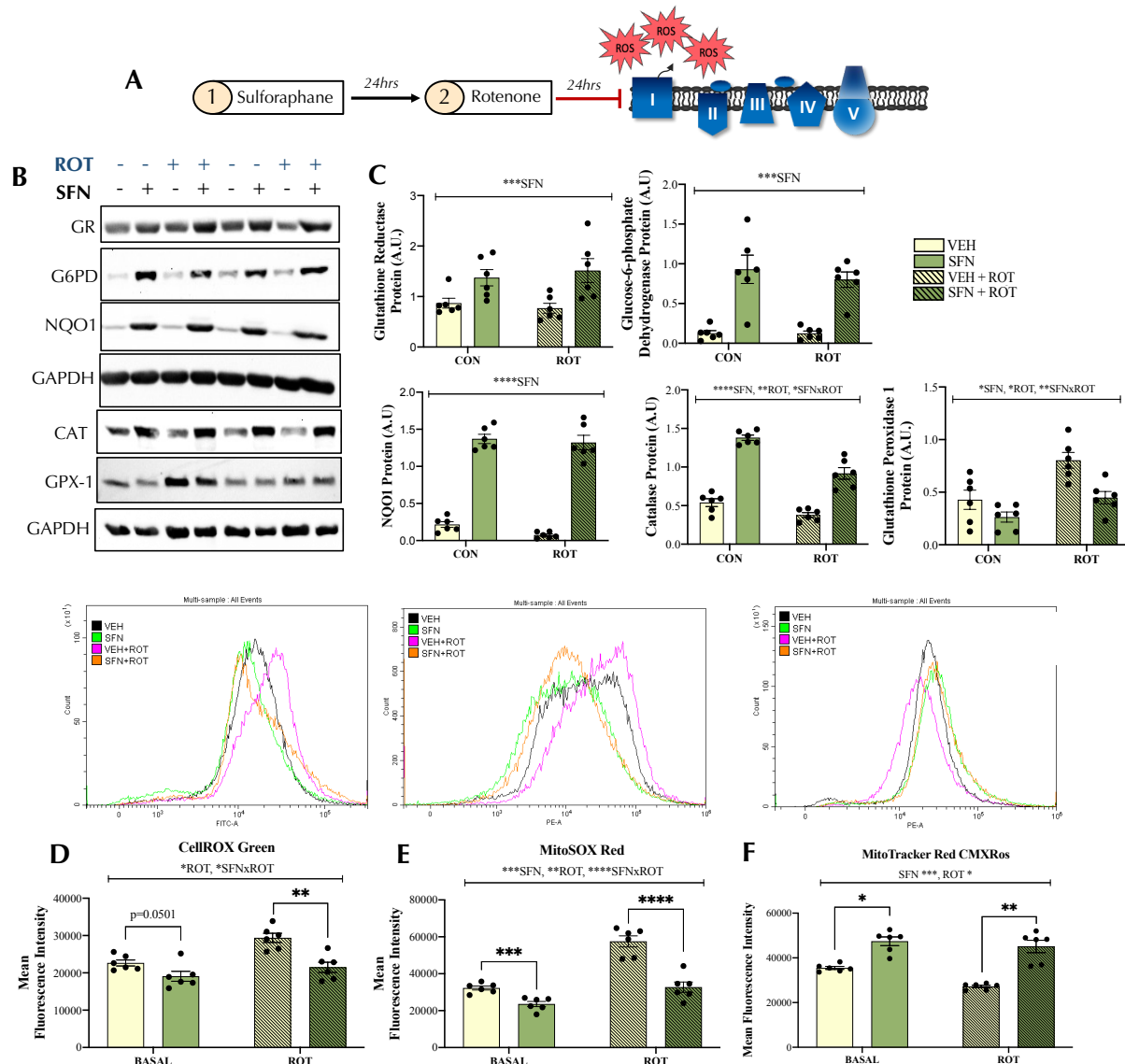


Figure 4: Antioxidant proteins and ROS with SFN pre-treatment followed by rotenone. **A)** Schematic depicting treatment. Myotubes were pre-treated with SFN or vehicle for 24hrs before co-incubation with 150nM rotenone (ROT), a complex 1 inhibitor, for 24hrs to induce oxidative stress. **B, C)** Representative western blots and corresponding quantifications of Glutathione Reductase (GR), Glucose-6-Phosphate Dehydrogenase (G6PD), NAD(P)H Quinone Oxidoreductase (NQO1), Catalase (CAT), and Glutathione Peroxidase-1 in whole cell lysates. All targets were normalized to GAPDH. **D, E, F)** Representative histograms and mean fluorescence intensity quantifications of CellROX Green, MitoSOX Red, and Mitotracker Red detected via flow cytometry. A two-way ANOVA was performed for all graphs to determine the effect of SFN and ROT. Statistics are shown as follows, “SFN” main effect of SFN, “ROT” main effect of rotenone “SFNxROT” reflects an interaction effect between SFN and ROT; * Denotes the p value for each effect. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. VEH=Vehicle, SFN=Sulforaphane, ROT=Rotenone. All values are represented as $\pm$ SEM.

Sulforaphane enhances PGC-1 α promoter activity, protein accumulation, and nuclear localization. To evaluate the molecular mechanisms underpinning Nrf-2 mediated mitochondrial biogenesis, PGC-1 α promoter activity was assessed to determine if there was an enhanced transcriptional drive for this regulator of mitochondrial biogenesis (Fig. 5A). Following transfection of a PGC-1 α -promoter-luciferase-reporter plasmid, there was approximately a 7-fold increase in luciferase activity (****PROM<0.0001) compared to the empty vector. After transfection of this plasmid and 24hrs of SFN treatment, there was an increase in promoter activity, evident through an interaction effect when combining the PGC-1 α promoter plasmid with SFN (***PROM+SFN; Fig. 5B). To determine what may be causing an increase in promoter activity, transcription factors previously reported to enhance PGC-1 α transcription were measured in nuclear and cytosolic fractions at the same timepoint promoter activity was observed (Fig. 5C, D). SFN treatment enhanced the nuclear accumulation of SP1 (**p<0.01) and CREB1 (**p<0.01) relative to vehicle-treated cells, however, no change was detected in USF-1 (p=0.117) or ATF-2 (p=0.103).

To determine the subcellular localization and accumulation of PGC-1 α , a CMV-promoter-GFP-PGC-1 α -reporter construct was employed. Following transfection, cells were co-treated with MG132, a proteasome inhibitor, to assess how treatment may augment PGC-1 α accumulation. PGC-1 α protein is very low basally, therefore, MG132 was required to begin with a more robust pool of PGC-1 α protein to assess any effect of SFN. We observed a main effect of SFN in PGC-1 α accumulation (***SFN<0.001) and nuclear localization (**SFN<0.01) basally, however, this was not significant following the post-hoc test. Upon co-treatment with MG132, there was a stark increase in PGC-1 α accumulation (**SFN+MG132<0.01) and translocation to the nucleus

(**SFN+MG132<0.01). MG132 treatment elicited a dramatic increase in PGC-1 α accumulation (****MG132<0.0001), however, this did not result in its nuclear localization (Fig. 6).

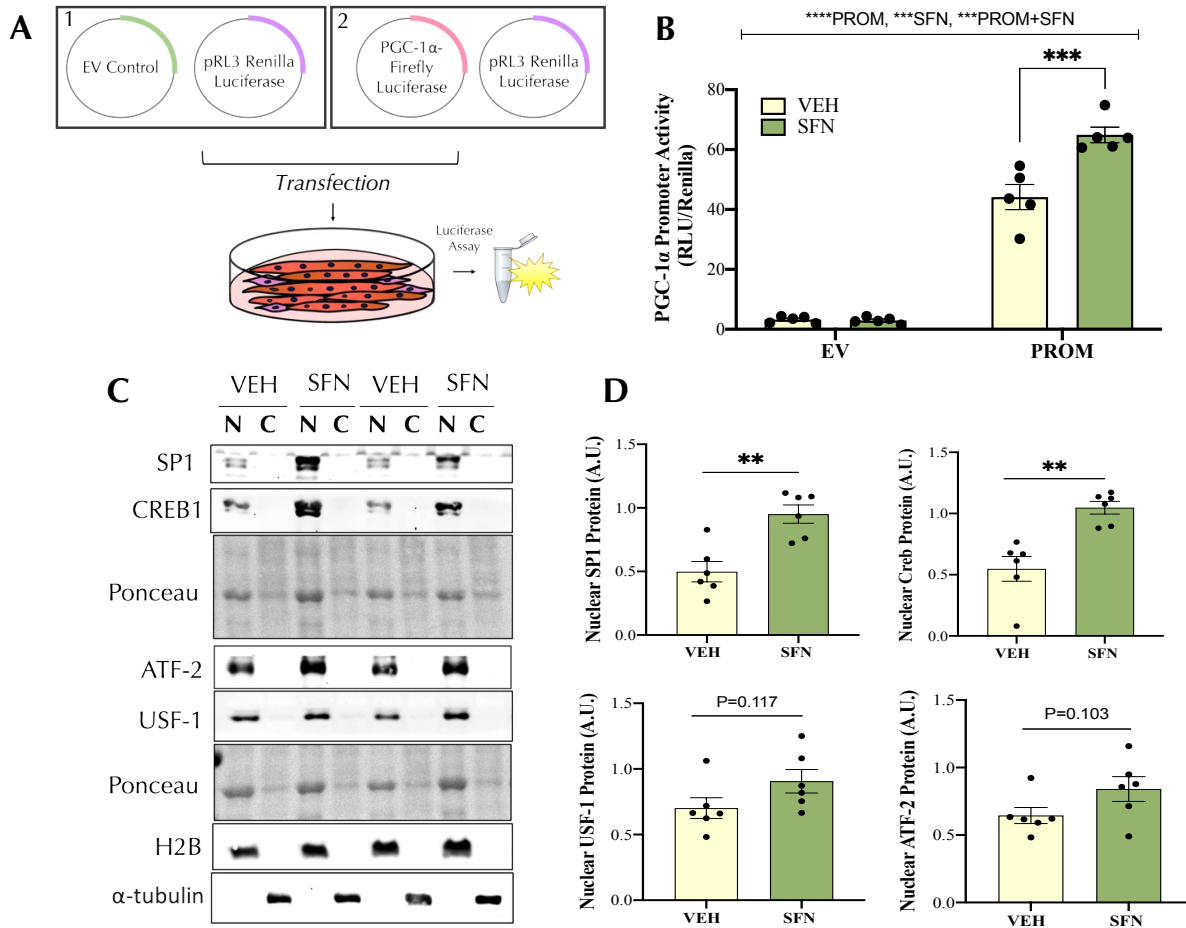


Figure 5: Transcriptional drive towards PGC-1 α following SFN treatment. **A)** Graphical representation of plasmids employed for transfection and subsequent luciferase assay. Myotubes were transfected with either an empty vector (EV) or a PGC-1 α -promoter-firefly-luciferase-reporter plasmid. In both instances, cells were co-transfected with a CMV-promoter-renilla-luciferase-reporter plasmid (pRL3) to control for transfection efficiency. **B)** PGC-1 α promoter activity was measured as relative light units (RLU), normalized to Renilla Luciferase (n=5). A two-way ANOVA was performed to determine the effect of the PGC-1 α promoter and SFN. Statistics are shown as follows, “PROM” main effect of PGC-1 α promoter, “SFN” main effect of SFN, “PROM+SFN” interaction effect between of PGC-1 α promoter and SFN treatment. * Denotes the p-value for each effect. **p<0.01, ***p<0.001, ****p<0.0001. **C, D)** Representative nuclear (N)-cytosolic (C) western blots and corresponding quantification of SP1 (**p<0.01), CREB1 (**p<0.01), USF-1 (p=0.117) and ATF-2 (p=0.103). All targets were normalized to ponceau, and purity was evaluated with H2B and α -tubulin. Significance was determined with a two-tailed unpaired T-test for each graph. All values are represented as $\pm$ SEM.

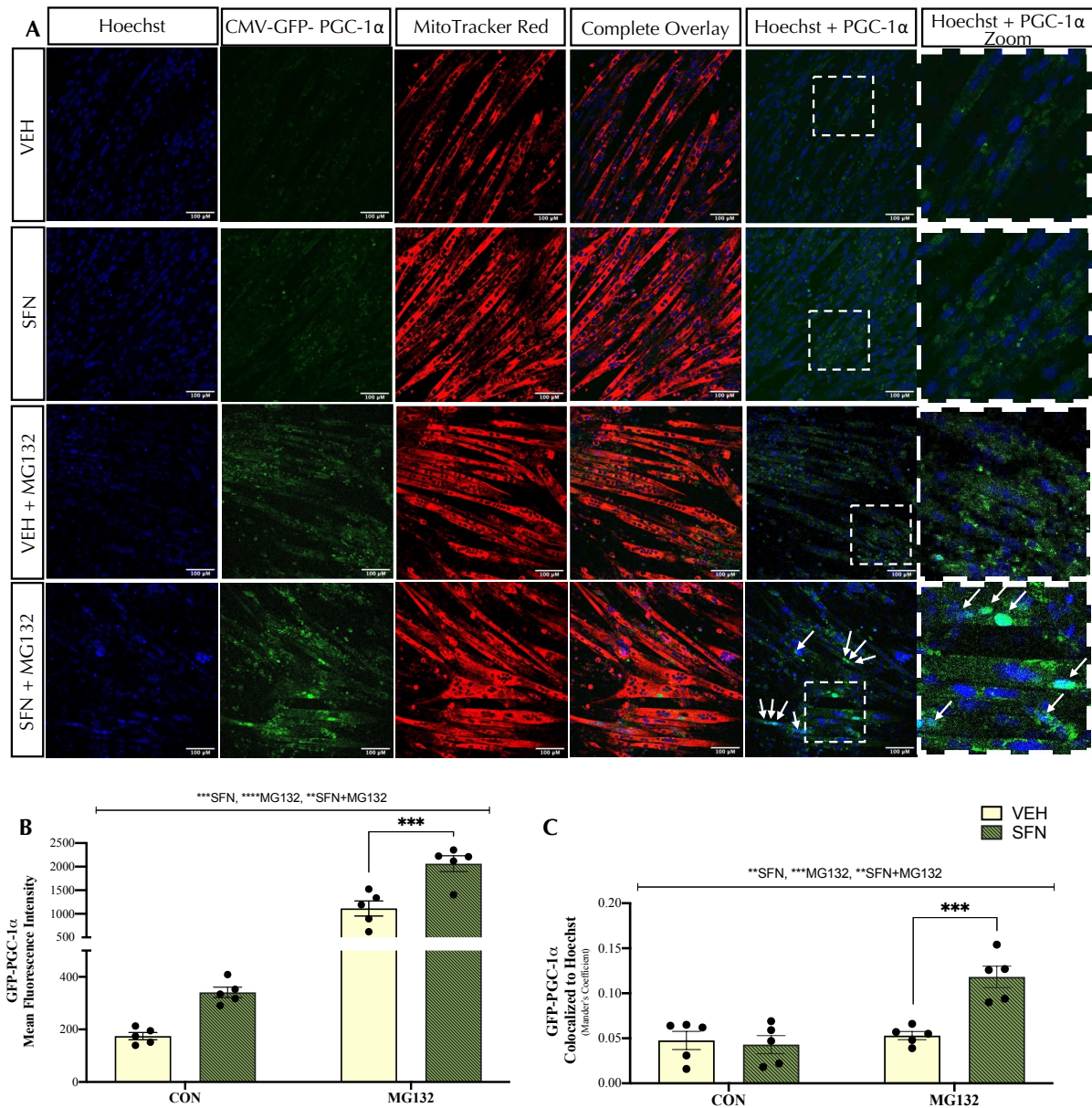


Figure 6: PGC-1 α accumulation and nuclear localization following SFN. **A)** Representative confocal images (20X magnification) of Vehicle (VEH), sulforaphane (SFN), VEH+MG132 and SFN+MG132 treated myotubes following 6hrs of treatment. Following treatment, cells were immediately stained with Hoechst and MitoTracker Red (n=5; roughly 15-20 myotubes per image). Hoechst and PGC-1 α overlay along with an inset panel enlarged by 2.8X, is provided to offer better visualization of nuclear PGC-1 α indicated by the white arrows. **B)** Mean fluorescence intensity of GFP was detected to measure PGC-1 α accumulation. **C)** Co-localization of GFP to Hoechst was determined using Mander's overlap coefficient. A two-way ANOVA was performed to evaluate the effect of SFN and MG132. Statistics are as follows, "SFN" main effect of SFN, "MG132" main effect of MG132, "SFN+MG132" interaction effect between SFN and MG132. * Denotes the p-value for each effect. **p<0.01, ***p<0.001, ****p<0.0001. All values are represented as $\pm$ SEM.

Effects of Sulforaphane on mitochondrial, lysosomal and antioxidant proteins when combined with CCA. Next to evaluate if there was any synergistic or additive effect of SFN treatment when combined with “exercise” an *in vitro* model of chronic contractile activity (CCA) was employed as previously outlined^{18–20}. Mitochondrial and lysosomal adaptations have been well documented both *in vivo* and *in vitro*, demonstrating that chronic muscle use enhances the synthesis of new mitochondria and lysosomes⁷. Consistent with previous reports, our CCA model elicited a marked increase in TFAM (**CCA<0.01), a protein involved in mtDNA replication, and TFE3 (***) (**CCA<0.001), a transcription factor which mediates the expression of lysosomal and autophagy-related genes. There was a main effect of SFN (****SFN<0.0001) treatment alone, as well as an interaction effect (**SFN+CCA<0.01) on TFAM protein expression. There was a significant increase in TFE3 protein with SFN treatment relative to untreated cells (\$p<0.01; Fig. 7A, B).

This was accompanied by an increase in mitochondrial and lysosomal-related proteins. There was a main effect of CCA and SFN on mitochondrial markers including COX I (**CCA<0.01, ****SFN<0.0001), UQCRC2 (*CCA<0.05, **SFN<0.01) and VDAC (*CCA<0.05, ****SFN<0.0001), as well as Mature Cathepsin B, a lysosomal protease which cleaves cellular cargo (*CCA<0.05, **SFN<0.01). There was also a main effect of SFN on Precursor Cathepsin B (***) (**SFN<0.001) and the lysosomal membrane protein vATPase, utilized as a measure of lysosomal mass (***) (**SFN<0.001; Fig. 7A, B).

Chronic exercise has been shown to enhance the levels of antioxidant proteins, which are essential for the reduction of ROS observed with training²². We were able to confirm that our model of CCA and SFN elicited an accumulation of antioxidant proteins, including NQO1 (****CCA<0.0001, ****SFN<0.0001), GR (*CCA<0.05, ***SFN<0.001), and GPX-1

(**CCA<0.01, ****SFN<0.0001). SFN also elicited a robust enhancement in CAT (****SFN<0.0001) and G6PD (****SFN<0.0001) protein. An interaction effect was observed for NQO1(****SFN+CCA<0.0001), HO-1 (**SFN+CCA<0.01), and GPX-1 protein (**SFN+CCA<0.01; Fig. 8A) B). There was a main effect of CCA and SFN on cellular (**CCA<0.01, **SFN<0.01) and mitochondrial ROS (**CCA<0.01, **SFN<0.01; Fig. 8C, D).

Effects of Sulforaphane on mitochondrial morphology when combined with CCA. Since our data revealed an effect of SFN on mitochondrial content, we sought to investigate if mitochondrial dynamics may be altered as well. To examine this, mitochondrial morphology was observed via confocal microscopy, and images were skeletonized to visualize mitochondrial length, branching and interconnectedness (Fig. 9C). While western blotting did not reveal any difference in MFN2, a fusion-related protein, there was significant alterations to DRP1, a protein recruited to the mitochondria for fission related processes. A two-way ANOVA revealed a main effect of SFN (****SFN<0.0001) and CCA (****CCA<0.0001), with an interaction effect between the two variables (****SFN+CCA<0.0001; Fig. 9A, B). Mitochondrial length (****SFN<0.0001) and the number of branch junctions (****SFN<0.0001) were enhanced following SFN treatment. In contrast to previous reports, we were unable to demonstrate that our model of CCA was able to alter mitochondrial morphology, though others have reported an increase in mitochondrial fusion

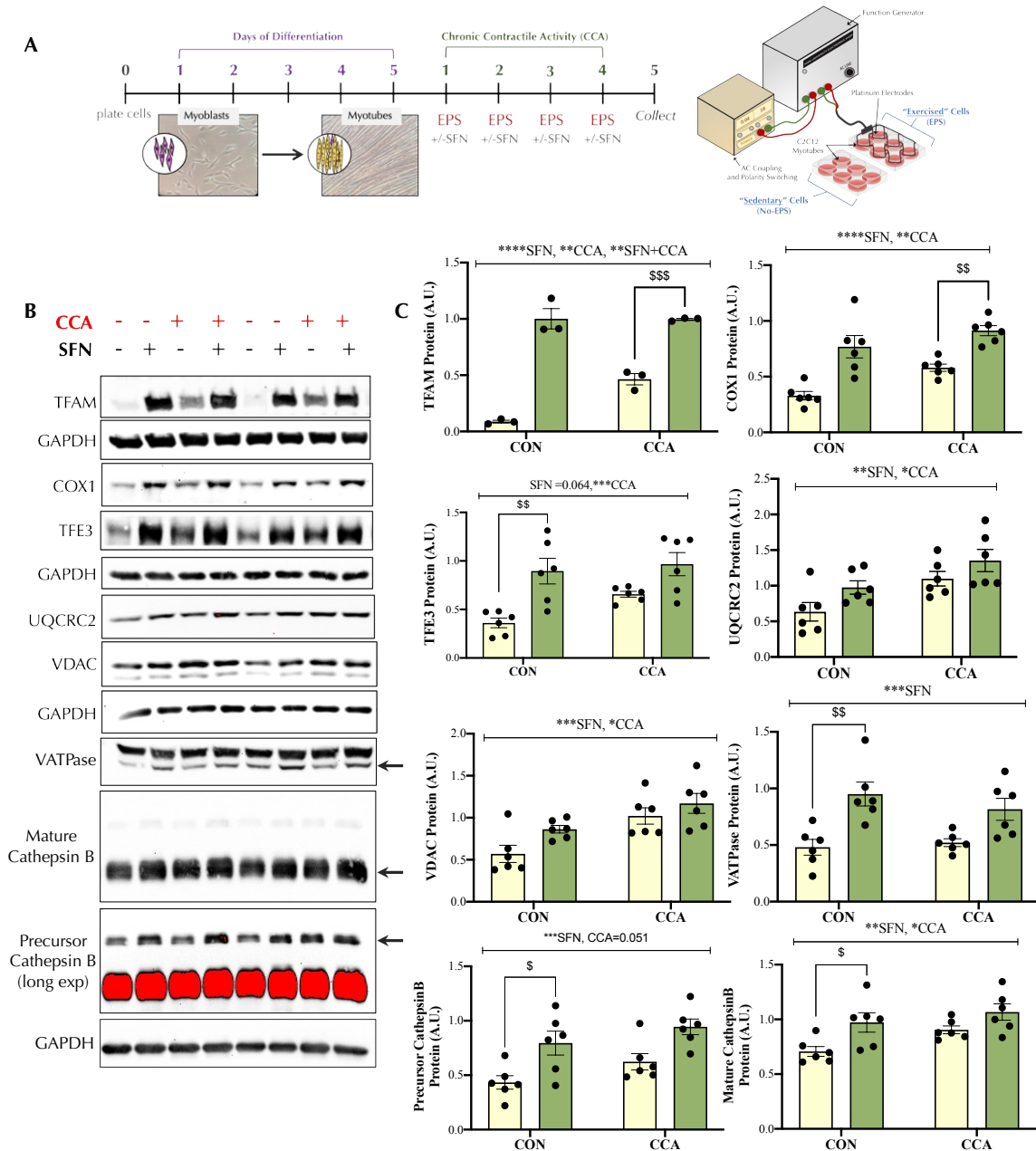


Figure 7. Mitochondrial and lysosomal proteins Following CCA. **A)** Schematic demonstrating the “exercise-in-a-dish” experimental model. Myoblasts were plated and differentiated into myotubes. Following differentiation, myotubes were subjected to 3 hours of Electrical Pulse Stimulation (EPS) followed by a 21-hour recovery, where they were supplemented with either vehicle or SFN. **B)** Representative western blots and corresponding quantifications of TFAM, COX I, TFE3, UQCRC2, VDAC, vATPase, and Cathepsin B. All targets were normalized to GAPDH. A two-way ANOVA was performed for all graphs to determine the effect of SFN and CCA. Statistics are shown as follows, “SFN” main effect of SFN, “CCA” main effect of CCA, and “SFN+CCA” interaction effect between SFN and CCA. * Denotes the p-value for each effect. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. SFN= Sulforaphane, CCA= Chronic Contractile Activity. All values are represented as $\pm$ SEM

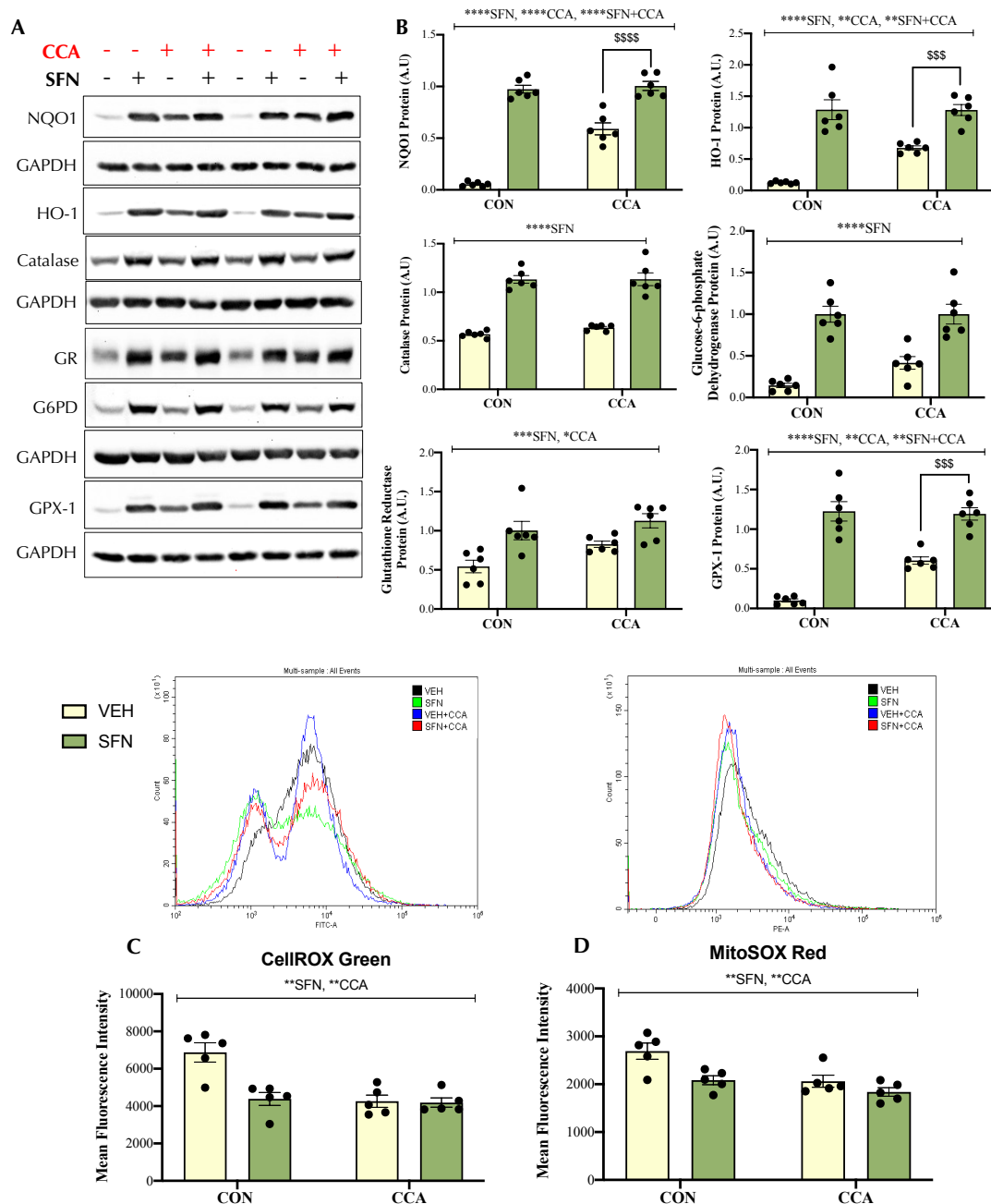


Figure 8. Antioxidant proteins and ROS following CCA. A, B) Representative western blots and the corresponding quantification for NQO1, Heme Oxygenase-1 (HO-1), Catalase (CAT), Glutathione reductase (GR), Glucose-6-phosphate-dehydrogenase (G6PD), and Glutathione peroxidase-1 (GPX-1) following CCA. All targets were normalized to GAPDH. A two-way ANOVA was performed for all graphs to determine the effect of SFN and CCA. Statistics are shown as follows, “SFN” main effect of SFN, “CCA” main effect of CCA, and “SFN+CCA” interaction effect between SFN and CCA. * Denotes the p-value for each effect. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. SFN= Sulforaphane, CCA= Chronic Contractile Activity. All values are represented as $\pm$ SEM

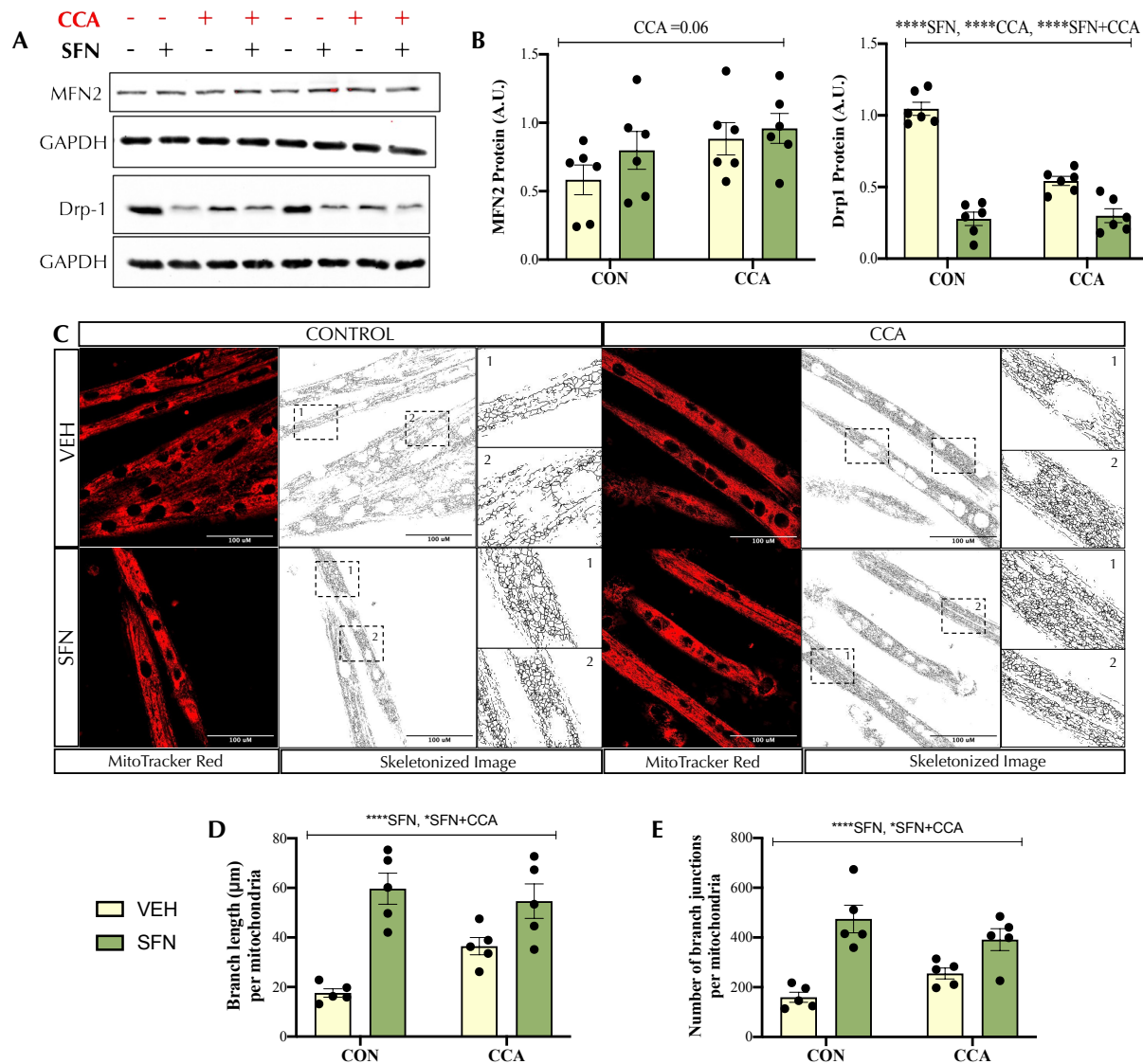


Figure 9. Mitochondrial Dynamics Following CCA. A, B) Representative western blots and corresponding quantifications of Mitofusin 2 (MFN2) and Dynamin-related protein-1 (Drp-1), both normalized to GAPDH (n=6). C) Representative confocal images (60X magnification) of myotubes stained with MitoTracker Red, subsequently skeletonized using ImageJ to illustrate mitochondrial morphology. Two inset panels numbered 1 and 2, are enlarged by 2.6X for each image to better visualize branching. D, E) Quantifications of mitochondrial branch length and the number of branch junctions per mitochondrion. One branch junction is defined as a point in the reticulum where 3 or more branches meet. A two-way ANOVA was performed for all graphs to determine the effect of SFN and CCA. Statistics are shown as follows, “SFN” main effect of SFN, “CCA” main effect of CCA, and “SFN+CCA” interaction effect between SFN and CCA. * Denotes the p-value for each effect. *P<0.05, ****P<0.0001. SFN= Sulforaphane, CCA= Chronic Contractile Activity. All values are represented as $\pm$ SEM

Sulforaphane activates upstream kinases consistent with acute contractile activity (ACA). To elucidate the precise mechanisms underlying Nrf-2 activation and alterations in mitochondrial content, an acute bout of contractile activity was performed to evaluate the expression of key kinases involved in mitochondrial biogenesis and Nrf-2 activation. AMPK (****ACA<0.0001) and JNK (****ACA<0.0001), two kinases which have been well documented to be upregulated with acute exercise, were significantly activated with our model of ACA (****ACA<0.0001). Other upstream kinases, including CAMK (****ACA<0.0001) and P38 MAPK (****ACA<0.0001) were also dramatically upregulated, demonstrating the sensitivity of these kinases to exercise-induced secondary messengers (Fig. S2). There was also a main effect of SFN on AMPK (*SFN<0.05) and JNK1 (*SFN<0.05) activation follow 3hrs of treatment (Fig. 10).

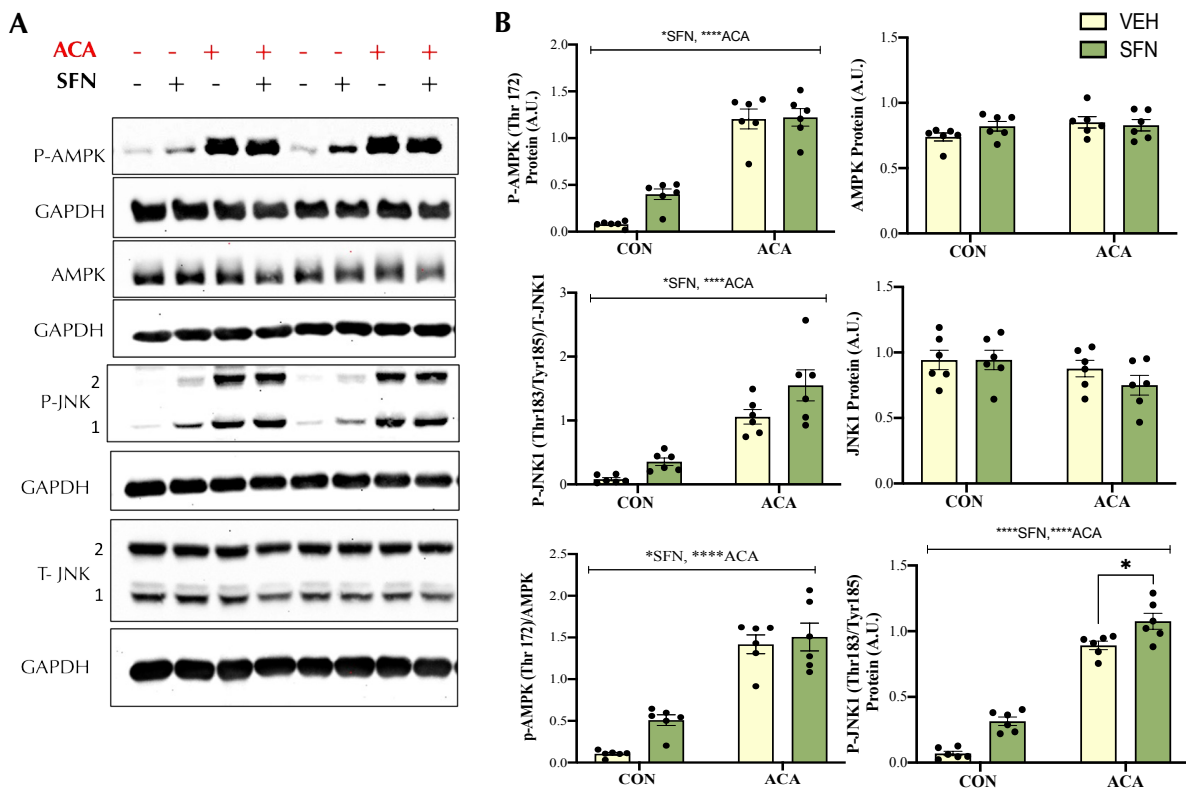


Figure 10. Upstream kinase activation in response to Acute Contractile Activity (ACA). A, B) Representative western blots and corresponding quantifications of phospho-AMPK, total-AMPK, phospho-JNK, and total-JNK. All targets were normalized to their corresponding GAPDH. To obtain a measure of kinase activation, phosphorylated targets were normalized to the total kinase protein present. A two-way ANOVA was performed for all graphs to determine the effect of SFN and ACA. Statistics are shown as follows, “SFN” main effect of SFN, “ACA” main effect of ACA. * Denotes the p-value for each effect. * $P < 0.05$, **** $P < 0.0001$. SFN= Sulforaphane, ACA= Acute Contractile Activity. All values are represented as $\pm$ SEM.

DISCUSSION

Mitochondria are critical organelles that play a pivotal role in the maintenance of skeletal muscle cellular homeostasis. However, mitochondrial phenotypes in muscle can be altered under a variety of disease conditions as well as with age ²², thereby highlighting the importance of investigating novel approaches to refresh and enhance the mitochondrial pool. The purpose of this study was to investigate the potential role of natural plant compound sulforaphane (SFN) in improving the mitochondrial phenotype through the activation of mitochondrial biogenesis and by increasing organelle antioxidant capacity. To further examine if the signaling cascades activated in response to SFN were common to those typically activated by exercise, we combined SFN treatment with a model of chronic contractile activity (CCA) of myotubes, which typically results in beneficial mitochondrial adaptations.

Previous work has shown that SFN increases the expression of antioxidant enzymes by activating the Nrf-2-Keap1 axis ^{9,23}. Upon SFN-mediated modifications to Keap1, Nrf-2 accumulates and translocates to the nucleus to bind to antioxidant response element (ARE) regions upstream of target genes ^{23–25}. We found that SFN induced a robust accumulation of whole cell as well as nuclear-localized Nrf-2 protein, likely mediating the upregulation of antioxidant proteins we observed. Cytosolic kinases AMPK ²⁶ and JNK ²⁷ have been reported to phosphorylate Nrf-2 and permit its release from Keap1. In both instances, these phosphorylation events enhance the translocation and nuclear retention of Nrf-2. Consistent with this, we observed increases in AMPK and JNK1 activation, similar to others ^{28,29}, suggesting that Nrf-2 release from Keap1 may be a mechanism driving Nrf-2 translocation. In addition, this translocation coincided with a marked reduction in Keap1 and an increase in p62 protein. Other groups have shown that Keap1 is removed through autophagy-mediated mechanisms ^{30,31} and that Nrf-2 signaling enhances autophagy

initiation ^{32,33}. These changes in protein levels suggest that Nrf-2 may elicit a p62-dependent positive feedback loop, to further confer Nrf-2 accumulation and signaling through the removal of Keap1 via autophagy ^{34,35}.

Nrf-2 regulates antioxidant enzyme expression by directly increasing target gene transcription. To investigate if this may have functional consequences to enhance mitochondrial preservation, cells were pre-treated with SFN, followed by rotenone to induce mitochondrial dysfunction and oxidative stress. Consistent with this signaling axis, Nrf-2 enhanced the expression of antioxidant enzymes including NQO1, HO-1, CAT, G6PD, GR and GPX-1. These changes in protein levels were sustained under rotenone-induced oxidative stress, and this served to blunt excessive cellular ROS. Importantly, this was accompanied by a reduction in mitochondrial ROS and an increase in membrane potential, suggesting that SFN protects mitochondrial function under oxidative stress. This enhancement was further exemplified when examining the corresponding increase in oxygen consumption and increased colocalization of MTG and MTR, indicating that the greater mitochondrial content also had an intact membrane potential. Consistent with these findings, we observed greater mitochondrial length and number of junctions per mitochondria, suggesting that SFN enhanced the extent of the mitochondrial reticulum. While SFN had no impact on MFN2 levels, it exerted a dramatic lowering effect on the pro-fission protein Drp-1, likely producing the more extensive organelle branching observed. Previous studies have showed that SFN reduces Drp-1 protein ³⁶ and recruitment to the mitochondria ³⁷.

This enhanced mitochondrial biogenesis is likely mediated in part by direct binding of Nrf-2 to ARE elements upstream of NRF-1, a transcription factor well known for its role in mediating the expression of TFAM ³⁸, as well as other NuGEMPs ³⁹. Indeed, Nrf-2 has been shown to increase NRF-1 and TFAM proteins, subsequently enhancing mtDNA copy number ³⁸.

Furthermore, overexpression of the downstream Nrf-2 target HO-1 has also been shown to increase PGC-1 α and TFAM protein ⁴⁰, whereas knockout significantly reduced total content and respiration ⁴¹, together suggesting that Nrf-2 signaling may play a role in mitochondrial biogenesis. To determine if Nrf-2-directed mitochondrial biogenesis was mediated in part by PGC-1 α , a PGC-1 α -promoter-luciferase-reporter construct was employed. Our results demonstrated an increase in promoter activity following SFN treatment, indicating a strong drive towards biogenesis. Isolation of nuclear fractions revealed a marked increase in Nrf-2 as noted above, which likely binds to putative ARE sites within the PGC-1 α promoter, along with both SP1 ^{19,42} and CREB ^{3,43}, which have both been previously reported to bind and enhance PGC-1 α promoter activity.

To assess if PGC-1 α was entering the nucleus to co-activate transcription factors involved in the expression of NuGEMPs, a CMV-promoter-GFP-PGC-1 α reporter plasmid was utilized. As PGC-1 α has been classified as an intrinsically disordered protein ⁴⁴, it is routinely targeted towards the proteasome for destruction, maintaining extremely low levels basally. To evaluate PGC-1 α accumulation and to ensure the reliability of our PGC-1 α measurements, the proteasome inhibitor MG132 was utilized. MG132 treatment resulted in a dramatic increase in whole cell PGC-1 α levels as expected. Nuclear translocation remained unchanged, suggesting that PGC-1 α levels are tightly regulated by proteasome activity. Interestingly, SFN enhanced PGC-1 α nuclear translocation in the presence of MG132. This suggests that SFN may help attenuate PGC-1 α decay, possibly via the marked induction of NQO1 (see Fig. 4), a well-documented downstream target of Nrf-2 ³¹, which has been shown to bind to PGC-1 α and protect it from the 20S proteasome, thereby increasing its half-life ⁴⁴. NQO1 also physically associates with the 20S proteasome, where it physically binds and blunts proteolytic activity, therefore further reducing PGC-1 α degradation ^{44,45}. These modifications could permit a high concentration gradient and sufficient time for PGC-

1 α PTMs necessary for nuclear localization, however the precise mechanisms underlying this finding must be further evaluated.

Exercise training *in vivo* or chronic contractile activity (CCA) in animal and cellular models have been reported to enhance the expression of antioxidant proteins, increase mitochondrial content, and reduce mitochondrial ROS emission ^{7,8}. To determine the potential additive effects of SFN when combined with exercise, C2C12 myotubes were chronically stimulated and supplemented with SFN during their recovery period between bouts of contractile activity. As expected, and shown previously, we established that our CCA model was effective in enhancing exercise-induced adaptations, including an increase in mitochondrial, lysosomal, and antioxidant-related proteins. However, CCA did not further enhance SFN-mediated adaptations. There was no difference between non-exercised and exercised cells treated with SFN for all proteins examined. This strongly suggests that SFN activates signaling pathways that are similar to those induced by exercise. In some instances, SFN treatment in presence of CCA resulted in a greater response than CCA alone, including mitochondrial markers TFAM and COX I. This may suggest that SFN is a strong stimulus for the activation of mtDNA transcription and/or replication, and future studies evaluating mtDNA copy number will be used to verify this.

SFN treatment also increased the levels of lysosomal-related proteins. This included TFE3, a transcription factor involved in lysosomal biogenesis, Cathepsin B, a protease involved in the degradation of cellular cargo, and vATPase, a lysosomal membrane complex responsible for acidification of the lysosomal lumen ⁴⁶. It is important to note the relationship between SFN and lysosomal content have been significantly underexplored, especially within the content of muscle research. However, two studies have demonstrated that SFN treatment enhanced lysosomal area and abundance of lysosomal proteins ^{47,48}. Furthermore, Nrf-2 overexpression, resulted in a marked

increase in lysosomal biogenesis, suggesting that Nrf-2 may regulate lysosomal content ⁴⁹. Interestingly, this may be driven by the ARE site found within the TFE3 promoter region ⁵⁰, which may mediate the enhancement in lysosome content observed. Together, this suggests that SFN increases cellular degradation capacity, which is useful for enhancing mitochondrial turnover to ensure the quality of the mitochondrial pool.

In conclusion, our results indicate that SFN increases lysosomal, mitochondrial, and antioxidant-related proteins similar to our *in vitro* model of chronic contractile activity. These are likely facilitated by Nrf-2, which translocates to the nucleus and enhances the expression of target genes. While further investigation is required to elucidate the precise mechanisms underlying these pathways, this study demonstrates that SFN can dramatically enhance the mitochondrial phenotype within skeletal muscle cells.

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FUTURE DIRECTIONS

1. **PGC-1 α transcription:** Since we observed an increase in PGC-1 α promoter activity and transcription factors (TFs) reported to bind to and enhance PGC-1 α promoter activity, it would be worth investigating if these are indeed binding using a Chromatin immunoprecipitation (ChIP) assay. This may be important to delineate if SFN-mediated PGC-1 α transcription is driven by the TFs identified or possibly Nrf-2. As mentioned earlier, two ARE sites exist upstream of the PGC-1 α promoter region, which may facilitate Nrf-2-mediated PGC-1 α transcription. Alternatively, a truncated PGC-1 α promoter plasmid can be utilized (i.e., without the ARE sites) to evaluate if PGC-1 α transcription persists.
2. **Nrf-2 accumulation:** We observed a robust accumulation of Nrf-2 protein in the nucleus, however the mechanisms mediating this increase remain unknown. While SFN has been shown to enhance Nrf-2 accumulation, it is currently unclear if this is due to 1) the release of Nrf-2 from Keap1, permitting its translocation or 2) nascent, newly translated Nrf-2 bypassing Keap1 and immediately translocating to the nucleus or 3) a combination of both. This can be investigated by performing an immunoprecipitation of Keap1 and seeing how much Nrf-2 remains bound following SFN as well as evaluating mRNA following treatment.
3. **Investigate the PGC-1 α -NQO1 axis:** Our results demonstrated that SFN reduced PGC-1 α protein decay in the presence of the proteasome inhibitor MG132. To evaluate if NQO1 may be mediating PGC-1 α accumulation, NQO1 protein can be measured with co-treatment. If there is an increase, two experiments can be completed to delineate the role of NQO1 in PGC-1 α stability 1) utilize the NQO1 inhibitor dicumarol and evaluate if PGC-1 α accumulation persists and 2) perform a co-immunoprecipitation assay to assess if NQO1 binds PGC-1 α to confer this protection as outlined by Adamovich ⁴⁴.

4. **Lysosomes and mitophagy**: We demonstrated that SFN enhanced the expression of lysosomal markers, which suggest an increase in content and degradation capacity. It would be interesting to evaluate if this is accompanied by an increase in mitophagy, providing another mechanism of SFN-mediated quality control, or if there is just a greater capacity for mitochondrial removal, proving more effective recycling of cellular components. This can be evaluated by examining mitophagy flux with mitochondrial-cytosolic samples or treating cells with Bafilomycin to examine autophagosome formation.

APPENDIX A: DATA AND STATISTICAL ANALYSIS

Table 1. Expression of upstream proteins of the Nrf-2-ARE antioxidant response following Sulforaphane (SFN)

A. Nrf-2 protein expression

NRF-2 PROTEIN (WHOLE CELL)		
N	VEH	SFN
1	0.06256991	1.16970796
2	0.0814786	1.41810933
3	0.03678749	1.35139273
4	0.03816761	0.92703088
5	0.04373133	1.08864647
6	0.10241223	1.24879257
X	0.06085786	1.200613323
SEM	0.01086655	0.073189082

Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

B. p62 protein expression

P62 PROTEIN		
N	VEH	SFN
1	0.24808923	0.608332052
2	0.4738185	0.831396857
3	0.32807331	0.706286025
4	0.37699621	0.907644995
5	0.26574245	0.307544304
6	0.34402906	0.643849669
X	0.33945813	0.667508984
SEM	0.03336395	0.085569551

Unpaired t test	
P value	0.0051
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

C. Keap1 protein expression

KEAP1 PROTEIN		
N	VEH	SFN
1	1.17699234	0.82425617
2	0.86271526	0.44993616
3	1.13203085	0.59349561
4	1.45487008	1.27102421
5	1.50132285	0.85102865
6	1.26169181	0.2671569
X	1.23160387	0.70948295
SEM	0.09530258	0.144338329

Unpaired t test	
P value	0.0129
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

D. Nuclear Nrf-2 protein expression

NUCLEAR NRF-2 PROTEIN		
N	VEH	SFN
1	0.17401552	1.88899113
2	0.16787843	1.63555124
3	0.13419849	1.57572071
4	0.10107122	1.30827341
5	0.10679202	1.55488169
6	0.22786064	1.86931109
X	0.15196939	1.638788212
SEM	0.01953291	0.088696228

Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

Table 2. Mitochondrial content following SFN

A. TFAM protein expression

TFAM PROTEIN		
N	VEH	SFN
1	0.19231825	0.98409868
2	0.04652291	0.61236413
3	0.0517099	0.37813226
4	0.0334408	0.44279
X	0.08099797	0.604346268
SEM	0.03730528	0.135876594

Unpaired t test	
P value	0.0099
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

B. COX I protein expression

COX I PROTEIN		
N	VEH	SFN
1	0.34697555	0.934642885
2	0.4576682	0.662683347
3	0.41009791	0.721438053
4	0.61918066	0.755187475
X	0.45848058	0.768487938
SEM	0.05816604	0.05858944

Unpaired t test	
P value	0.0095
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

C. MFN2 protein expression

MFN2 PROTEIN		
N	VEH	SFN
1	0.38668712	1.223950856
2	1.1701081	1.247259917
3	0.8488105	1.215849278
4	0.8344769	1.162393931
X	0.81002065	1.212363498
SEM	0.1609806	0.017937706

Unpaired t test	
P value	0.0476
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

D. MitoTracker Green mean fluorescence intensity

MITOTRACKER GREEN		
N	VEH	SFN
1	638077.167	762797.567
2	555587.533	751002.367
3	472254.467	824899.467
4	575378.9	762177.367
X	560324.517	775219.192
SEM	34218.5156	16780.37123

Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

E. MitoTracker Red mean fluorescence intensity

MITOTRACKER RED		
N	VEH	SFN
1	157929.9	193214.8
2	152792.033	217801.733
3	150873.467	192856
4	142850.4	173446.533
X	151111.45	194329.7665
SEM	3130.72413	9085.050866

Unpaired t test	
P value	0.0041
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

Table 3. Mitochondrial respiration and functional parameters following SFN treatment

A. Basal respiration

BASAL RESPIRATION				
N	24hr		48hr	
	VEH	SFN	VEH	SFN
1	1735.33	2314.49	799.55	2002.64
2	1745.18	2825.93	1791.07	2109.94
3	1609.82	2068.55	1223.76	2647.4
4	1444.2	1855.56	1262.42	2885.1
5	1520.51478	3703.45957	1345.85053	2635.10652
X	1611.008956	2553.597914	1284.530106	2456.037304
SEM	58.93786064	329.8520574	158.0712536	170.0195168

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.7120	0.5822	ns	No
Time	2.443	0.3137	ns	No
SFN	60.72	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary
24hr:VEH vs. 24hr:SFN	-942.6	-1767 to -117.9	Yes	*
24hr:VEH vs. 48hr:VEH	326.5	-498.2 to 1151	No	ns
24hr:VEH vs. 48hr:SFN	-845.0	-1670 to -20.36	Yes	*
24hr:SFN vs. 48hr:VEH	1269	444.4 to 2094	Yes	**
24hr:SFN vs. 48hr:SFN	97.56	-727.1 to 922.2	No	ns
48hr:VEH vs. 48hr:SFN	-1172	-1996 to -346.8	Yes	**

B. ATP production

ATP PRODUCTION				
	24hr		48hr	
N	VEH	SFN	VEH	SFN
1	1506.43	2028.22	691.19	1667.07
2	1460.57	2515.67	1506.46	1737.71
3	1389.09	1877.94	970.83	2309.41
4	1285.4	1670.02	1010.11	2375.21
5	862.92436	3167.86182	1014.81211	2335.9719
X	1300.882872	2251.942364	1038.680422	2085.07438
SEM	115.6670817	268.0507245	131.4452304	156.9784043

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.1462	0.7926	ns	No
Time	2.961	0.2464	ns	No
SFN	64.17	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
24hr:VEH vs. 24hr:SFN	-951.1	-1672 to -229.7	Yes	**	0.0081
24hr:VEH vs. 48hr:VEH	262.2	-459.2 to 983.6	No	ns	0.7292
24hr:VEH vs. 48hr:SFN	-784.2	-1506 to -62.81	Yes	*	0.0308
24hr:SFN vs. 48hr:VEH	1213	491.9 to 1935	Yes	***	0.0010
24hr:SFN vs. 48hr:SFN	166.9	-554.5 to 888.2	No	ns	0.9098
48hr:VEH vs. 48hr:SFN	-1046	-1768 to -325.0	Yes	**	0.0038

C. Maximal respiration

MAXIMAL RESPIRATION				
	24hr		48hr	
N	VEH	SFN	VEH	SFN
1	4785.85	7571.34	4964.78	6108.51
2	5873.1	8249.61	6028.81	5878.43
3	4542	7293.52	4193.71	7112.13
4	4487.12	6280.48	5342.08	9186.7
5	6331.43443	10892.936	6053.81515	9570.35096
X	5203.900886	8057.5772	5316.63903	7571.224192
SEM	377.2143629	776.3510997	349.0012714	768.8342598

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.7190	0.6265	ns	No
CCA	0.2796	0.7610	ns	No
SFN	52.27	0.0006	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
24hr:VEH vs. 24hr:SFN	-2854	-5296 to -411.0	Yes	*	0.0194
24hr:VEH vs. 48hr:VEH	-112.7	-2555 to 2330	No	ns	0.9991
24hr:VEH vs. 48hr:SFN	-2367	-4810 to 75.39	No	ns	0.0592
24hr:SFN vs. 48hr:VEH	2741	298.2 to 5184	Yes	*	0.0253
24hr:SFN vs. 48hr:SFN	486.4	-1956 to 2929	No	ns	0.9397
48hr:VEH vs. 48hr:SFN	-2255	-4697 to 188.1	No	ns	0.0758

D. Spare respiratory capacity

SPARE RESPIRATORY CAPACITY				
	24hr		48hr	
N	VEH	SFN	VEH	SFN
1	2619.1	5256.85	3948.37	4661.04
2	3910.28	5423.68	3768.49	4859.31
3	2932.18	5234.67	2969.95	4464.72
4	2729.1	4446.16	3636.68	6301.6
5	4821.672	7930.168	4338.03714	7462.2657
X	3402.4664	5658.3056	3732.30543	5549.7871
SEM	421.9166808	592.7199428	224.19123	577.35595

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.6112	0.6526	ns	No
Time	0.1558	0.8198	ns	No
SFN	52.77	0.0006	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
24hr:VEH vs. 24hr:SFN	-2256	-4189 to -322.9	Yes	*	0.0196
24hr:VEH vs. 48hr:VEH	-329.8	-2263 to 1603	No	ns	0.9606
24hr:VEH vs. 48hr:SFN	-2147	-4080 to -214.4	Yes	*	0.0269
24hr:SFN vs. 48hr:VEH	1926	-6.970 to 3859	No	ns	0.0510
24hr:SFN vs. 48hr:SFN	108.5	-1824 to 2041	No	ns	0.9985
48hr:VEH vs. 48hr:SFN	-1817	-3750 to 115.5	No	ns	0.0691

E. MitoTracker Red co-localized to MitoTracker Green

MTR-MTG CO-LOC		
N	VEH	SFN
1	0.791	0.849
2	0.688	0.979
3	0.817	0.972
4	0.781	0.93
5	0.746	0.979
6	0.673	0.933
7	0.769	0.97
8	0.65	0.986
9	0.798	0.905
X	0.74588889	0.944777778
SEM	0.02021856	0.01515272

Unpaired t test	
P value	<0.0001
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

Table 4. Antioxidant proteins and ROS with SFN pre-treatment followed by rotenone

A. Glutathione reductase protein expression

GLUTATHIONE REDUCTASE PROTEIN				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	0.77891927	1.78563205	0.98354631	2.44888201
2	0.83995513	1.44857108	1.12711501	1.84387739
3	1.34694931	1.87476637	0.53233906	1.642725
4	0.6940876	1.17388848	0.62193925	0.96200485
5	0.73143871	0.91634441	0.75248424	1.18204159
6	0.82139822	1.03667535	0.5893644	1.00465561
X	0.868791373	1.37264629	0.767798045	1.514031075
SEM	0.098179565	0.162176748	0.097372959	0.236200503

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.611	0.4550	ns	No
ROT	0.04473	0.9002	ns	No
SFN	42.85	0.0008	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:CON vs. CON:SFN	-0.5039	-1.133 to 0.1258	No	ns	0.1467
CON:CON vs. ROT:CON	0.1010	-0.5287 to 0.7306	No	ns	0.9691
CON:CON vs. ROT:SFN	-0.6452	-1.275 to -0.01560	Yes	*	0.0434
CON:SFN vs. ROT:CON	0.6048	-0.02480 to 1.234	No	ns	0.0625
CON:SFN vs. ROT:SFN	-0.1414	-0.7710 to 0.4883	No	ns	0.9217
ROT:CON vs. ROT:SFN	-0.7462	-1.376 to -0.1166	Yes	*	0.0167

B. Glucose-6-phosphate-dehydrogenase protein expression

G6PD PROTEIN				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	0.13479717	1.55997356	0.15804425	0.83127277
2	0.10668026	1.16680142	0.16630357	0.77133868
3	0.27266073	0.92807918	0.23503187	0.88927485
4	0.13390433	0.89469939	0.07989093	1.03175871
5	0.02851418	0.23347777	0.06900364	0.92510814
6	0.07669316	0.80649817	0.03832153	0.35467222
X	0.125541638	0.931588248	0.124432632	0.800570895
SEM	0.033643167	0.931588248	0.030324119	0.096167992

Source of Variation	% of total variation	P value	P value summary	Significant?
ROT	0.5643	0.5527	ns	No
SFN	71.01	0.0003	***	Yes
ROT x SFN	0.5455	0.5603	ns	No

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.8060	-1.350 to -0.2623	Yes	*	0.0104
CON:VEH vs. ROT:VEH	0.001109	-0.5426 to 0.5448	No	ns	>0.9999
CON:VEH vs. ROT:SFN	-0.6750	-1.219 to -0.1313	Yes	*	0.0217
CON:SFN vs. ROT:VEH	0.8072	0.2634 to 1.351	Yes	*	0.0103
CON:SFN vs. ROT:SFN	0.1310	-0.4127 to 0.6747	No	ns	0.8113
ROT:VEH vs. ROT:SFN	-0.6761	-1.220 to -0.1324	Yes	*	0.0216

C. NQO1 protein expression

NQO1 PROTEIN				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	0.26100822	1.59110957	0.04264502	1.20195987
2	0.15465014	1.26758376	0.0251984	1.03613115
3	0.2562663	1.21695438	0.05609325	1.21912298
4	0.35769652	1.36751305	0.12422732	1.24601678
5	0.09848376	1.26400823	0.12004995	1.56414308
6	0.16224977	1.51358252	0.07484287	1.65914438
X	0.215059118	1.370125252	0.073842802	1.321086373
SEM	0.038376497	0.061821727	0.016662659	0.097430721

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.1389	0.4614	ns	No
ROT	0.5917	0.1368	ns	No
SFN	94.34	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:CON vs. CON:SFN	-1.155	-1.398 to -0.9121	Yes	****	<0.0001
CON:CON vs. ROT:CON	0.1412	-0.1017 to 0.3841	No	ns	0.3867
CON:CON vs. ROT:SFN	-1.106	-1.349 to -0.8631	Yes	****	<0.0001
CON:SFN vs. ROT:CON	1.296	1.053 to 1.539	Yes	****	<0.0001
CON:SFN vs. ROT:SFN	0.04904	-0.1939 to 0.2920	No	ns	0.9413
ROT:CON vs. ROT:SFN	-1.247	-1.490 to -1.004	Yes	****	<0.0001

D. Catalase protein expression

CATALASE PROTEIN				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	0.68655066	1.28358929	0.41919956	1.07845707
2	0.44678396	1.40520563	0.30841098	1.16800217
3	0.59017269	1.31868293	0.44888277	0.73781245
4	0.60234935	1.48873849	0.34958866	0.69812235
5	0.34021034	1.32431058	0.27681189	0.92910034
6	0.5654384	1.46270696	0.46491862	0.89385382
X	0.538584233	1.38053898	0.377968747	0.917558033
SEM	0.05070242	0.034377415	0.031709873	0.075225522

Source of Variation	% of total variation	P value	P value summary	Significant?
ROT	14.97	0.0015	**	Yes
SFN	73.49	<0.0001	****	Yes
ROT x SFN	3.520	0.0255	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.8420	-1.093 to -0.5912	Yes	***	0.0002
CON:VEH vs. ROT:VEH	0.1606	-0.09012 to 0.4114	No	ns	0.2024
CON:VEH vs. ROT:SFN	-0.3790	-0.6297 to -0.1282	Yes	**	0.0096
CON:SFN vs. ROT:VEH	1.003	0.7518 to 1.253	Yes	****	<0.0001
CON:SFN vs. ROT:SFN	0.4630	0.2122 to 0.7137	Yes	**	0.0039
ROT:VEH vs. ROT:SFN	-0.5396	-0.7903 to -0.2889	Yes	**	0.0020

E. Glutathione peroxidase-1 protein expression

GPX-1 PROTEIN				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	0.39744904	0.28589806	0.91100564	0.66812955
2	0.53614322	0.37819246	0.67275878	0.50941852
3	0.69363023	0.38114858	0.8207936	0.22847003
4	0.21638091	0.12265901	0.7446025	0.42350336
5	0.12635066	0.11650284	0.57530701	0.47372118
6	0.59959092	0.29654403	1.0938035	0.38531388
X	0.428257497	0.26349083	0.803045172	0.448092753
SEM	0.090995558	0.048316139	0.075003474	0.059333471

Source of Variation	% of total variation	P value	P value summary	Significant?
ROT	30.85	0.0136	*	Yes
SFN	26.63	0.0160	*	Yes
ROT x SFN	3.566	0.0228	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:CON vs. CON:SFN	0.1648	0.01184 to 0.3177	Yes	*	0.0379
CON:CON vs. ROT:CON	-0.3748	-0.5277 to -0.2219	Yes	**	0.0011
CON:CON vs. ROT:SFN	-0.01984	-0.1728 to 0.1331	No	ns	0.9606
CON:SFN vs. ROT:CON	-0.5396	-0.6925 to -0.3866	Yes	***	0.0002
CON:SFN vs. ROT:SFN	-0.1846	-0.3375 to -0.03167	Yes	*	0.0243
ROT:CON vs. ROT:SFN	0.3550	0.2020 to 0.5079	Yes	**	0.0014

F. CellROX Green mean fluorescence intensity

CELLROX GREEN MEAN FLUORESCENCE INTENSITY				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	25613.2	25199	26496.6	26661.8667
2	24447.9	19536	30108.8	23011.9
3	21115.3	18902.2	25666.8667	17752.8
4	22606	17337.2	31060.8	22843.0333
5	21468.9	15871.5	33871.5667	20288.6333
6	20687.7	17622	29037.6667	18382.3667
X	22656.5	19077.98333	29373.71668	21490.1
SEM	808.6120566	1330.905375	1234.891617	1365.801527

Source of Variation	% of total variation	P value	P value summary	Significant?
ROT	23.84	0.0110	*	Yes
SFN	37.58	0.0065	**	Yes
ROT x SFN	5.302	0.0257	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
BASAL:VEH vs. BASAL:SFN	3579	-1.361 to 7158	No	ns	0.0501
BASAL:VEH vs. ROT:VEH	-6717	-10297 to -3137	Yes	**	0.0037
BASAL:VEH vs. ROT:SFN	1166	-2413 to 4746	No	ns	0.6514
BASAL:SFN vs. ROT:VEH	-10296	-13876 to -6716	Yes	***	0.0005
BASAL:SFN vs. ROT:SFN	-2412	-5992 to 1168	No	ns	0.1770
ROT:VEH vs. ROT:SFN	7884	4304 to 11463	Yes	**	0.0018

G. MitoSOX Red mean fluorescence intensity

MITOSOX RED MEAN FLUORESCENCE INTENSITY				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	33705.7	17992.6	63058.6	29907.1
2	30388.8	25922.7	61977.9	44153.4
3	35791	26323.5	64713.6	34549.7
4	28466.1	22452	58719.9	34494.6
5	31558	26921.9	48026.7	29116.8
6	33442.8	21737.4	48546.3	23976.6
X	32225.4	23558.35	57507.16667	32699.7
SEM	1069.855333	1415.004315	3024.266587	2796.185917

Source of Variation	% of total variation	P value	P value summary	Significant?
ROT	39.92	0.0015	**	Yes
SFN	37.75	0.0005	***	Yes
ROT x SFN	8.777	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
BASAL:VEH vs. BASAL:SFN	8667	5343 to 11991	Yes	***	0.0008
BASAL:VEH vs. ROT:VEH	-25282	-28606 to -21958	Yes	****	<0.0001
BASAL:VEH vs. ROT:SFN	-474.3	-3798 to 2850	No	ns	0.9490
BASAL:SFN vs. ROT:VEH	-33949	-37273 to -30625	Yes	****	<0.0001
BASAL:SFN vs. ROT:SFN	-9141	-12465 to -5817	Yes	***	0.0006
ROT:VEH vs. ROT:SFN	24807	21484 to 28131	Yes	****	<0.0001

H. MitoTracker Red mean fluorescence intensity

MTR FLUORESCENCE INTENSITY				
	CON		ROT	
N	VEH	SFN	VEH	SFN
1	34183.3	49233.6	25319.1	52010.5667
2	33687.4	43797.3	28680.5	48981.3667
3	38238.7	49708.8	25572.0333	48168.4
4	35080.2	49476.8	27912.1	48329.5
5	36247.8	39691	27657.5	36897.9667
6	35485.3	52628.6	27933.1667	36251.5667
X	35487.11667	47422.68333	27179.06667	45106.56113
SEM	664.6892113	1938.633915	566.6050206	2758.221361

Source of Variation	% of total variation	P value	P value summary	Significant?
ROT	8.797	0.0243	*	Yes
SFN	69.50	0.0007	***	Yes
ROT x SFN	2.798	0.1145	ns	No

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
BASAL:VEH vs. BASAL:SFN	-11936	-20124 to -3747	Yes	*	0.0112
BASAL:VEH vs. ROT:VEH	8308	119.5 to 16497	Yes	*	0.0474
BASAL:VEH vs. ROT:SFN	-9619	-17808 to -1431	Yes	*	0.0271
BASAL:SFN vs. ROT:VEH	20244	12055 to 28432	Yes	**	0.0010
BASAL:SFN vs. ROT:SFN	2316	-5872 to 10505	No	ns	0.7343
ROT:VEH vs. ROT:SFN	-17927	-26116 to -9739	Yes	**	0.0018

Table 5. Transcriptional drive towards PGC-1 α following SFN treatment

A. Luciferase activity: Sample luminescence reading of Luciferase reporter assay. Samples were plated in duplicates, normalized to Renilla to obtain a ratio (red). These values were averaged (highlighted in yellow).

	1	2	3	4	5	6	7	8	9	10	11	12	
A	EV VEH 1907	EV VEH 1519	EV VEH	EV VEH 2429	EV VEH 2295	EV VEH							Well ID
	879	838		654	673								Luciferase activity:Lum
	2.17	1.813	1.9915	3.714	3.41	3.562							Renilla Activity:Lum
													Firefly/Renilla ratio
B	EV SFN 2629	EV SFN 2287	EV SFN	EV SFN 2510	EV SFN 2200	EV SFN							Well ID
	695	723		806	921								Luciferase activity:Lum
	3.783	3.163	3.473	3.114	2.389	2.7515							Renilla Activity:Lum
													Firefly/Renilla ratio
C	PROM VEH 28926	PROM VEH 27822	PROM VEH	PROM VEH 27512	PROM VEH 27536	PROM VEH							Well ID
	647	653		627	696								Luciferase activity:Lum
	44.708	42.606	43.857	43.879	39.563	41.721							Renilla Activity:Lum
													Firefly/Renilla ratio
D	PROM SFN 39354	PROM SFN 37208	PROM SFN	PROM SFN 41006	PROM SFN 40982	PROM SFN							Well ID
	601	593		653	701								Luciferase activity:Lum
	65.481	62.745	64.113	62.796	58.462	60.629							Renilla Activity:Lum
													Firefly/Renilla ratio
E	N=4			N=5									Well ID
													Luciferase activity:Lum
													Renilla Activity:Lum
													Firefly/Renilla ratio

PGC-1 PROMOTER ACTIVITY				
N	EV		PROMOTER	
	VEH	SFN	VEH	SFN
1				
2	3.617	2.5745	91.5355	124.756
3	4.3795	2.665	50.5285	63.9495
4	4.0745	4.36	30.227	61.0835
5	1.9915	3.473	43.657	64.113
6	3.562	2.7515	41.721	60.629
X	3.5249	3.1648	51.5338	74.9062
SEM	0.411920848	0.338567667	10.52012685	12.48288924

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	3.743	0.0006	***	Yes
PROM	89.38	<0.0001	****	Yes
SFN	3.550	0.0008	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
EV:VEH vs. EV:SFN	0.2747	-9.764 to 10.31	No	ns	0.9998
EV:VEH vs. PROM:VEH	-40.91	-50.95 to -30.87	Yes	****	<0.0001
EV:VEH vs. PROM:SFN	-61.69	-71.73 to -51.65	Yes	****	<0.0001
EV:SFN vs. PROM:VEH	-41.19	-51.23 to -31.15	Yes	****	<0.0001
EV:SFN vs. PROM:SFN	-61.97	-72.01 to -51.93	Yes	****	<0.0001
PROM:VEH vs. PROM:SFN	-20.78	-30.82 to -10.74	Yes	***	0.0001

B. Nuclear SP1 protein expression

SP1 PROTEIN		
N	VEH	SFN
1	0.97305511	1.19077298
2	0.31016562	0.97084426
3	0.49632661	1.14585395
4	0.55354913	1.09650521
5	0.80944411	1.08804326
6	0.70839408	1.39945197
X	0.64182244	1.148578605
SEM	0.09681621	0.058506666

Unpaired t test	
P value	0.0012
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

C. Nuclear CREB protein expression

CREB PROTEIN		
N	VEH	SFN
1	0.23613443	1.24447485
2	0.56344934	1.07650075
3	0.5491851	1.05917769
4	0.99797144	1.47725722
5	0.95341654	1.24189753
6	1.06916374	1.26880261
X	0.7282201	1.228018442
SEM	0.13429731	0.062105171

Unpaired t test	
P value	0.0070
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed

D. Nuclear ATF-2 protein expression

ATF-2 PROTEIN		
N	VEH	SFN
1	1.21230296	1.8024394
2	1.54371597	1.49601832
3	1.23185056	1.48078678
4	1.10831969	1.41305224
5	1.13215807	1.53556914
6	1.84240735	2.21382905
X	1.34512577	1.656949155
SEM	0.11812882	0.124154924

Unpaired t test	
P value	0.0988
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed

E. Nuclear USF-1 protein expression

USF-1 PROTEIN		
N	VEH	SFN
1	1.2753858	1.62969952
2	1.35336194	1.69809785
3	1.49614074	1.81613182
4	1.31504145	1.64732698
5	1.45939002	2.10628363
6	2.08174801	2.56699819
X	1.49684466	1.910756332
SEM	0.12195614	0.149605

Unpaired t test	
P value	0.0576
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed

Table 6. PGC-1 α accumulation and nuclear localization following SFN.

A. GFP-PGC-1 α mean fluorescence intensity

GFP MEAN FLUORESCENCE				
N	CON		MG132	
	VEH	SFN	VEH	SFN
1	139.54	334.15	620.4	2116.93
2	151.45	408.94	895.5	1403.4
3	195.22	317.08	1523.54	2211.71
4	174.05	290.94	1190.47	2354.36
5	213.06	353.03	1335.91	2224.25
X	174.664	340.828	1113.164	2062.13
SEM	13.545	19.856	160.342	168.965

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	6.248	0.0041	**	Yes
MG-132	72.13	<0.0001	****	Yes
SFN	12.68	0.0002	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-166.2	-639.9 to 307.6	No	ns	0.7496
CON:VEH vs. MG132:VEH	-938.5	-1412 to -464.8	Yes	***	0.0002
CON:VEH vs. MG132:SFN	-1887	-2361 to -1414	Yes	****	<0.0001
CON:SFN vs. MG132:VEH	-772.3	-1246 to -298.6	Yes	**	0.0013
CON:SFN vs. MG132:SFN	-1721	-2195 to -1248	Yes	****	<0.0001
MG132:VEH vs. MG132:SFN	-949.0	-1423 to -475.2	Yes	***	0.0002

B. Nuclear GFP-PGC-1 α (Mander's coefficient)

GFP-HOECHST OVERLAP				
N	CON		MG132	
	VEH	SFN	VEH	SFN
1	0.016	0.022	0.057	0.154
2	0.062	0.018	0.066	0.094
3	0.065	0.069	0.048	0.09
4	0.031	0.059	0.039	0.127
5	0.064	0.047	0.055	0.126
X	0.0476	0.043	0.053	0.1182
SEM	0.0101	0.01	0.0045	0.0118

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	23.35	0.0021	**	Yes
MG132	31.14	0.0006	***	Yes
SFN	17.60	0.0059	**	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	0.004600	-0.03399 to 0.04319	No	ns	0.9858
CON:VEH vs. MG132:VEH	-0.005400	-0.04399 to 0.03319	No	ns	0.9775
CON:VEH vs. MG132:SFN	-0.07060	-0.1092 to -0.03201	Yes	***	0.0004
CON:SFN vs. MG132:VEH	-0.01000	-0.04859 to 0.02859	No	ns	0.8790
CON:SFN vs. MG132:SFN	-0.07520	-0.1138 to -0.03661	Yes	***	0.0002
MG132:VEH vs. MG132:SFN	-0.06520	-0.1038 to -0.02661	Yes	***	0.0009

Table 7. Mitochondrial and lysosomal proteins Following CCA

A. TFAM protein expression

TFAM PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.06720434	0.90797708	0.36452014	0.97961723
2	0.0849262	0.91302432	0.50077736	1.00327777
3	0.11036943	1.18260992	0.52624795	1.00653573
X	0.08749999	1.001203773	0.463848483	0.99647691
SEM	0.012526965	0.090714775	0.050205503	0.00848214

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.896	0.0066	**	Yes
CCA	5.607	0.0075	**	Yes
SFN	84.93	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.9137	-1.151 to -0.6764	Yes	****	<0.0001
CON:VEH vs. CCA:VEH	-0.3763	-0.6136 to -0.1391	Yes	**	0.0042
CON:VEH vs. CCA:SFN	-0.9090	-1.146 to -0.6717	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	0.5374	0.3001 to 0.7746	Yes	***	0.0004
CON:SFN vs. CCA:SFN	0.004727	-0.2325 to 0.2420	No	ns	0.9999
CCA:VEH vs. CCA:SFN	-0.5326	-0.7699 to -0.2954	Yes	***	0.0004

B. COX I protein expression

COX I PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.29710279	1.19063295	0.60133364	1.07813572
2	0.30651589	0.71043627	0.5437474	0.90275065
3	0.35879295	0.82357209	0.70275428	0.94350345
4	0.21010502	0.58724834	0.46758085	0.96387021
5	0.31227496	0.48517805	0.61286593	0.76668639
6	0.4899186	0.8137666	0.55308288	0.82215846
X	0.329118368	0.768472383	0.580227497	0.912850813
SEM	0.037736385	0.099962178	0.032290579	0.04492691

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.081	0.3856	ns	No
CCA	14.85	0.0037	**	Yes
SFN	56.58	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.4394	-0.6775 to -0.2012	Yes	***	0.0003
CON:VEH vs. CCA:VEH	-0.2511	-0.4892 to -0.01297	Yes	*	0.0365
CON:VEH vs. CCA:SFN	-0.5837	-0.8219 to -0.3456	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	0.1882	-0.04989 to 0.4264	No	ns	0.1539
CON:SFN vs. CCA:SFN	-0.1444	-0.3825 to 0.09376	No	ns	0.3512
CCA:VEH vs. CCA:SFN	-0.3326	-0.5708 to -0.09449	Yes	**	0.0044

C. TFE3 protein expression

TFE3 PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.4745162	1.18518869	0.7179209	1.2170404
2	0.20396118	0.59494345	0.54039741	0.69667415
3	0.22609293	0.47977173	0.60047826	0.56218491
4	0.36087956	1.31353805	0.73878501	1.23209844
5	0.48042166	0.92269152	0.67985165	0.89578644
6	0.42017447	0.8744534	0.6653313	1.19609924
X	0.361007667	0.895097807	0.657127422	0.966647263
SEM	0.049500909	0.132037993	0.030431632	0.119346246

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	3.155	0.2444	ns	No
SFN	8.457	0.0636	ns	No
CCA	44.53	0.0002	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.5341	-0.9046 to -0.1635	Yes	**	0.0033
CON:VEH vs. CCA:VEH	-0.2961	-0.6667 to 0.07443	No	ns	0.1475
CON:VEH vs. CCA:SFN	-0.6056	-0.9762 to -0.2351	Yes	***	0.0010
CON:SFN vs. CCA:VEH	0.2380	-0.1326 to 0.6085	No	ns	0.3036
CON:SFN vs. CCA:SFN	-0.07155	-0.4421 to 0.2990	No	ns	0.9480
CCA:VEH vs. CCA:SFN	-0.3095	-0.6801 to 0.06103	No	ns	0.1228

D. UQCRC2 protein expression

UQCRC2 PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.69212012	1.230624	0.91316281	1.43351892
2	0.7177049	1.28188252	1.49959602	1.66893318
3	1.19700334	0.95044555	1.28149974	1.91729911
4	0.48009811	0.86248086	0.96768714	1.01943661
5	0.38346301	0.75984497	0.83913527	1.03712234
6	0.33384737	0.76702523	1.08852639	1.04667254
X	0.634039475	0.975383855	1.098267895	1.35383045
SEM	0.12960239	0.093510836	0.102175994	0.155949368

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.3228	0.7305	ns	No
SFN	31.15	0.0026	**	Yes
CCA	15.63	0.0246	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.3413	-0.8273 to 0.1447	No	ns	0.2338
CON:VEH vs. CCA:VEH	-0.4642	-0.9502 to 0.02178	No	ns	0.0644
CON:VEH vs. CCA:SFN	-0.7198	-1.206 to -0.2338	Yes	**	0.0026
CON:SFN vs. CCA:VEH	-0.1229	-0.6089 to 0.3631	No	ns	0.8929
CON:SFN vs. CCA:SFN	-0.3784	-0.8645 to 0.1076	No	ns	0.1632
CCA:VEH vs. CCA:SFN	-0.2556	-0.7416 to 0.2304	No	ns	0.4720

E. VDAC protein expression

VDAC PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.4215916	0.85590284	0.81988122	1.28146697
2	0.63214833	1.00766243	1.41339081	1.620341
3	1.04569838	0.86925142	1.12858902	1.29880685
4	0.5348614	0.96429827	1.10405767	1.08204155
5	0.40268496	0.76247144	0.83630913	0.84118157
6	0.38097244	0.71784772	0.82387411	0.90005376
X	0.569659518	0.862905687	1.021016993	1.170648617
SEM	0.102816371	0.045626429	0.097615336	0.118399015

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.360	0.4592	ns	No
SFN	37.99	0.0007	***	Yes
CCA	12.93	0.0305	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.2932	-0.6698 to 0.08331	No	ns	0.1631
CON:VEH vs. CCA:VEH	-0.4514	-0.8279 to -0.07480	Yes	*	0.0153
CON:VEH vs. CCA:SFN	-0.6010	-0.9775 to -0.2244	Yes	**	0.0012
CON:SFN vs. CCA:VEH	-0.1581	-0.5347 to 0.2184	No	ns	0.6488
CON:SFN vs. CCA:SFN	-0.3077	-0.6843 to 0.06882	No	ns	0.1346
CCA:VEH vs. CCA:SFN	-0.1496	-0.5262 to 0.2269	No	ns	0.6864

F. vATPase protein expression

VATPASE PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.37476967	0.88123826	0.51754238	0.97387654
2	0.45018368	0.81642274	0.4049002	0.6772449
3	0.54272759	0.89027338	0.50633208	0.56096556
4	0.2261185	0.67556611	0.47655601	0.5959418
5	0.5701471	1.01206488	0.56735409	0.93644287
6	0.71851454	1.42742331	0.65487094	1.15087438
X	0.48041018	0.950498113	0.521259283	0.815891008
SEM	0.069610267	0.105393844	0.034521134	0.097347046

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	2.661	0.2950	ns	No
CCA	0.7599	0.5719	ns	No
SFN	50.55	0.0001	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.4701	-0.7930 to -0.1472	Yes	**	0.0031
CON:VEH vs. CCA:VEH	-0.04085	-0.3638 to 0.2821	No	ns	0.9843
CON:VEH vs. CCA:SFN	-0.3355	-0.6584 to -0.01256	Yes	*	0.0399
CON:SFN vs. CCA:VEH	0.4292	0.1063 to 0.7522	Yes	**	0.0068
CON:SFN vs. CCA:SFN	0.1346	-0.1883 to 0.4575	No	ns	0.6539
CCA:VEH vs. CCA:SFN	-0.2946	-0.6176 to 0.02829	No	ns	0.0816

G. Precursor Cathepsin B protein expression

PRECURSOR CATHEPSIN B PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.37173435	0.82401965	0.48476762	0.96796238
2	0.48745471	0.97605492	0.66034665	1.22296028
3	0.68007706	1.13784389	0.97414947	1.00658875
4	0.22025191	0.40505524	0.50188695	0.69585906
5	0.4141131	0.55794042	0.541213	0.8472487
6	0.42680699	0.86657348	0.57628215	0.92342034
X	0.433406353	0.794581267	0.62310764	0.944006585
SEM	0.061487586	0.110233212	0.074687992	0.071570163

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.1455	0.8076	ns	No
CCA	10.32	0.0508	ns	No
SFN	41.74	0.0005	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.3612	-0.6842 to -0.03819	Yes	*	0.0250
CON:VEH vs. CCA:VEH	-0.1897	-0.5127 to 0.1333	No	ns	0.3780
CON:VEH vs. CCA:SFN	-0.5106	-0.8336 to -0.1876	Yes	**	0.0014
CON:SFN vs. CCA:VEH	0.1715	-0.1515 to 0.4945	No	ns	0.4640
CON:SFN vs. CCA:SFN	-0.1494	-0.4724 to 0.1736	No	ns	0.5766
CCA:VEH vs. CCA:SFN	-0.3209	-0.6439 to 0.002088	No	ns	0.0519

H. Mature cathepsin B protein expression

MATURE CATHEPSIN B PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.75890861	1.01596069	0.87485226	0.99902446
2	0.69705238	1.02843684	0.97673531	1.1056497
3	0.89787881	1.31309444	1.04365811	1.34482795
4	0.61875374	0.71845776	0.8110239	0.83402722
5	0.61014805	0.75203816	0.84257632	0.93880306
6	0.65933245	1.00324196	0.87900432	1.17931293
X	0.70701234	0.971871642	0.904641703	1.066940887
SEM	0.044222195	0.088526063	0.03590921	0.074526905

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.720	0.4359	ns	No
CCA	14.01	0.0345	*	Yes
SFN	29.84	0.0035	**	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.2649	-0.5201 to -0.009586	Yes	*	0.0403
CON:VEH vs. CCA:VEH	-0.1976	-0.4529 to 0.05764	No	ns	0.1668
CON:VEH vs. CCA:SFN	-0.3599	-0.6152 to -0.1047	Yes	**	0.0041
CON:SFN vs. CCA:VEH	0.06723	-0.1880 to 0.3225	No	ns	0.8810
CON:SFN vs. CCA:SFN	-0.09507	-0.3503 to 0.1602	No	ns	0.7271
CCA:VEH vs. CCA:SFN	-0.1623	-0.4176 to 0.09297	No	ns	0.3118

Table 8. Antioxidants proteins and ROS following CCA

A. NQO1 protein expression

NQO1 PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.08823892	1.06124701	0.54051005	1.01215853
2	0.05572468	0.94106707	0.68584786	1.1366004
3	0.07042123	0.91063109	0.57154654	0.90478049
4	0.04606522	1.11104381	0.50712718	1.13196385
5	0.02964275	0.93970849	0.41720998	0.91044904
6	0.03531565	0.87864269	0.81648772	0.93343593
X	0.054234742	0.97372336	0.589788222	1.00489804
SEM	0.009036861	0.037310279	0.057734251	0.043815871

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	10.21	<0.0001	****	Yes
SFN	12.89	<0.0001	****	Yes
CCA	71.50	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.9195	-1.082 to -0.7572	Yes	****	<0.0001
CON:VEH vs. CCA:VEH	-0.5356	-0.6979 to -0.3732	Yes	****	<0.0001
CON:VEH vs. CCA:SFN	-0.9507	-1.113 to -0.7883	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	0.3839	0.2216 to 0.5463	Yes	****	<0.0001
CON:SFN vs. CCA:SFN	-0.03117	-0.1935 to 0.1311	No	ns	0.9488
CCA:VEH vs. CCA:SFN	-0.4151	-0.5774 to -0.2528	Yes	****	<0.0001

B. HO-1 protein expression

HO-1 PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.14037989	1.5164251	0.5913369	1.48019946
2	0.11073886	1.10554797	0.67893551	1.34210434
3	0.12289149	1.17203554	0.77988262	1.51480816
4	0.15432366	1.96498496	0.74550736	1.25221396
5	0.10374973	1.01774009	0.68450764	1.15635935
6	0.12092217	0.93942908	0.58375606	0.93842111
X	0.125500967	1.286027123	0.677321015	1.280684397
SEM	0.007683187	0.15829739	0.032336864	0.087902227

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	7.058	0.0067	**	Yes
CCA	6.790	0.0076	**	Yes
SFN	70.74	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-1.161	-1.525 to -0.7962	Yes	****	<0.0001
CON:VEH vs. CCA:VEH	-0.5518	-0.9162 to -0.1875	Yes	**	0.0021
CON:VEH vs. CCA:SFN	-1.155	-1.520 to -0.7908	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	0.6087	0.2444 to 0.9730	Yes	***	0.0008
CON:SFN vs. CCA:SFN	0.005343	-0.3590 to 0.3697	No	ns	>0.9999
CCA:VEH vs. CCA:SFN	-0.6034	-0.9677 to -0.2390	Yes	***	0.0008

C. Catalase protein expression

CAT PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.58982052	1.2939809	0.65849163	1.21984517
2	0.61330135	1.19027926	0.65958284	1.41509952
3	0.55157252	1.11778301	0.60115222	1.08906735
4	0.56181943	1.07173225	0.65469804	1.06125517
5	0.57182079	1.09487136	0.64488	1.06483673
6	0.51729266	1.02346142	0.59244817	0.95448228
X	0.567604545	1.132018033	0.635208817	1.134097703
SEM	0.013442846	0.03942409	0.012379915	0.066006868

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.3395	0.4168	ns	No
CCA	0.3840	0.3884	ns	No
SFN	89.40	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.5644	-0.7208 to -0.4080	Yes	****	<0.0001
CON:VEH vs. CCA:VEH	-0.06760	-0.2240 to 0.08880	No	ns	0.6280
CON:VEH vs. CCA:SFN	-0.5665	-0.7229 to -0.4101	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	0.4968	0.3404 to 0.6532	Yes	****	<0.0001
CON:SFN vs. CCA:SFN	-0.002080	-0.1585 to 0.1543	No	ns	>0.9999
CCA:VEH vs. CCA:SFN	-0.4989	-0.6553 to -0.3425	Yes	****	<0.0001

D. Glutathione reductase protein expression

GR PROTEIN				
	CON		CCA	
	VEH	SFN	VEH	SFN
1	0.71294391	1.54322669	0.84840828	1.28363165
2	0.51487086	0.99965749	0.91400509	1.29192141
3	0.7647057	0.94380099	0.96855594	1.19425753
4	0.63857734	0.93210513	0.76548137	1.30028978
5	0.30819217	0.91893142	0.73507418	0.87157856
6	0.32024099	0.67820252	0.72943306	0.82077958
X	0.543255162	1.00265404	0.82682632	1.127076418
SEM	0.080149138	0.117323891	0.040751405	0.090424276

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.850	0.3693	ns	No
CCA	12.16	0.0289	*	Yes
SFN	42.14	0.0003	***	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.4594	-0.8023 to -0.1165	Yes	**	0.0064
CON:VEH vs. CCA:VEH	-0.2836	-0.6265 to 0.05938	No	ns	0.1282
CON:VEH vs. CCA:SFN	-0.5838	-0.9268 to -0.2409	Yes	***	0.0006
CON:SFN vs. CCA:VEH	0.1758	-0.1671 to 0.5188	No	ns	0.4933
CON:SFN vs. CCA:SFN	-0.1244	-0.4674 to 0.2185	No	ns	0.7424
CCA:VEH vs. CCA:SFN	-0.3003	-0.6432 to 0.04270	No	ns	0.0995

E. Glucose-6-phosphate-dehydrogenase protein expression

G6PD PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.15867382	1.3780761	0.48388865	1.50694614
2	0.1928202	1.14485288	0.7048634	1.13048114
3	0.22823927	0.98566265	0.3547881	0.72420581
4	0.1451071	0.89105261	0.13579293	0.8179391
5	0.0712631	0.89873867	0.40723995	1.01702063
6	0.07405756	0.70262022	0.40330037	0.80849925
X	0.145026842	1.000167188	0.4149789	1.000848678
SEM	0.025737449	1.000167188	0.075397965	0.118347826

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	2.578	0.1327	ns	No
CCA	2.604	0.1308	ns	No
SFN	73.83	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.8551	-1.195 to -0.5152	Yes	****	<0.0001
CON:VEH vs. CCA:VEH	-0.2700	-0.6099 to 0.07003	No	ns	0.1513
CON:VEH vs. CCA:SFN	-0.8558	-1.196 to -0.5158	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	0.5852	0.2452 to 0.9252	Yes	***	0.0006
CON:SFN vs. CCA:SFN	-0.0006815	-0.3407 to 0.3393	No	ns	>0.9999
CCA:VEH vs. CCA:SFN	-0.5859	-0.9259 to -0.2459	Yes	***	0.0006

F. Glutathione peroxidase-1 protein expression

GPX-1 PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.12220605	0.86549995	0.50238106	1.19394778
2	0.1530239	1.10269946	0.56847856	0.90534677
3	0.15643744	1.00852917	0.60866526	1.06486898
4	0.06880694	1.70640695	0.61614775	1.45650417
5	0.0473376	1.3961723	0.81330102	1.32174759
6	0.05529353	1.26818748	0.51748412	1.208014
X	0.100517577	1.224582552	0.604409628	1.191738215
SEM	0.020193418	0.123014098	0.045843364	0.078610664

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	7.358	0.0024	**	Yes
CCA	5.667	0.0063	**	Yes
SFN	74.81	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-1.124	-1.430 to -0.8186	Yes	****	<0.0001
CON:VEH vs. CCA:VEH	-0.5039	-0.8094 to -0.1984	Yes	***	0.0009
CON:VEH vs. CCA:SFN	-1.091	-1.397 to -0.7858	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	0.6202	0.3147 to 0.9256	Yes	****	<0.0001
CON:SFN vs. CCA:SFN	0.03284	-0.2726 to 0.3383	No	ns	0.9902
CCA:VEH vs. CCA:SFN	-0.5873	-0.8928 to -0.2819	Yes	***	0.0002

G. CellROX Green mean fluorescence intensity

CELLROX GREEN MEAN FLUORESCENCE INTENSITY				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	6572.3	4477.3	4737.5	3831.7
2	7811.8	4938.9	3667.2	3873.3
3	4987.6	3044	3549.2	4283.4
4	7363.5	4926.5	5275.3	3823.3
5	7628.2	4533.1	4070.1	3823.3
X	6872.68	4383.96	4259.86	4188.5
SEM	516.5953865	348.4792642	327.9901471	4188.5

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	20.00	0.0052	**	Yes
CCA	26.99	0.0017	**	Yes
SFN	22.43	0.0035	**	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	2489	976.5 to 4001	Yes	**	0.0012
CON :VEH vs. CCA:VEH	2613	1101 to 4125	Yes	***	0.0008
CON :VEH vs. CCA:SFN	2684	1172 to 4196	Yes	***	0.0006
CON :SFN vs. CCA:VEH	124.1	-1388 to 1636	No	ns	0.9953
CON :SFN vs. CCA:SFN	195.5	-1317 to 1708	No	ns	0.9821
CCA:VEH vs. CCA:SFN	71.36	-1441 to 1584	No	ns	0.9991

H. MitoSOX Red mean fluorescence intensity

MITOSOX RED MEAN FLUORESCENCE INTENSITY				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	2584.4	2003.7	1916.7	1697.3
2	2816.9	2097.4	2026.5	1598.6
3	2091.7	1773.8	1960.8	1986
4	2886.1	2231.2	2554	1822.9
5	3074.8	2312.3	1852.2	2084
X	2690.78	2083.68	2062.04	1837.76
SEM	169.0770635	93.97869652	126.2326844	89.37465301

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	5.671	0.1417	ns	No
CCA	29.60	0.0028	**	Yes
SFN	26.75	0.0040	**	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	607.1	106.0 to 1108	Yes	*	0.0151
CON :VEH vs. CCA:VEH	628.7	127.7 to 1130	Yes	*	0.0118
CON :VEH vs. CCA:SFN	853.0	352.0 to 1354	Yes	***	0.0009
CON :SFN vs. CCA:VEH	21.64	-479.4 to 522.7	No	ns	0.9993
CON :SFN vs. CCA:SFN	245.9	-255.1 to 747.0	No	ns	0.5147
CCA:VEH vs. CCA:SFN	224.3	-276.8 to 725.3	No	ns	0.5875

Table 9. Mitochondrial Dynamics Following CCA

A. MFN2 protein expression

MFN2 PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	0.70654093	0.91657747	0.94889846	0.97431311
2	0.74067926	0.963409	0.98241406	1.13603743
3	0.87019246	1.31590589	1.37852159	1.34473164
4	0.68041544	0.71945014	0.70661691	0.84307157
5	0.25853382	0.41356553	0.57072145	0.55692902
6	0.24089941	0.46200351	0.71375846	0.8978536
X	0.582876887	0.798485257	0.883488488	0.958822728
SEM	0.108689989	0.138624124	0.117903571	0.10942346

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.352	0.5631	ns	No
CCA	14.60	0.0676	ns	No
SFN	5.818	0.2368	ns	No

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-0.2156	-0.6877 to 0.2565	No	ns	0.5866
CON:VEH vs. CCA:VEH	-0.3006	-0.7727 to 0.1715	No	ns	0.3106
CON:VEH vs. CCA:SFN	-0.3759	-0.8481 to 0.09617	No	ns	0.1496
CON:SFN vs. CCA:VEH	-0.08500	-0.5571 to 0.3871	No	ns	0.9572
CON:SFN vs. CCA:SFN	-0.1603	-0.6325 to 0.3118	No	ns	0.7783
CCA:VEH vs. CCA:SFN	-0.07533	-0.5475 to 0.3968	No	ns	0.9695

B. Drp-1 protein expression

DRP-1 PROTEIN				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	1.16971875	0.58558439	0.32082478	0.41480383
2	0.98991864	0.47771551	0.37471356	0.28832528
3	0.95947619	0.54246057	0.39047572	0.47078508
4	1.20358906	0.64958395	0.29286886	0.20623239
5	1.01377236	0.43152403	0.19418336	0.1800863
6	0.94093948	0.56757915	0.09406813	0.23791308
X	1.046235747	0.277855735	0.542407933	0.299690993
SEM	0.045770489	0.046517159	0.031866831	0.0481351

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	16.42	<0.0001	****	Yes
CCA	13.81	<0.0001	****	Yes
SFN	60.75	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	0.7684	0.5959 to 0.9408	Yes	****	<0.0001
CON:VEH vs. CCA:VEH	0.5038	0.3314 to 0.6763	Yes	****	<0.0001
CON:VEH vs. CCA:SFN	0.7465	0.5741 to 0.9190	Yes	****	<0.0001
CON:SFN vs. CCA:VEH	-0.2646	-0.4370 to -0.09211	Yes	**	0.0019
CON:SFN vs. CCA:SFN	-0.02184	-0.1943 to 0.1506	No	ns	0.9843
CCA:VEH vs. CCA:SFN	0.2427	0.07028 to 0.4152	Yes	**	0.0041

C. Mitochondrial branch length

MITOCHONDRIAL BRANCH LENGTH (μm)/MITO				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	22.877	60.157	38.878	67.325
2	13.18	42.065	26.199	53.386
3	15.912	49.742	47.498	44.617
4	16.258	71.123	36.266	35.159
5	19.749	75.363	33.538	72.747
X	17.5952	59.69	36.4758	54.6468
SEM	1.683025888	6.269680359	3.475246472	6.965324288

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	9.477	0.0313	*	Yes
CCA	3.170	0.1911	ns	No
SFN	60.14	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-42.09	-62.60 to -21.59	Yes	***	0.0001
CON:VEH vs. CCA:VEH	-18.88	-39.39 to 1.625	No	ns	0.0767
CON:VEH vs. CCA:SFN	-37.05	-57.56 to -16.55	Yes	***	0.0005
CON:SFN vs. CCA:VEH	23.21	2.709 to 43.72	Yes	*	0.0239
CON:SFN vs. CCA:SFN	5.043	-15.46 to 25.55	No	ns	0.8942
CCA:VEH vs. CCA:SFN	-18.17	-38.68 to 2.334	No	ns	0.0920

D. Number of branch junctions

NUMBER OF BRANCH JUNCTIONS/MITO				
	CON		CCA	
N	VEH	SFN	VEH	SFN
1	217.857	511.545	283.25	399.143
2	114	359	197.778	440.5
3	124.5	415.2	314	407.667
4	146	413.165	271.091	226.143
5	195.625	673.5	210.455	484.667
X	159.5964	474.482	255.3148	391.624
SEM	20.233646	55.49416754	22.13154038	44.01348532

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	9.655	0.0338	*	Yes
CCA	0.05007	0.8693	ns	No
SFN	61.64	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON:VEH vs. CON:SFN	-314.9	-470.5 to -159.3	Yes	***	0.0001
CON:VEH vs. CCA:VEH	-95.72	-251.3 to 59.89	No	ns	0.3273
CON:VEH vs. CCA:SFN	-232.0	-387.6 to -76.42	Yes	**	0.0030
CON:SFN vs. CCA:VEH	219.2	63.56 to 374.8	Yes	**	0.0048
CON:SFN vs. CCA:SFN	82.86	-72.75 to 238.5	No	ns	0.4473
CCA:VEH vs. CCA:SFN	-136.3	-291.9 to 19.29	No	ns	0.0969

Table 10. Upstream kinase activation in response to Acute Contractile Activity (ACA)

A. Phosphorylated AMPK protein expression

P-AMPK PROTEIN				
	CON		ACA	
N	VEH	SFN	VEH	SFN
1	0.08466987	0.46749247	1.38403534	1.35473872
2	0.0840036	0.42029926	1.18037174	1.51407239
3	0.07945967	0.49683225	1.16389399	1.20758733
4	0.01971294	0.13305969	0.723299	0.84753106
5	0.11704707	0.50388881	1.41400151	1.29519844
6	0.09522277	0.38258724	1.36386537	1.11240426
X	0.08001932	0.400693287	1.204911158	1.221922033
SEM	0.013262571	0.056772679	0.10572063	0.09322549

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	2.068	0.0603	ns	No
ACA	84.94	<0.0001	****	Yes
SFN	2.557	0.0386	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	-0.3207	-0.6226 to -0.01879	Yes	*	0.0348
CON :VEH vs. ACA:VEH	-1.125	-1.427 to -0.8230	Yes	****	<0.0001
CON :VEH vs. ACA:SFN	-1.142	-1.444 to -0.8400	Yes	****	<0.0001
CON :SFN vs. ACA:VEH	-0.8042	-1.106 to -0.5023	Yes	****	<0.0001
CON :SFN vs. ACA:SFN	-0.8212	-1.123 to -0.5193	Yes	****	<0.0001
ACA:VEH vs. ACA:SFN	-0.01701	-0.3189 to 0.2849	No	ns	0.9986

B. Total AMPK protein expression

AMPK PROTEIN				
	CON		ACA	
N	VEH	SFN	VEH	SFN
1	0.7486041	0.78218114	0.86119569	0.70254422
2	0.78340882	0.77286683	0.82457817	0.73291201
3	0.76688067	0.88919823	0.72063273	0.9473492
4	0.5914682	0.68094666	0.78958602	0.78055288
5	0.75279545	0.88817301	0.87107457	0.85636675
6	0.79041409	0.91302498	1.03932948	0.95242881
X	0.738928555	0.821065142	0.85106611	0.828692312
SEM	0.030244029	0.037000192	0.043757027	0.04380284

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	7.225	0.1965	ns	No
ACA	9.488	0.1414	ns	No
SFN	2.362	0.4537	ns	No

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	-0.08214	-0.2369 to 0.07266	No	ns	0.4644
CON :VEH vs. ACA:VEH	-0.1121	-0.2669 to 0.04266	No	ns	0.2113
CON :VEH vs. ACA:SFN	-0.08976	-0.2446 to 0.06503	No	ns	0.3888
CON :SFN vs. ACA:VEH	-0.03000	-0.1848 to 0.1248	No	ns	0.9475
CON :SFN vs. ACA:SFN	-0.007627	-0.1624 to 0.1472	No	ns	0.9990
ACA:VEH vs. ACA:SFN	0.02237	-0.1324 to 0.1772	No	ns	0.9770

C. Phosphorylated/total AMPK protein expression

P-AMPK/AMPK				
	CON		ACA	
N	VEH	SFN	VEH	SFN
1	0.03332883	0.20307769	0.91604839	1.08580863
2	0.15548323	0.64533309	1.62328412	1.5124343
3	0.12047201	0.50176583	1.31225506	1.16796578
4	0.11310367	0.59767801	1.60710899	1.92833231
5	0.1072283	0.54381848	1.43148555	2.06583105
6	0.10361413	0.55874183	1.61510008	1.27470137
X	0.105538362	0.508402488	1.417547032	1.505845573
SEM	0.016325351	0.064232839	0.112556691	0.166947459

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.506	0.1534	ns	No
ACA	81.15	<0.0001	****	Yes
SFN	3.671	0.0312	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	-0.4029	-0.8224 to 0.01666	No	ns	0.0626
CON :VEH vs. ACA:VEH	-1.312	-1.732 to -0.8925	Yes	****	<0.0001
CON :VEH vs. ACA:SFN	-1.400	-1.820 to -0.9808	Yes	****	<0.0001
CON :SFN vs. ACA:VEH	-0.9091	-1.329 to -0.4896	Yes	****	<0.0001
CON :SFN vs. ACA:SFN	-0.9974	-1.417 to -0.5779	Yes	****	<0.0001
ACA:VEH vs. ACA:SFN	-0.08830	-0.5078 to 0.3312	No	ns	0.9342

D. Phosphorylated JNK1 protein expression

P-JNK1 PROTEIN				
	CON		ACA	
N	VEH	SFN	VEH	SFN
1	0.0468243	0.40368192	0.90863742	1.28953562
2	0.1269465	0.3803216	0.93741109	1.07681351
3	0.11377658	0.36290145	0.96140076	1.20002231
4	0.02232499	0.28851792	0.75542667	0.88250113
5	0.04551543	0.2196717	0.84363657	0.98398028
6	0.06266577	0.23408939	0.9467658	1.02147503
X	0.069675595	0.314863997	0.892213052	1.075721313
SEM	0.016952843	0.032021422	0.032220983	0.060514903

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.1351	0.4357	ns	No
ACA	89.06	<0.0001	****	Yes
SFN	6.529	<0.0001	****	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	-0.2452	-0.3987 to -0.09172	Yes	**	0.0012
CON :VEH vs. ACA:VEH	-0.8225	-0.9760 to -0.6691	Yes	****	<0.0001
CON :VEH vs. ACA:SFN	-1.006	-1.160 to -0.8526	Yes	****	<0.0001
CON :SFN vs. ACA:VEH	-0.5773	-0.7308 to -0.4239	Yes	****	<0.0001
CON :SFN vs. ACA:SFN	-0.7609	-0.9143 to -0.6074	Yes	****	<0.0001
ACA:VEH vs. ACA:SFN	-0.1835	-0.3370 to -0.03004	Yes	*	0.0156

E. Total JNK1 protein expression

JNK1 PROTEIN				
	CON		ACA	
N	VEH	SFN	VEH	SFN
1	0.84082482	0.91125705	0.92944707	0.75207872
2	0.83472074	0.88328072	0.75698145	0.63034236
3	0.70814583	0.64401674	0.64456214	0.4673564
4	0.97938149	1.10159641	1.01469756	0.95255352
5	1.19027739	0.9651837	1.04278985	0.93651333
6	1.10213918	1.1536132	0.8730412	0.76304241
X	0.942581575	0.94315797	0.876919878	0.750314457
SEM	0.074187734	0.07392963	0.06262189	0.07532028

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	3.171	0.3857	ns	No
ACA	13.10	0.0865	ns	No
SFN	3.113	0.3899	ns	No

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	-0.0005764	-0.2844 to 0.2832	No	ns	>0.9999
CON :VEH vs. ACA:VEH	0.06566	-0.2182 to 0.3495	No	ns	0.9152
CON :VEH vs. ACA:SFN	0.1923	-0.09155 to 0.4761	No	ns	0.2611
CON :SFN vs. ACA:VEH	0.06624	-0.2176 to 0.3501	No	ns	0.9132
CON :SFN vs. ACA:SFN	0.1928	-0.09097 to 0.4767	No	ns	0.2588
ACA:VEH vs. ACA:SFN	0.1266	-0.1572 to 0.4104	No	ns	0.6046

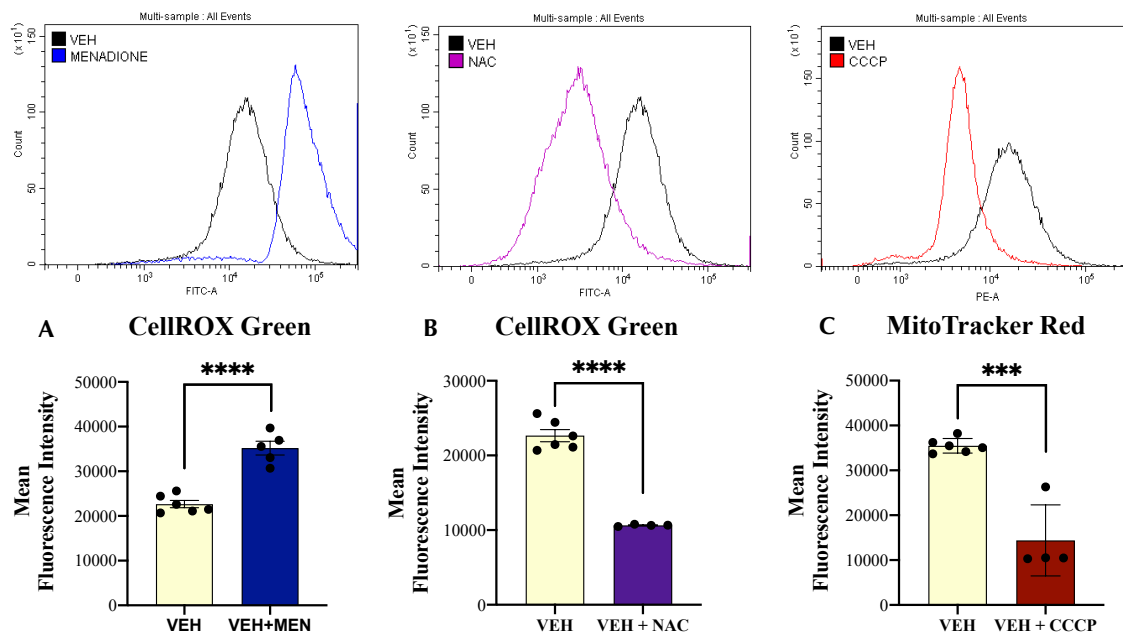
F. Phosphorylated/total JNK1 protein expression

P-JNK1/JNK1				
	CON		ACA	
N	VEH	SFN	VEH	SFN
1	0.05568853	0.44299457	0.97761072	1.71462853
2	0.1520826	0.4305784	1.23835412	1.70829947
3	0.1606683	0.56349691	1.49155637	2.56768136
4	0.02279499	0.26190892	0.74448457	0.92645832
5	0.03823934	0.22759574	0.80901878	1.05068476
6	0.05685831	0.20291844	1.08444573	1.3386871
X	0.081055345	0.354915497	1.057578382	1.551073257
SEM	0.024385477	0.059147458	0.113716468	0.242940513

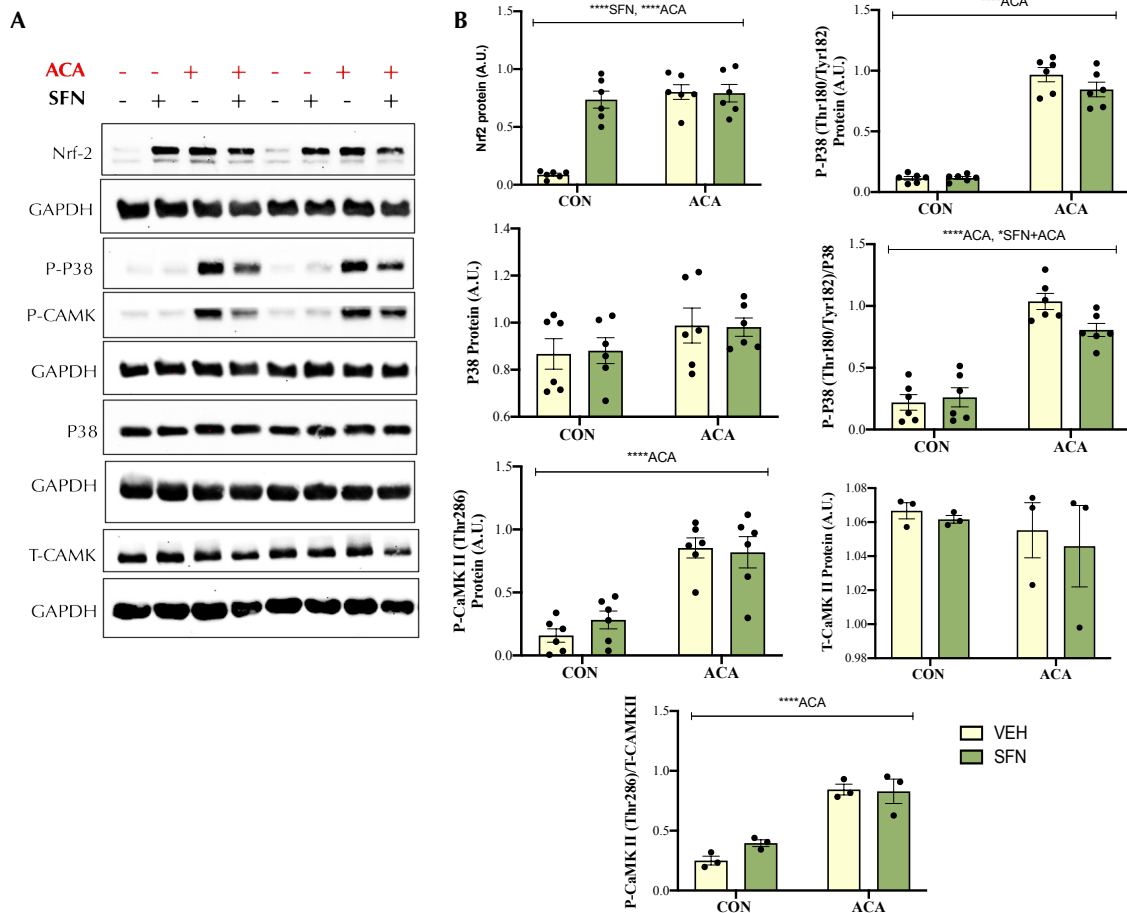
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.7013	0.4351	ns	No
ACA	68.63	<0.0001	****	Yes
SFN	8.560	0.0115	*	Yes

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
CON :VEH vs. CON :SFN	-0.2739	-0.8196 to 0.2719	No	ns	0.5112
CON :VEH vs. ACA:VEH	-0.9765	-1.522 to -0.4307	Yes	***	0.0004
CON :VEH vs. ACA:SFN	-1.470	-2.016 to -0.9242	Yes	****	<0.0001
CON :SFN vs. ACA:VEH	-0.7027	-1.248 to -0.1569	Yes	**	0.0088
CON :SFN vs. ACA:SFN	-1.196	-1.742 to -0.6504	Yes	****	<0.0001
ACA:VEH vs. ACA:SFN	-0.4935	-1.039 to 0.05228	No	ns	0.0853

APPENDIX B: ADDITIONAL DATA
(STATISTIC TABLES NOT SHOWN)



Supplementary Figure 1. Controls for flow cytometry stains. **A)** Myotubes were treated with 100 μ M menadione (MEN) for one hour, which acts as positive control for CellROX Green (n=4-6). **B)** N-acetyl cysteine (NAC), a widely used antioxidant that reduces ROS, was utilized as a negative control for CellROX Green (n=4-6). Cells were treated with 20mM NAC overnight. Both NAC and MEN were resuspended in Differentiation Media (DM) and added directly to cells. **C)** The mitochondrial uncoupler, Carbonyl cyanide m-chlorophenylhydrazone (CCCP), increases proton permeability thereby dissipating mitochondrial membrane potential. CCCP acts as a negative control for MitoTracker Red, which accumulates in mitochondria with their membrane potential intact. 30 μ M CCCP was resuspended in DM and cells were treated for 30 minutes prior to analysis (n=4-6); VEH=Vehicle, SFN=Sulforaphane. An unpaired two-tailed T-test was performed for each graph (***P<0.001, ****P<0.0001). All values are represented as $\pm$ SEM.



Supplementary Figure 2. Upstream signaling proteins following Acute Contractile Activity (ACA). **A, B)** Representative western blots and the corresponding quantification for Nrf-2, phospho-P38, total-P38, phospho-CAMK and total-CAMK following ACA. All targets were normalized to GAPDH. To obtain a measure of kinase activation, phosphorylated targets were normalized to total kinase protein present. A two-way ANOVA was performed for all graphs to determine the effect of SFN and ACA. Statistics are shown as follows, “SFN” main effect of SFN, “ACA” main effect of ACA. * Denotes the p value for each effect. ****P<0.0001. SFN= Sulforaphane, ACA= Acute Contractile Activity. All values are represented as $\pm$ SEM.

APPENDIX C: LABORATORY METHODS AND PROTOCOLS

WESTERN BLOTTING

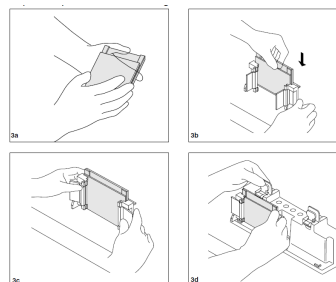
Gel Electrophoresis-SDS PAGE (Protein BIORAD System)

Reagents:

1. Acrylamide/Bis-Acrylamide, 30% Solution 37.5:1 (BioShop 10.502)
 - a. Store at 4°C
2. Under Tris Buffer
 - a. 1M Tris, pH 8.8 (60.5g/500ml)
 - b. Store at 4°C
3. Over Tris Buffer
 - a. 1M Tris, pH 6.8 (12.1g/100ml)
 - b. Bromophenol Blue (for colour)
 - c. Store at 4°C
4. Ammonium Persulfate (APS)
 - a. 10% (w/v) APS in ddH₂O (1g/10ml)
 - b. Stored at 4°C
5. Sodium Dodecyl Sulfate (SDS)
 - a. 10% (w/v) in ddH₂O (1g/10ml)
 - b. Store at room temperature
6. TEMED (Sigma T-9281)
7. Electrophoresis Buffer, pH 8.3 (10L)
 - a. 25mM Tris 30.34g, 192mM Glycine 144g, 0.1% SDS 10g
 - b. Volume to 10L with ddH₂O
 - c. Store at room temperature
8. 6X SDS
 - a. Warm 100% glycerol in water bath at 65°C for 30 minutes
 - b. Combine 1.2g SDS, 0.06g Bromophenol Blue, 3mls of 1M Tris, pH 6.8 and 1ml of ddH₂O and stir at 4°C for 5 minutes
 - c. Add 3mls of 100% glycerol, stir and aliquot mixture.
 - d. Store at -20°C
 - e. Add 5% (v/v) β-mercaptoethanol (Sigma M6250) to 6X SDS just prior to use
9. *tetra*-Amyl alcohol ReagentPlus, 99% (Sigma 152463)

Procedure:

1. *Prepare gel electrophoresis rack:*
 - a. Clean glass plates thoroughly with soap followed by 95% ethanol then ddH₂O.
 - b. Dry carefully with a kimwipe.
 - c. Assemble glass plates as shown below:
 - d. Check the seal by adding a small volume of ddH₂O then pour off and let dry.



2. *Prepare separating gels:*

a. Mini Protean 3 Bio-Rad System volumes:

	8%	10%	12%	15%	18%
Acrylamide	2.7 ml	3.3 ml	4.0 ml	5.0 ml	6.0 ml
ddH₂O	4.1 ml	3.5 ml	2.8 ml	1.8 ml	0.8 ml
Under Tris	3.0 ml	3.0 ml	3.0 ml	3.0 ml	3.0 ml
SDS	100µl	100µl	100µl	100µl	100µl
APS	100µl	100µl	100µl	100µl	100µl
TEMED	10µl	10µl	10µl	10µl	10µl

- Mix the contents of the separating gel without adding APS or TEMED. Stir.
- Add APS and TEMED. Stir.
- Slowly pour the entire volume of the solution into the space between the two plates while keeping plates tilted to prevent bubble formation.
- Add *tert*-Amyl alcohol to coat top surface of gel solution.
- Allow 30 minutes for gel polymerization.
- Remove *tert*-Amyl alcohol by pouring it off and remove any remainder with a kimwipe. Rinse with ddH₂O.

3. *Prepare stacking gel:*

- For a single mini gel use the following volumes →
- Mix the contents of the stacking gel without adding APS or TEMED. Stir.
- Add APS and TEMED. Stir.
- Using a Pasteur pipette slowly add the entire volume from the beaker in between the plates.
- Add comb for desired number of wells.
- Allow 30 minutes for gel polymerization.

Acrylamide	500 µl
Over Tris	625 µl
ddH₂O	3.75 ml
SDS	50 µl
APS	50 µl
TEMED	7.5 µl

4. *Prepare samples:*

- Turn on the block heater to 95°C.
- Pipette required volume of sample into new eppendorf with same amount of lysis buffer and 5 µl of sample dye. Keep samples on ice until all samples are prepared (use pipette plan).
- Briefly spin each sample to bring volume to the bottom of the eppendorf.
- Incubate each sample at 95 °C for 5 minutes in the heating block to denature the proteins.
- Briefly spin again to return volume to the bottom of the eppendorf.

5. *Assemble Mini-PROTEAN gel caster system:*

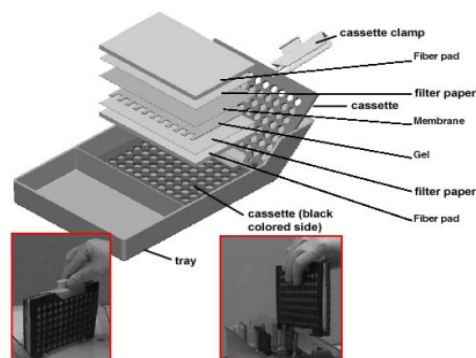
- See images below
- If you are only running one gel a rectangular plastic pseudo plate must be clamped on the other side of the caster.
- Fill with electrophoresis buffer between the plates and outside of the plates in the chamber.
- Slowly remove the comb using both hands (one on each side) by pulling the comb straight upwards.
- Fix any wells that are deformed using a small spatula.

- f. Clean out the wells using a syringe filled with electrophoresis buffer.
 - g. Withdraw the entire volume of the sample using a Hamilton syringe. Inject volume slowly into the bottom of the well.
6. *Gel electrophoresis:*
- a. Immediately after all samples are loaded place the lid on the gel chamber.
 - b. Place positive and negative plugs into the power supply and turn on power supply.
 - c. Set power supply to 120V. Gel will run for ~2 hours depending on percent gel made.
 - d. When the bromophenol blue has run off the bottom of the gel (or when gel has separated the desired amount) turn off the power supply. Remove plugs from power supply and remove lid.
 - e. Prepare for electrotransfer of proteins from the gel to nitrocellulose membrane.

Western Blotting-Transfer and Immunodetection:

Reagents:

1. Transfer Buffer
 - a. 0.025M Tris-HCl pH 8.3 12.14g
 - b. 0.15M Glycine 45.05g
 - c. 20% Methanol 800ml
 - d. make up to 4L with ddH₂O
 - e. store at 4°C
2. Ponceau S stain
 - a. 0.1% (w/v) Ponceau S
 - b. 0.5% (v/v) Acetic Acid
 - c. Store at room temperature
3. Wash Buffer
 - a. Tris-HCl pH 7.5 12g
 - b. NaCl 58.5g
 - c. 0.1% Tween 10ml
 - d. Store at room temperature
4. Blocking Solution
 - a. 5% (w/v) skim milk powder in wash buffer OR
 - b. 5% (w/v) BSA in wash buffer
5. Enhanced Chemiluminescence Fluid (ECL; Santa Cruz sc-2048)
6. Film/Developer/Fixer



Procedure:

1. *Transfer Procedure:*
 - a. Remove electrophoresis plates from chamber and separate the plates.
 - b. Cut away unnecessary parts of the gel using a spatula and measure remaining gel size.
 - c. Using a paper cutter cut 6 pieces of Whatman paper per gel to the same size as the gel. Wearing gloves cut nitrocellulose membrane (GE Healthcare RPN303D) to the dimensions of the gel.
 - d. Assemble Whatman paper, nitrocellulose membrane and gel as shown above:

- e. Close the cassette and place in the transfer chamber with the black side of the cassette facing the back side of the chamber.
 - f. Place ice pack in the chamber.
 - g. Place lid on the chamber and connect the leads to the power supply.
 - h. Turn on the power supply and run at 120V for 2 hours. This can vary depending on the size of the protein of interest.
2. *Removal of transfer membrane:*
- a. Turn off the power supply and disconnect leads from the power supply then remove the lid from the chamber.
 - b. Remove the cassette from the chamber.
 - c. With gloves on, remove the Whatman paper and gel and place the nitrocellulose membrane in a plastic dish.
 - d. Add Ponceau S stain on the membrane and gently swirl.
 - e. Drain off the remaining Ponceau S and save for reuse.
 - f. Rinse the membrane with ddH₂O to reduce the red background. Wrap membrane in saran wrap and scan image.
 - g. Cut the membrane while protein bands are still visible at the desired molecular weight.
 - h. Rotate membrane at room temperature in wash buffer until remaining Ponceau S has been removed.
 - i. Incubate membrane for 1 hour with rotation in blocking solution.
 - j. Incubate membrane with desired antibody diluted in blocking solution overnight at 4°C. Membrane is placed face up into the solution on a glass plate covered in parafilm. To maintain a moist environment overnight, wet a small kimwipe and form it into a ball and place in each corner of the dish. Cover the dish with saran wrap.
3. *Immunodetection:*
- a. Wash the blots in wash buffer with gentle rotation for 5 minutes 3X.
 - b. Incubate the blots for 1 hour in room temperature with the appropriate secondary antibody diluted in blocking solution.
 - c. Membrane is placed face up in solution on a glass plate covered with parafilm. Place moist kimwipes in each corner of the dish and cover the dish with saran wrap.
 - d. Following the incubation, wash the membrane 3X for 5 minutes with wash buffer.
4. *Enhanced Chemiluminescence Detection:*
- a. Mix ECL fluids “A” and “B” in a 1:1 ratio in a disposable Rohr tube.
 - b. Place blots on saran wrap face up and apply ECL solution for 2 minutes.
 - c. Dab off excess ECL on a kimwipe and place blots face down on a fresh piece of saran wrap and wrap tightly.
 - d. Expose blot to film (time will vary depending on protein and antibody).
 - e. Place film into developer (time will vary).
 - f. Once image appears place film into fixer for 2 minutes. Wash with fresh water when complete.

ISOLATING CYTOPLASMIC AND NUCLEAR FRACTIONS

Materials required:

1. Ice
2. Ice cold 1xPBS
3. Labelled 2mL and 1.5mL Eppendorf tubes
4. Cell scrapers (as many as conditions)

Stock solutions:

1. 1 M Tris = (200mL ddH₂O + 24.22 g)
2. 1 M NaCl = (200mL ddH₂O + 11.68 g)
3. 250 mM EDTA = (200mL ddH₂O + 18.61 g); Add NaOH pellets to dissolve
4. 250 mM DTT = (30mL ddH₂O + 1.92 g)
5. 86 1 M MgCl₂ = (50mL ddH₂O + 10.165 g)

Cytoplasmic buffer (200 mL)

1. 100 mM Tris → 2mL from 1 M stock
2. 10 mM NaCl → 2mL from 1 M stock
3. 3 mM MgCl₂ → 600μL from 1 M stock
4. 0.5% NP-40 → 1mL added directly
- Top up with ddH₂O and mix thoroughly
- Working buffer must be prepared with Cocktail [Phosphatase (Cocktail 2, 3) and Protease] Inhibitors at 0.2% concentration for each.

Nuclear buffer (200 mL)

1. 50 mM Tris → 10mL from 1 M stock
2. 5 mM MgCl₂ → 1mL from 1 M stock
3. 0.1 mM EDTA → 80μL from 250 mM
4. stock 1 mM DTT → 800μL from 250 mM stock
5. 40% glycerol → 80mL
- Top up with ddH₂O and mix thoroughly

Procedure:

1. Prepare the working cytoplasmic buffer with appropriate concentrations of cocktail inhibitors. Place on ice.
2. Culture cells at the appropriate density. High cell yield is recommended for this protocol. Use two 6-well plates or one 15cm² cell culture dish per condition. (Myoblasts can be cultured at 4x10⁴ in a 6-well plate or 8x10⁴ in a dish, and then differentiated to myotubes once 80-85% confluency has been reached).
3. After treatment, collect the cells with ice cold PBS in 15 mL falcon tubes. Scraping the cells instead of trypsinizing helps maintain the pellet size. Use of trypsin-EDTA to collect cell pellet requires an additional washing step to remove the media from the suspension, which reduces pellet size and results in lesser mitochondrial yield.
4. Centrifuge at 1400xg for 5 min. Aspirate the supernatant.
5. Resuspend the pellet in cytoplasmic buffer. Volume depends on pellet size, approximately 3X the size of the pellet is adequate.
6. Incubate on ice for 5 min.
7. Rock the samples at 4°C for 5 min.
8. Centrifuge the samples at 2500xg for 5 min at 4°C.

9. Collect the supernatant and transfer to properly labelled Eppendorf tubes to be stored as the cytoplasmic fraction at -80°C until use.
10. Resuspend the remaining nuclear pellet with cytoplasmic buffer.
11. Rock the samples at 4°C for 3 min.
12. Centrifuge the samples at 3200xg for 3 min at 4°C.
13. Collect and discard supernatant.
14. Repeat steps 10-13 approximately 10-12 times to ensure thorough washing and purity of nuclear pellet.
15. Resuspend the final pellet following discarded cytoplasmic buffer with 150-200µL of nuclear buffer. Transfer each sample to properly labelled Eppendorf tubes.
16. Prepare 0.15 unit/µL of Benzonase (0.5µL Benzonase in 7.83µL of sterile H₂O)
17. Add 1.5µL of Benzonase to each nuclear sample. Incubate on ice for 10-20 min.
18. Samples are to be stored as the nuclear fraction at -80°C until use.

MITOCHONDRIAL RESPIRATION IN ADHERENT CELLS USING THE SEAHORSE XF

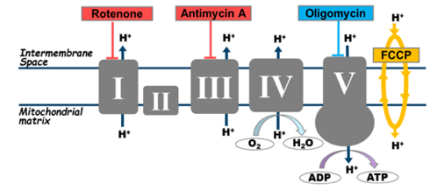
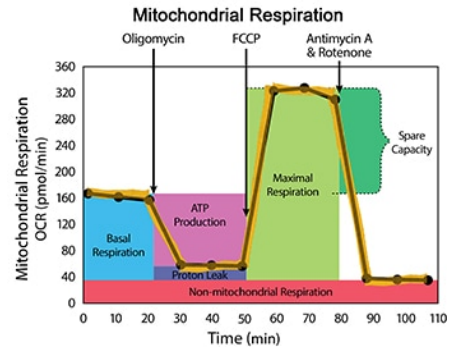
Materials & reagents:

- 96-well Cell Culture Microplate (Agilent; 102416-100)
- Extracellular Flux Assay Kit Cartridge (Agilent; 102416-100)
- Seahorse XF Calibrant Solution, pH 7.4
- Seahorse XF Mito Stress Kit (Agilent; 103015-100)
- Seahorse XF DMEM Medium, pH 7.4
 - With 5mM HEPES; Without phenol red, sodium bicarbonate, glucose, pyruvate, & L-glutamine Agilent, Cat# 103575-100 4° C bottom shelf
- 200mM Glutamine Solution, 50mL (Agilent; 103579-100)
- 1.0M Glucose Solution, 50mL (Agilent; 103577-100)
- Sterile H₂O, Molecular grade (Wisent; 809-115-CL)

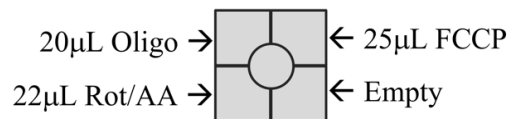
Procedure:

1. *Preparation*
 - Plate cells on 96-well Seahorse Plate.
 - Mix 75µL of suspended cells in 15mL of Growth Media.
 - Add 80µL of diluted cells to each well using a multichannel pipette.
 keep the 4 corners of the plate blank!
2. *Day before running experimental plate:*
 - Hydrate Assay Kit Cartridge with 200µL of sterile H₂O in cartridge utility plate.
 - Aliquot 50mL of XF Calibrant solution in 50mL conical tube.
 - Place both the Cartridge and Calibrant Aliquot in incubator beside seahorse machine overnight.
 Add water to incubator tray if needed
- Day of experiment:*
 - 1) Place XF DMEM, 200mM Glutamine, and 1.0M Glucose in water bath to warm.
 - 2) Calibrate cartridge by aspirating out the sterile H₂O and adding 200µL of XF Calibrant solution.
 - 3) Place the cartridge back in the 37°C incubator.

- 4) Prepare Seahorse Media (50 mL):
 - i. 47.75 mL XF DMEM
 - ii. 1.0 mL 1M Glucose
 - iii. 1.25 mL 200mM Glutamine
- 5) Wash cells in seahorse plate with 80µL of Seahorse Media (Repeat for 2 washes).
- 6) Add 160µL of Seahorse Media to each well (including negative control corners). Place Cell plate with Seahorse Media back in incubator @ 37°C.



- 7) Prep Mito Stress Kit Substrates in Seahorse Media:
 - i. Oligomycin (100µM) → 630µL
 - ii. FCCP (100µM) → 720µL
 - iii. Rotenone/ AA (50µM) → 540µL
 - **Oligomycin:** blocks ATP synthase to look at ATP production lost vs background respiration.
 - **FCCP:** uncouples mitochondria to look at maximal respiration.
 - **Rotenone/Antimycin A:** block complex I and III to stop electron flow to show spare capacity vs background.
- 8) Prepare substrate working stocks in 15mL tube:
 - i. 600µL of 100µM Oligomycin → 2,400µL Seahorse Media
 - ii. 300µL of 100µM FCCP → 2,700µL Seahorse Media
 - iii. 300µL of 50µM Rot/AA → 2,700µL Seahorse Media
- 9) Load ports in Cartridge (keep Cartridge on the Calibrant-loaded Utility plate, cell plate should remain in incubator until all calibration steps have been completed).



- 10) Running the Plate in the Seahorse Reader Using Wave Software
 - i. Open Wave program.
 - ii. Load template: "Mito Stress Test" and set plate layout according to samples.
 - iii. Click "Run" – Insert XF calibrant-loaded utility plate with cartridge placed on top, loaded with substrates as per above.
 - iv. Allow for Calibration steps to complete (~15-20 minutes).
 - v. Follow prompt on screen and swap Utility plate with Seahorse plate containing cells.
 - vi. Click "Continue".
- 11) Normalization Plate with MitoTracker Green and Hoescht (Immediately following completion of Seahorse experiment)
 - i. In a 15mL tube add:
 1. 12mL of Seahorse Media

2. 12.1µL of 20mM Hoescht stock
3. 3.6µL of 1mM MitoTracker Green stock
- ii. Add 100µL of dye mixture to cells and place in incubator for 30min @ 37°C. Protect from light.
- iii. After incubation, run plate on plate reader → read: Fluorescence intensity:
 1. MitoTracker Green → 485/20 Excitation, 528/20 Emission
 2. Hoescht → 360/40 Excitation, 460/40 Emission
- iv. Open Seahorse File, click “Normalize” and input fluorescence values for MitoTracker Green/Hoescht, click “Apply”.
- v. Click “Export” and select “Mito Stress Test Report Generator”.

GATING STRATEGIES, COMPENSATION, AND READING FLOW CYTOMETRY DATA

Materials required:

1. Ice
2. Ice cold 1xPBS
3. Labelled 1.5mL Eppendorf tubes
4. Cell scrapers (as many as conditions)

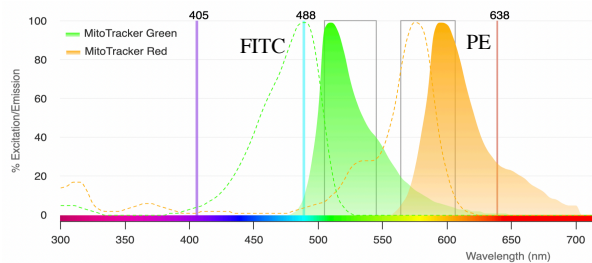
Procedure (tube mode sample collection):

1. Daily setup, clean, and quality control (QC)

1. Turn on the computer and open the CytoFlex software, ensure the sheath and waste fluid icons are green, indicating waste is low and there is adequate sheath fluid available.
2. Click “initialize”. The machine will prompt you to begin daily executions, click “yes” and proceed.
 - a. Open the right side of the machine and ensure the daily clean (DC) bottle is at least halfway full of solution.
 - b. DC solution must be replaced once per month and is prepared with equal parts ddH₂O and Contrad 70 solution (Beckman).
3. Prepare a small round bottom tube with 2mL sterile ddH₂O. Place the tube on the vial stand and click “start”. A 10-minute H₂O clean will occur.
4. Prepare another small round bottom tube with 2mL of FlowClean Cleaning Agent (Beckman) and perform the daily clean for 5 minutes.
5. Retrieve QC beads from the 4°C (Beckman), and vortex vigorously for a minimum of 2 minutes to disperse clumped beads.
6. Obtain a sterile 1.5mL eppendorf tube and aliquot roughly 200µL of QC solution. Vortex this for 1 minute.
7. Open the “QC standardization” protocol and select the lot number corresponding to the beads being used. Do not proceed with the experiment if QC fails. This is an important step to ensure all the lasers are properly aligned and functional.

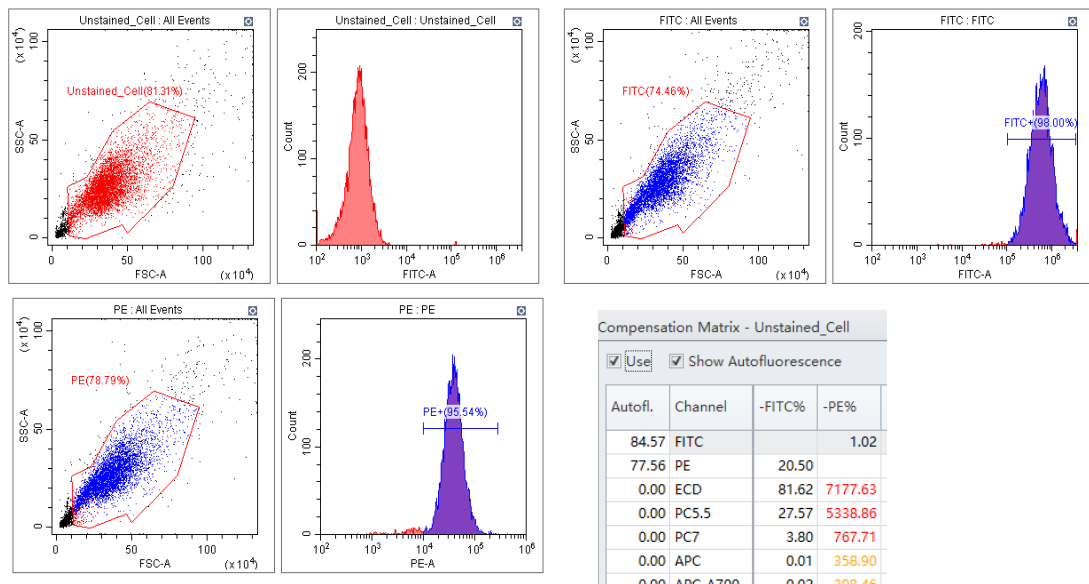
2. Compensation: correcting for fluorescence spillover

- Rationale:** Fluorescent molecules emit light at distinguished wavelengths which are detected by specific filters. Yet the emission spectra of two fluorophores may still overlap, making it important that each detector/laser is fitted with filters such as FITC and PE, which eliminate neighbouring light and provide a narrower spectral region. However, even with filters in place, some FITC fluorescence may be detected by the PE filter and some of the PE fluorescence may be detected by the FITC filter, resulting in fluorescence spillover. To account for this, compensation experiments were completed prior to handling experimental samples when applying more than one dye.



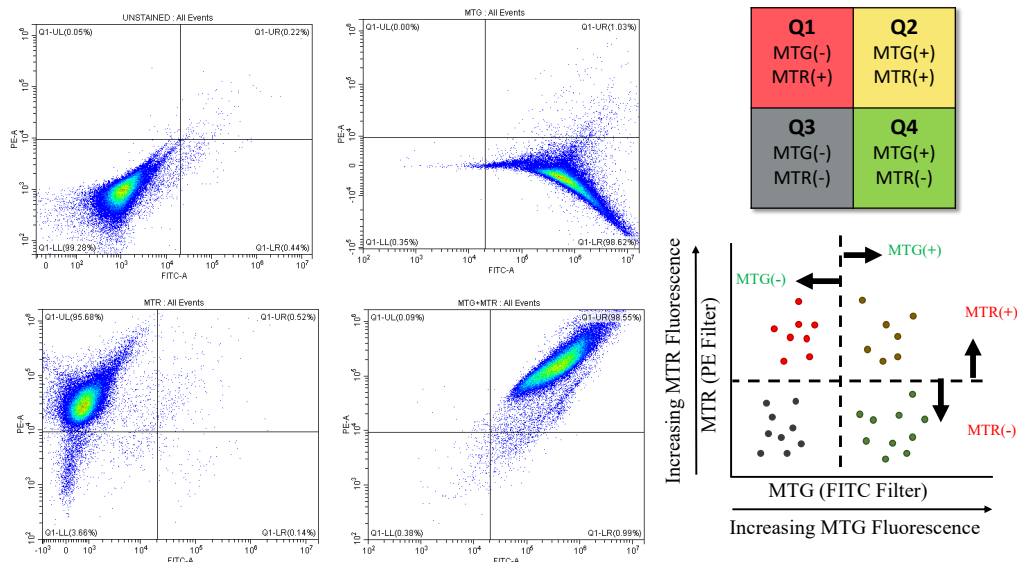
The importance of compensation is exemplified when observing the spectral overlap between MTG and MTR as shown on the left

- Unstained and single stained (e.g., MitoTracker Green, MitoTracker Red) cells were prepared, and resuspended in ice cold PBS. Debris was gated out of the cell population and applied to both FITC and PE filters.
- A gate was placed on the positive peak, corresponding to stained cells. Fluorescent spillover in both channels were calculated and saved to the compensation library. These parameters were applied to the new experimental file.
 - Check the compensation matrix and ensure values are below 25 for the most reliable results. If values are too high the CytoFlex software will alert you by displaying the values in yellow and red.



3. Experimental samples: gating strategies and reading data

1. Following treatments and staining, aspirate 6-well cell culture plates with myotubes. wash once with warm PBS and trypsinize with 250 μ L trypsin/well for 5 minutes at 37°C.
2. Add 250 μ L of phenol free media/well to neutralize trypsin. Gently scrape cells for collection and centrifuge in a 1.5mL Eppendorf tube at 1.4 RPM for 3 minutes.
3. Aspirate media and resuspend each pellet in ice cold PBS. Volume should be approximately 5X the pellet size to produce a very dilute solution.
4. Place samples on ice, and cover with aluminum foil to protect from light.
5. Vortex each sample vigorously before placing on the vial stand and running your sample. Ensure the flow rate is extremely low, as myotubes are large and can clog the tubing system.
6. Record 50,000 events (1 event = 1 cell) for each condition.
7. Begin the experiment with unstained cells, followed by single stains and finally the double stained control cells, as shown below with the representative dot plots
- Ensure a backflush (minimum 10 seconds) is completed in between samples to remove any remaining sample.



8. Apply gates to dot plots encompassing all events, starting with unstained cells. Depending on the stain used, your gating strategy may differ, but when staining for MitoTracker Green (MTG) and MitoTracker Red (MTR), this should be extremely well retained by healthy cells, meaning staining should be close to 100%. Ensure almost all cells are within the corresponding quadrant for each stain, modifying the gates as needed after seeing all stains.

****The same gate must be applied to every condition to minimize bias****

9. After the experiment, perform the daily clean as completed prior to the experiment (see above) and shut down the cytometer.

PREPARING LB MEDIA AND AGAR PLATES

Lysogeny Broth (LB Media; 500mL):

- Yeast extract (2.5g)
- Biotryptone (5.0g)
- NaCl (5.0g)

Agar Plates Media (500mL):

- Yeast extract (2.5g)
- Biotryptone (5.0g)
- NaCl (5.0g)
- Agar (7.5g)

1. Obtain two beakers, measure everything for each corresponding solution.
2. Add 500mL of ddH₂O, add a stir bar, and mix both solutions on a stir plate (high speed). Ensure there are no clumps.
 - NOTE: the agar may be slightly cloudy, this is okay.
3. Pour the solutions into glass bottles (~250mL into a 500mL bottle), cap very loosely and autoclave.
 - NOTE: for agar, autoclave each bottle with a stir bar, this will help with the cool down process later.
4. Place LB media on the shelf, this will be used later.
5. Agar bottles must be cooled down before use (warm to the touch). Place bottles on a stir plate, spin them at a high speed with the cap loosened to prevent pre-mature setting.

While the agar is cooling prepare antibiotics & petri dishes:

6. Add antibiotics in a ratio of 2μL antibiotic: 1mL LB media added (∴ 1mL antibiotic: 500mL LB media). Ampicillin or Kanamycin can be added, depending on DNA resistance.
 - Kanamycin → 25mg/mL
 - Ampicillin → 50mg/mL

NOTE: dilute each antibiotic in sterile H₂O, vortex until clear, and place on the orbital shaker at 4°C until ready to use; this must be prepared fresh.
7. Set up a large Kim-wipe in the fume hood and lay out petri dishes. Label with the date, antibiotic, and initials.
8. Add the appropriate antibiotic to agar, place on stir plate for one minute then place in the fume hood.
9. Spark flame the Bunsen burner, open agar bottle, and flame the neck of the bottle to sterilize. Pour the agar into the dish. Flame the neck of the bottle for every pour and work close to the flame to ensure your workspace is sterile.
10. Push away any bubbles to the side with a sterile P10 tip. Leave the lids halfway on the dish to ensure the agar solidifies without any condensation.
11. Once the agar has completely solidified, wrap the plates with parafilm and place inverted in the 4°C.

PLASMID DNA AMPLIFICATION AND ELUTION

DAY 1: TRANSFORMING BACTERIA AND STREAKING AGAR PLATES

1. Place *E. coli* cells and your plasmid of interest on ice. Add 1µL of the plasmid (100ng) directly to the bacteria (~1mL of cells, previously aliquoted). Stir gently with a pipette tip.
2. Incubate on ice for 30 minutes.
3. Heat shock the bacteria-DNA solution for exactly 30 seconds at 42°C. Immediately place this on ice and incubate for five minutes.
4. Add 475µL of outgrowth media, mix gently by pushing against the tube with your finger.
5. Incubate the cells at 30°C and 250 RPM for one hour.
6. Following incubation, set up the Bunsen burner again in the fume hood. Dip the inoculation wand in the bacteria-DNA solution. Streak agar plates throughout all 4 quadrants of the plate to isolate individual colonies. Place the dish in the 37°C incubator overnight (~16hrs).

DAY 2: COLONY COLLECTION AND SMALL AMPLIFICATION

1. Prepare the appropriate antibiotic for each plasmid (as previously described) and add it to the LB that was prepared previously.
2. Mix the LB media by hand. Aliquot an arbitrary volume of LB media into a 15mL falcon tube (~5mL).
3. Obtain a sterile P10 and gently collect 1-3 colonies. Place the whole tip in the falcon tube.
4. Agitate overnight at 37°C and 250 RPM.
 - Incubate an extra 15mL tube with only LB media (no colonies) as a control. This tube should be clear, while the other tubes containing cells should be cloudy.

DAY 3: LARGER AMPLIFICATION

1. Autoclave 500mL flasks and centrifugation bottles (1 per colony). These flasks will be used for a second, larger amplification step.
2. Prepare the antibiotic as described previously, place on the orbital shaker at 4°C.
3. Light the Bunsen burner. Flame the neck of the LB bottle and pour 250mL into each large flask. Repeat this for each colony.
4. Add the correct proportion of antibiotic to each flask, mix by hand.
5. Pour all contents of the 15mL falcon tube (containing colony of interest) into the corresponding flask.
6. Place the flask on the shaker at 250 RPM at 37°C overnight.

DAY 4: ELUTING DNA WITH THE QIAGEN MAXI-DNA PLASMID KIT (CAT NO. 12123)

1. Save a bacterial stab of each plasmid to avoid performing a bacterial transformation again.
 - Obtain a cryotube, add 500uL 50% glycerol and 1mL of the overnight liquid culture.
 - Label (date, plasmid, antibiotic, and initials) and snap freeze in liquid nitrogen before storing in -80°C.

2. Verify that the LB media containing your colony is cloudy before pouring the contents of the flasks into the corresponding centrifugation bottles. Spin this down at 6000xg for 15 minutes at 4°C.
 - Use rotor JA-10, kept in the 4°C; ensure the teeth of the rotor are aligned and the cap is tightened until it is immovable.
3. While your bacteria are spinning, assemble the DNA isolation setup:
 - Obtain a large Pyrex and place a tube holder on the back.
 - Place the QIAfilter cartridges in the rack with the caps screwed on (one cartridge/centrifugation bottle).
 - Place a cheese cloth in each tube in a bowl shape.
4. Following centrifugation, resuspend the pellet with 10mL of buffer P1. Perform resuspension with a sarpette and vigorously vortex until a homogenous solution is formed.
5. Add 10mL of buffer P2, screw on the caps tightly, and mix each bottle by hand using a quick pulsating flick. Incubate at room temperature for 5 minutes.
6. Add 10mL of buffer P3 to each bottle. Mix again with quick pulsating flicks.
7. Pour the lysate over the corresponding QIAfilter cartridge, over the cheese cloth.
 - Do NOT insert the plunger.
 - Incubate for 10 minutes at room temperature.
8. Place the Hi-speed maxi tips on the tube holders and equilibrate with 10mL of buffer QBT.
9. Once the QBT has cleared, remove the cheese cloth in the cartridges (this should contain cellular debris) and use a plunger to squeeze the liquid into the equilibrated maxi tips.
10. Pour 60mL of buffer QC into each of the Hi-speed maxi tips.
11. Organize 50mL falcon tubes corresponding to each maxi tip. Add 15mL of buffer QF to each maxi tip.
12. Precipitate DNA by adding 10.5mL of isopropanol to each tube. Mix by hand and incubate for 5 minutes at room temperature.
13. During the incubation, setup 30mL syringes with maxi module filters. Pull the plungers off before putting the filters on.
14. Pour the contents of the falcon tube into the syringe setup, filter all the way down using the plunger. The DNA should now be trapped in the filter.
15. Take the filter off, pull out the plunger and put the filter back on. Add 2mL of 70% EtOH to each syringe and plunge through. Remove the filter, remove plunger, put the filter back on and repeat this step 5 times.
16. Remove the filter and transfer them to a 5mL syringe.
17. Set-up 15mL tubes and elute the solution with 1mL of buffer TE. Plunge through and repeat this step once more.
18. Add 100uL 3M sodium acetate (pH = 7.0) and 2.5mL 70% EtOH to each tube.
19. Mix by hand. You should be able to see some DNA at this point.
20. Cap the tubes and place in liquid nitrogen for 10 minutes.
21. Thaw the tubes then centrifuge at 15000xg for 10 minutes at 4°C.

- Use the JA-25-50 rotor found in the 4°C.
- 22. Discard the supernatant by pouring away from the pellet.
- 23. Wash the pellet with 300µL of 80% EtOH.
- 24. Spin down at 15000xg for 2 minutes at 4°C.
- 25. Discard the supernatant and dry the DNA pellet by carefully inverting the tubes.
- 26. Resuspend DNA with 50µL of sterile H₂O (depending on the size this volume may vary).
Mix with gentle pipetting.
- 27. Transfer all contents to a sterile Eppendorf tube.
- 28. Measure [DNA] using the NanoDrop and store at -20°C.

LUCIFERASE ASSAY

Materials required:

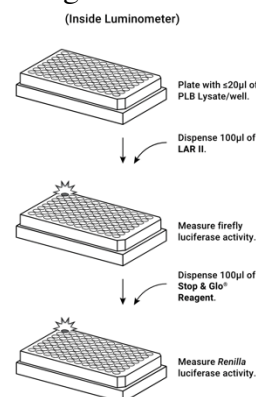
1. ddH₂O
2. White opaque 96-well plate
3. Steady-Glo[®] Luciferase Assay System kit (Promega, Cat no. E1910)

Reagent preparation

1. *1X passive lysis buffer (PLB)*: add 1 volume of 5x PLB to 4 volumes of ddH₂O, mix well.
2. *LAR II*: resuspend the lyophilized luciferase assay substrate in luciferase assay buffer II
3. *1X Stop & Glo[®] reagent*: add 2.1mL of 50X Stop & Glo[®] Substrate to 105mL of Stop & Glo[®] Buffer in a sterile Eppendorf tube, producing a final working concentration of 1X.

Measurement of Luciferase activity (as shown on the right):

1. Collect cells after treatment as whole cells, snap frozen in liquid nitrogen.
2. Resuspend each pellet with ~500µL 1X PLB. Place on ice.
3. Add 20µL of cell-1X-PLB lysate into a white opaque 96-well plate.
4. Add 100µL of LAR II directly on top of the sample.
 - Samples must be plated in duplicates and averaged for the final reading.
 - Add 20µL 1X PLB (no sample) to evaluate background signal.
5. READ: measure firefly luciferase activity.
6. Immediately add 1X Stop & Glo[®] reagent into each well to quench the firefly luciferase activity and initiate renilla luciferase activity.
7. READ: quench and read.
8. Correct firefly luciferase values to renilla luciferase readings for each well. Average duplicates per sample.



APPENDIX D: OTHER CONTRIBUTIONS TO THE LITERATURE

PEER-REVIEWED PUBLICATIONS

1. Moradi N., Sanfrancesco, V., **Champsi, S.**, Hood, D.A. Regulation of lysosomes in skeletal muscle during exercise, disuse, and aging. *Free Radical Biology & Medicine*. Submitted.
2. Moradi N., **Champsi S.**, Hood D.A. Sulforaphane, Urolithin A and ZLN005 induce time dependent alterations in antioxidant capacity, mitophagy and mitochondrial biogenesis in muscle cells. *Sports Medicine and Health Science*. April 2024.
3. D'Souza N.C., Aiken A.J., Hoffman E.G., Atherley S.C., **Champsi S.**, Aleali N., Shakeri D., El-Zahed M., Akbarian N., Mansouri M.N., Bavani P.Z., Liggins R.L., Chan O., Riddell M.C. Evaluating the effectiveness of a novel somatostatin receptor 2 antagonist, ZT-01, for hypoglycemia prevention in a rodent model of type 2 diabetes. *Frontiers in Pharmacology*. January 2024.

BOOK CHAPTERS

1. Hood D.A., Memme J.M., Oliveira A.N., Moradi, N., **Champsi, S.** Skeletal muscle book, volume II, section VII, chapter 24: Mitochondrial Biogenesis in Skeletal Muscle. *Springer Nature*. Submitted.

PUBLISHED ABSTRACTS AND CONFERENCE PROCEEDINGS

1. **Champsi S**, Hood D.A. Sulforaphane Treatment Mimics Training-induced Mitochondrial Adaptations in Skeletal Muscle Cells. *International Biochemistry of Exercise*. Limerick, Ireland. July 2024 – poster presentation.
2. **Champsi S**, Hood D.A. Induction of the Nrf-2 Pathway Mitigates Oxidative Stress and Enhances the Mitochondrial Phenotype in Skeletal Muscle Cells. *Muscle Health Awareness Day*. Toronto, ON. May 2024 – poster presentation.
3. **Champsi S**, Hood D.A. Activation of the Nrf-2 Pathway Improves the Mitochondrial Phenotype in Skeletal Muscle Cells. *American Journal of Physiology Summit*. Long beach, CA, USA. Apr 2024 – poster presentation.
4. **Champsi S**, Hood D.A. Effect of Sulforaphane on Mitochondria in Skeletal Muscle Cells. *Canadian Society for Exercise Physiology Conference*. Journal of Applied Physiology, Nutrition and Metabolism. In Press, Calgary, AB. October 2023 – poster presentation
5. **Champsi S**, Moradi N, Hood D.A. Characterizing the Role of Sulforaphane in Mitochondrial Function and Content in Skeletal Muscle Cells. *Muscle Health Awareness Day*. Toronto, ON. May 2023 – poster presentation.

ORAL PRESENTATIONS

1. Sulforaphane Impacts the Antioxidant Response and Mitochondria in Skeletal Muscle Cells. *Ontario Exercise Physiology conference*. Kingston, ON. July 2023 – oral presentation.
2. The Impact of Sulforaphane on Mitochondria in Skeletal Muscle Cells. KAHS Graduate Seminar 2023. York University, Toronto, ON, Jan 2024.

JUNIOR REVIEWER

Nutrients (2024)
Journal of Biochemistry and Molecular Biology. (2024)
Therapeutic Advances in Respiratory Disease. (2023)
Antioxidants. (2023)