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The role of SIRT1 in skeletal muscle function and repair of older mice

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Abstract

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Human skeletal muscle is a highly metabolic tissue necessary for mobility and coordination. Responsible for approximately one-fifth of the resting human metabolism, skeletal muscle is also an important regulator of metabolites like glucose and contributes to the regulation of body temperature. Although there is a gradual decline in muscle mass associated with aging, a certain percentage of the population suffer from severe muscle mass and strength deterioration, classified as sarcopenia (5-13% for people aged 60-70 years old, 11-50% for those 80 or older). Sarcopenia is linked to increased morbidity and mortality rates in the elderly population, while annual healthcare costs related to sarcopenia total in the millions of dollars. Because the prevalence of sarcopenia is expected to increase as a larger percentage of the population transitions into old age, it becomes imperative to understand the mechanisms of aging and longevity so that more effective interventions can be taken against age-related muscle deterioration. Our laboratory has previously demonstrated that resveratrol, a known activator of the protein sirtuin 1 (SIRT1), was effective in enhancing human muscle adaptations to exercise in elderly populations. A rich body of literature has long supported the association of SIRT1 with longevity, but there are still gaps in our knowledge of how SIRT1 expression affects the functionality and performance of muscles in aging skeletal muscle. Furthermore, SIRT1 has been shown to be important in the function of muscle satellite cells—which are muscle stem cells that are responsible for the majority of muscle regeneration. However, there is little knowledge about how SIRT1 expression affects muscle regeneration and performance after injuries. To investigate the role of SIRT1 in the performance of aging and injured skeletal muscle, we have employed the use of several transgenic mouse models with differential expression of SIRT1. Using these

models, we performed a series of functional muscle tests, before and after cardiotoxin (CTX) induced muscle injuries, to identify and compare muscle aptitude and recovery capability. Skeletal muscle sections from each model were also taken to identify differences in muscle fiber size and type distribution. Additionally, both mitochondria and satellite cells were isolated from these models to assess whether SIRT1 expression contributed to differences in metabolic or regenerative capacities. We found that there was little functional difference between young wild-type (YWT, aged 20-30 weeks) and aged (80+ weeks old) wild-type (WT-80), SIRT1 overexpressor (OE-80), and SIRT1 muscle-knockout (MKO-80) mice in either force production or fatigability in the absence of intervention. Mice lacking SIRT1 expression in their satellite cells (SKO-80), however, did show a reduction in force production. Interestingly, both the OE-80 and MKO-80 mice showed significant ($P < 0.05$) increases for p53 expression and reduced fatigability after recovering from injury, with the SIRT1 overexpressor model showing some signs of muscle potentiation. MKO-80 mice showed a significant increase in satellite cell regeneration ($P < 0.05$) *in vitro* when analyzed with EdU, but no difference in proliferation when compared *in vivo* with BrdU, indicating that SIRT1 expression in adult skeletal muscle may be an early factor in limiting the proliferation of satellite cells. The mitochondrial and structural profiles of each model were found to have minimal differences. Overall, our data indicate that although SIRT1 expression in skeletal muscle does not seem to be necessary for normal muscle function after injury, it does exert some influence in muscle repair. Altering SIRT1 expression either positively or negatively in skeletal muscle improves muscle fatigability in injury-recovered muscles, indicating a potential regulatory role for SIRT1 in skeletal muscle, but not an essential requirement for its deacetylation activity. Interestingly, these alterations of SIRT1 expression in aged skeletal muscle also resulted in a significant increase of p53 expression, indicating a potential benefit for p53 expression to muscle recovery. SIRT1 expression in satellite cells was shown to be necessary to achieve normal contractile force, but did not affect fatigability in those muscle. Our work has indicated a complex role for SIRT1 in skeletal muscle regeneration. We have shown for the first time that

SIRT1 is required in satellite cells for proper function, but is not essential for muscles to recover their functionality after injury. We have also provided evidence for a potentially new target for muscle recovery, the protein p53, and new insights into the role of SIRT1 in muscle recovery.

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List of Abbreviations

ΔΨm	mitochondrial membrane potential
°C	degrees Celsius
APC	allophycocyanin
ANOVA	analysis of variance
ASM	Appendicular Skeletal Muscle Mass
Atg-7	autophagy-related protein 7
Atg-12	autophagy-related protein 12
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphocyte 2 protein
Bim	Bcl-2-like protein 11
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CD31	cluster of differentiation 31
CD45	cluster of differentiation 45
CDC	Center for Disease Control
C. elegans	*Caenorhabditis elegans*
CSA	cross sectional area
CTX	cardiotoxin (*Naja mossambica*)
DCFH-DA	2'7'-dichlorodihydrofluorescein diacetate
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
EdU	5-ethyl-2'-deoxyuridine
EGCG	epigallocatechin-3-gallate
ESC	embryonic stem cell
FACS	fluorescence-activated cell sorting

FBS	fetal bovine serum
FOXO	forkhead box O
FOXO3	forkhead box O3
FSC	forward
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GTE	green tea extract
H_2O_2	hydrogen peroxide
h	hour
Hz	Hertz
IADL	Instrumental Activities of Daily Living
IgG	immunoglobulin G
IgM	immunoglobulin M
IFM	interfibrillar mitochondria
JC-1	5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimi-dazolylcarbocyanine iodide
kDa	kiloDalton
kg	kilogram
LC3	microtubule-associated proteins 1A/1B light chain 3B
m/s	meters per second
M	molar
Min	minutes
mL	milliliter
mM	millimolar
MCK	muscle creatine kinase
Mfn1	mitofusin-1
Mfn2	mitofusin-2
MHC	myosin heavy chain
MKO	muscle knockout (of SIRT1)
MKO-80	muscle knockout aged 80+ weeks
MnSOD	manganese-dependent superoxide dismutase
mRNA	messenger ribonucleic acid

NAD+	nicotine adenine dinucleotide (oxidized)
NADH	nicotine adenine dinucleotide (reduced)
NAM	nicotinamide
NHANES III	Third National Health and Nutrition Examination Survey
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nm	nanometer
OE	overexpressor (of SIRT1)
OE-80	overexpressor model aged 80+ weeks
Opa1	dynamin-like 120 kDa protein
PacBlue	Pacific Blue
PAGE	polyacrylamide gel electrophoresis
PE-Cy7	polyethylene glycol – cyanine 7
p53	tumor protein p53
Pax7	paired box 7
PBS	phosphate buffered saline
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Pum2	pumilio homolog 2
RNA	ribonucleic acid
Sca-1	stem cells antigen-1
SD	standard deviations
SDS	sodium dodecyl sulfate
SIRT1	sirtuin 1
Sir2	silent information regulator
Sir2p	silent information regulator 2 protein
SKO	satellite cell knockout (of SIRT1)
SKO-80	satellite cell knockout model aged 80+ weeks
SRT1720	small-molecule activator of SIRT1 1720
SSC	side scatter
SSM	subsarcolemmal mitochondria
TA	tibialis anterior

U	unit
UPR	unfolded protein response
VCAM-1	vascular cell adhesion protein 1
WT	wild-type
WT-80	wild-type model aged 80+ weeks
XO	xanthine oxidase
YWT	young wild-type model (aged 20-30 weeks)
µL	microliter
µM	micromolar

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Specific Aims

Muscle function peaks for the average person by 30 years of age; afterwards, most people experience an average decline of up to 1% muscle mass per year—a loss that can be exacerbated in old age (~65 years old) to up to a 3% loss per year [1]. This age-related loss of muscle mass is generally referred to as sarcopenia. A clinical working definition was established in 2010 by the European Working Group on Sarcopenia in Older People to characterize sarcopenic individuals as those with low muscle mass coupled with low muscle strength, where muscle mass is two or more standard deviations below the mean average of healthy young adults (when matched for sex) [2]. The focus of researchers on sarcopenic muscle wasting has increased over the last decade as evidence has accumulated to establish the growing relevance of sarcopenia to overall health. Sarcopenia is viewed most prevalently in the context of personal mobility, with the resultant loss of balance, coordination, and stability from muscle wasting acting as both a direct (impairing muscle performance) and indirect (increasing the likelihood of fall-related injuries) cause of physical disability [3]. Furthermore, prolonged immobility resulting from an injury or surgery can compound age-related muscle loss, possibly leading to permanent disability [4]. Loss of mobility and independence may often result in the need for assisted care or extended hospital stays; therefore, it is not surprising that estimates have suggested healthcare expenditures related to sarcopenia had cost the United States $18.5 million in the year 2000 alone [5].

Beyond the detriment sarcopenia brings to independence and quality of life, the loss of mobility resulting from age-related muscle loss also reduces the likelihood the elderly can benefit from physical exercise. The American College of Sports Medicine promotes the paradigm that “Exercise is Medicine,” a sentiment that is echoed by research touting the benefits of exercise in treating and preventing chronic disease [6,7]. Routine exercise is correlated with a reduced risk of developing cancer [8] and is recommended by the American Heart Association to help prevent the development of cardiovascular disease [9]. Furthermore, physical exercise is also strongly

recommended for the prevention and treatment of type 2 diabetes [10], signifying the importance of muscle not only for mobility and exercise, but also as a major metabolic regulator. Skeletal muscle mass accounts for roughly 20% of the body's resting metabolism and acts to regulate body heat and multiple metabolites like glucose, amino acids, and fatty acids [11]. Therefore, the cumulative effect of muscle loss with aging has far-reaching consequences for overall health, necessitating further characterization of the processes that underlie both sarcopenia and aging in general.

Interestingly, sarcopenia selectively causes marked atrophy and weakness in type II muscle fibers; whereas type I, oxidative fibers are largely spared from the most negative effects of aging [12]. It is largely unknown why type I fibers are selectively preserved in sarcopenia. The protein sirtuin 1 (SIRT1) is an NAD+-dependent deacetylase known to promote longevity in several model organisms [13] and is well characterized as metabolic regulator highly associated with oxidative, type I muscle fibers [14]. The long-term goal of our work was to identify the physiological and functional relevance of SIRT1 to skeletal muscle function in aging and muscle recovery and to determine whether SIRT1 could function as a suitable target for interventions against age-related muscle deterioration. The **central hypothesis** of this dissertation was that the preservation of type I fibers in aging was related to the high expression of SIRT1 in those fibers and that a loss of SIRT1 would exacerbate the fatigability and attenuate force output in muscle of aged mice. Furthermore, we hypothesized that increasing SIRT1 would improve muscle functionality by improving mitochondrial health in skeletal muscle—through increasing the abundance of mitochondria and the enzymatic activity of mitochondrial respiratory complexes—and by increasing muscle satellite cell abundance. We tested our **hypothesis** by investigating the following specific aims: (1) elucidate the role of SIRT1 in regulating muscle function and fatigue resistance in regenerating skeletal muscle of aged mice, (2) determine the role of SIRT1 in regulating mitochondrial biogenesis and function in regenerating/aging skeletal muscle, and (3) determine if SIRT1 activation can improve proliferation in satellite cells in aging mice.

To address **Specific Aim 1**, we utilized 5 mouse models: YWT (young wild-type, aged 20-30 weeks); WT-80 (wild-type mice, aged 80+ weeks); OE-80 (overexpressor mice, aged 80+ weeks), MKO-80 (muscle knockouts of SIRT1, aged 80+ weeks); and SKO-80 (satellite cell knockouts of SIRT1, aged 80+ weeks), all of which shared a common C57BL/6J background. These models were functionally compared via their skeletal muscle force generation and fatigability, factors which were induced by electrical stimulation of the hindlimbs. Our working hypothesis was that the loss of SIRT1 would exacerbate skeletal muscle fatigability and reduce muscle force after injury, while overexpression of SIRT1 would protect against these effects.

To address **Specific Aim 2**, we isolated mitochondria from skeletal muscles of our aged, transgenic mouse models and compared the number and size of mitochondria, as well as the mitochondrial membrane potential and complex activity. Our working hypothesis was that the loss of SIRT1 in the skeletal muscle knockout and satellite cell knockouts models of aged mice would reduce mitochondrial activity and increase mitochondrial dysfunction in mature skeletal muscle.

To address **Specific Aim 3**, we induced muscular injury in our mice through the administration of cardiotoxin (CTX) isolated from the Mozambique spitting cobra (Naja mossambica). We then administered 5-bromo-2'-deoxyuridine (BrdU) through drinking water with ad libitum access and allowed the mice to recover for 3 weeks. After the mice recovered from their injuries, we evaluated their muscle performances and the number of BrdU positive nuclei as a measurement of proliferation. Additionally, we isolated satellite cells from each model and labeled the cells with 5-ethynyl-2'-deoxyuridine (EdU). EdU labeled cells were then allowed to proliferate and were subsequently quantified as a measurement of satellite cell proliferation. Our working hypothesis was that overexpression of SIRT1 would improve muscle satellite cell-mediated skeletal muscle regeneration and muscle function after injury by increasing the proliferation of satellite cells.

The data generated from this study was significant because it expanded upon previous work, which mainly characterized structural, biochemical, or metabolic differences in skeletal

muscle associated with altered SIRT1 expression, and did not investigate the translational effects these differences had on actual muscle performance. Furthermore, our findings were novel in that they highlighted a potentially important association between the expression of SIRT1 and p53 during skeletal muscle repair, enhancing our understanding of the repair process of skeletal muscle in aging mice.

References

1. Mitchell WK, Williams J, Atherton P, Larvin M, Lund J, Narici M. Sarcopenia, Dynapenia, and the Impact of Advancing Age on Human Skeletal Muscle Size and Strength; a Quantitative Review. Front Physiol. 2012;3. doi:10.3389/fphys.2012.00260

2. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, et al. Sarcopenia: European consensus on definition and diagnosis. Age Ageing. 2010;39: 412–423. doi:10.1093/ageing/afq034

3. Sarcopenia: Aging-Related Loss of Muscle Mass and Function | Physiological Reviews [Internet]. [cited 20 Mar 2019]. Available: https://www.physiology.org/doi/abs/10.1152/physrev.00061.2017?rfr_dat=cr_pub%3Dpubmed&url_ver=Z39.88-2003&rfr_id=ori%3Arid%3Acrossref.org&journalCode=physrev

4. Hida T, Ishiguro N, Shimokata H, Sakai Y, Matsui Y, Takemura M, et al. High prevalence of sarcopenia and reduced leg muscle mass in Japanese patients immediately after a hip fracture. Geriatrics & Gerontology International. 2013;13: 413–420. doi:10.1111/j.1447-0594.2012.00918.x

5. Janssen I, Shepard DS, Katzmarzyk PT, Roubenoff R. The healthcare costs of sarcopenia in the United States. J Am Geriatr Soc. 2004;52: 80–85.

6. Kujala UM. Benefits of exercise therapy for chronic diseases. Br J Sports Med. 2006;40: 3–4. doi:10.1136/bjsm.2005.021717

7. Booth FW, Roberts CK, Laye MJ. Lack of exercise is a major cause of chronic diseases. Compr Physiol. 2012;2: 1143–1211. doi:10.1002/cphy.c110025

8. Brown JC, Winters-Stone K, Lee A, Schmitz KH. Cancer, Physical Activity, and Exercise. Compr Physiol. 2012;2: 2775–2809. doi:10.1002/cphy.c120005

9. Agarwal SK. Cardiovascular benefits of exercise. Int J Gen Med. 2012;5: 541–545. doi:10.2147/IJGM.S30113

10. Colberg SR, Sigal RJ, Fernhall B, Regensteiner JG, Blissmer BJ, Rubin RR, et al. Exercise and Type 2 Diabetes. Diabetes Care. 2010;33: e147–e167. doi:10.2337/dc10-9990

11. Leon AS. Attenuation of Adverse Effects of Aging on Skeletal Muscle by Regular Exercise and Nutritional Support. American Journal of Lifestyle Medicine. 2017;11: 4–16. doi:10.1177/1559827615589319

12. Nilwik R, Snijders T, Leenders M, Groen BBL, van Kranenburg J, Verdijk LB, et al. The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. Experimental Gerontology. 2013;48: 492–498. doi:10.1016/j.exger.2013.02.012

13. Guarente L. Sirtuins, Aging, and Metabolism. Cold Spring Harb Symp Quant Biol. 2011;76: 81–90. doi:10.1101/sqb.2011.76.010629

14. Chalkiadaki A, Igarashi M, Nasamu AS, Knezevic J, Guarente L. Muscle-Specific SIRT1 Gain-of-Function Increases Slow-Twitch Fibers and Ameliorates Pathophysiology in a Mouse Model of Duchenne Muscular Dystrophy. PLOS Genetics. 2014;10: e1004490. doi:10.1371/journal.pgen.1004490

Chapter 1:

Literature Review

1.1 Aging Skeletal Muscle: Sarcopenia

In a letter dated August 4th, 1836, Charles Darwin wrote to his sister, "…I shall act, as I now think—that a man who dares to waste one hour of time has not discovered the value of life." This small sentiment of Darwin unintentionally illustrates the underlying nature of longevity research: time is a fundamentally valuable, but limited resource. Scientific study and modern medicine have long benefitted human health; the Center of Disease Control (CDC) FastStats, for example, detail a progressive increase in life expectancy (at birth) for the United States from 1900 (47.3 years) to 2015 (78.7 years) [1]. Modern science, however, credits Dr. Nathan W. Shock as the "father of gerontology," due in part to his landmark, longitudinal study detailing the age-related changes in physiological function across the heart, kidneys, lungs, nerves, and brain [2]. His study was the first to markedly observe differential rates of functional decline between individuals and spurred forward new investigations into the process of aging. The intricacies of aging have been examined to mechanistic levels across a vast spectrum of models and tissues, including skeletal muscle. Currently, the majority of investigations into aging skeletal muscle are framed within the context of sarcopenia.

The term 'sarcopenia'—derived from the Greek *sarx*, meaning 'flesh,' and *penia*, meaning 'poverty,'—was established by Dr. Irwin H. Rosenberg in an effort to draw serious attention to the dramatic decrease in lean muscle mass observed with aging [3]. Although a cursory internet search generally defines sarcopenia as the loss of muscle mass with aging, more recent literature has attempted to define and refine the differences between general and clinical sarcopenia. The first attempt at a clinical definition came from a study investigating elderly Hispanic and non-Hispanic white subjects living in New Mexico. The investigators defined the cutoff values for sarcopenia as two standard deviations (SD) below the mean muscle mass relative to reference data for sex-matched subjects 18-40 years old and quantified according to the Appendicular Skeletal Muscle Mass (ASM) index to account for height differences among subjects [4]. In this

initial study, balance abnormalities, histories of bone fractures, and falling accidents were associated with sarcopenia [4]. Furthermore, sarcopenia showed significant correlation with both age and obesity, independent of biological sex. This finding would lead to another investigation, establishing the existence of sarcopenic obesity (defined in that particular study as sarcopenia coupled with body fat greater than the 60th percentile of the study) [5]. Sarcopenic obese patients were identified as being significantly more likely to be male and were 2.5 times as likely to report functional decline according to the Instrumental Activities of Daily Living (IADL) index. Importantly, both obese and non-obese subjects that reported functional IADL drops were reported to have over a 20% increase in death rates relative to the elderly who did not show IADL drops. However, a growing accrual of conflicting reports initially disputed these claims by providing strong evidence that low relative muscle mass alone did not contribute to increased rates of disability or death [6,7]. Notably, analyses based on the Health, Aging, and Body Composition (HABC) longitudinal study following 3,075 men and women, ages 70-79, indicated that the decline of lean body mass alone could not account for an increase in persistent lower extremity limitation, when normalized only to height [8]. To the contrary, subjects defined as sarcopenic by the ASM in some cases showed no decline in lower extremity performance, despite their relatively low muscle mass, whereas including obesity as a factor allowed for better prediction of functionality loss for subjects classified as sarcopenic. Furthermore, a later evaluation of the HABC study also showed evidence that an age-associated decline of muscle strength, evaluated as the torque generated by the mid-thigh muscle of elderly subjects, occurred independently from the loss of mass [9]. Loss of muscle strength was up to 5 times greater when adjusted to the relative loss of muscle CSA, and could occur in subjects that lost weight, in weight-stable subjects, and even in subjects that showed small increases in muscle CSA. The growing body of work reporting similarly conflicting results began to illustrate the need for a better definition of sarcopenia [10].

In 2011, the European Working Group on Sarcopenia in Older People (EWGSOP) convened to create a consensus on the working definition of sarcopenia [6]. EWGSOP recommended that beyond the criterion of low muscle mass (defined by 2 SD below mean muscle mass of sex-matched, healthy young adults), subjects should present at least one of two criteria: low muscle strength (handgrip: < 30 kg men/ 20 kg women) or low physical performance (gait on 6m course: < 1 m/s men, < 1.175 m/s women).These working definitions set a baseline for consensus in scientific research that aimed to reduce the variability seen in the prevalence of sarcopenia.

Despite the lack of a universal adoption of the EWGSOP standard, meta-analysis of previously published literature—recontextualized with these standards—has identified a clear link with sarcopenia to mortality and functional decline [11]. Although clear epidemiological studies are lacking for other commonly reported outcomes of sarcopenia—including falls, fractures, and increased frequency and length of hospitalization—evidence does suggest that injuries can worsen the sarcopenic state. The most commonly studied injuries related to sarcopenia are hip fractures, which are cited as the most frequently occurring fall-related injuries, outside of superficial injuries, to persons aged ≥ 75 years old [12]. Such falls may continue to harm elderly patients during hospitalization, as extended bouts of muscle disuse and/or immobility have long been attributed to reductions in skeletal muscle and bone mass [13–15]. One cross-sectional study reported that at least 75% of elderly men and 50% of elderly women (> 70 years) hospitalized with hip fractures were attributed with sarcopenia—rates generally over 20% more than age-matched patients without hip fractures [16]. Longitudinal studies have also shown that hospitalized geriatrics with sarcopenia are significantly more likely to experience mortality in as few as 2 years post-hospitalization [17,18]. Sarcopenic geriatrics who do survive are faced with the grim aspect of rising healthcare costs. A 2004 study estimated that spending in 2000 for sarcopenia in the United States likely amounted to $18.5 billion in healthcare costs, just in

consideration of spending related to disability services, including hospitalization, rehabilitation, or at-home health services [19].

1.2 Selective Atrophy of Type II Fibers with Sarcopenia

Interestingly, sarcopenic muscle wasting does not appear to affect skeletal muscles homogenously. Skeletal muscle across mammals consists of heterogenous populations of myofibers, most easily differentiated by their contractile and metabolic properties [20]. Contraction properties are dependent on the expression of myosin heavy chain isoforms, which have differential rates of ATP hydrolysis—determinants of the twitch characteristics (i.e. whether the fiber is a fast-twitch or slow-twitch fiber). Type II fibers are pale muscle fibers reliant on glycolysis for their metabolism, are capable of undergoing marked hypertrophy, and are characterized by fast-twitch contractions. In contrast, type I fibers are a red, due to their rich myoglobin content, which is used to sequester oxygen. This oxygen is necessary for respiration, as these fibers are reliant on oxidative phosphorylation for their energy needs. Furthermore, type I fibers are characterized as slow-twitch fibers and undergo ATP hydrolysis at a slower, but more continuous rate.

Characterization of skeletal muscle fibers from human subjects aged 65-86 years old has shown that type II fibers are smaller in their mean individual CSA and cover about 13% less total CSA than do type I fibers [21]. The smaller occupied area also correlates with fewer muscle satellite cells (MSCs) per fiber, totaling to about 0.9% fewer MSCs overall. In a separate study comparing the characteristics of the vastus lateralis muscle in young (24-26 years old) and elderly (70-72 years old) men, type II fibers were shown to decrease in in CSA up to 29% in the elderly, relative to a mean decline of 20% across all fiber types [22]. Furthermore, the quantity of type II fibers decreased by about 10%, without significant change in type I fiber quantity. Overall, these reductions were shown to generate a 14% reduction of CSA in aged quadricep muscles relative

to the control young men. This selective atrophy of type II fibers is also exacerbated with prolonged immobility resulting from hip fractures in elderly female patients [23].

1.3 Loss of Muscle Satellite Cells with Type II Fibers

Associated with the loss of type II fibers is a reduction of satellite cells and myonuclei. Muscle satellite cells (MSCs) are mononucleated stem cells that derive their name from their physiological location around myofibers, situated between the fiber's plasma membrane and the muscle basal lamina [24]. The position of these muscle stem cells is critical to their function *in vivo:* damage to the host skeletal muscle activates MSCs to initiate muscle repair [25,26]. Vital to MSCs is the paired box 7 (Pax7) transcription factor. Mice models knocked out for Pax7 completely lack MSC populations [27] and tamoxifen-induced inhibition of Pax7 in adult satellite cells severely reduces muscle repair and leads to marked muscular atrophy [28,29].

Importantly, satellite cells contribute to muscle repair through a mechanism of asymmetric division, a process that maintains two distinct fates for MSCs: proliferation or differentiation [30]. Generally, Pax7-expressing MSCs operate in a state of quiescence with little to no activity beyond basal metabolism [24]. Upon activation by injury, MSCs will upregulate the expression of myogenic differentiation factors, which drive the asymmetric division: co-expression of Pax7 and MyoD (myogenic differentiation 1) commit satellite cell populations to proliferation, whereas expression of MyoD and downregulation of Pax7 commit these sells to differentiation [31]. Importantly, the pool of satellite cells available for muscle repair declines in association with aging [32]. Associated with the decline of satellite cell number is a loss of regenerative capacity for muscle repair, although this decline occurs without an associated loss of differentiation potential, indicating that age-related dysfunction in regeneration is associated with satellite cell abundance and/or proliferative capacity [33]. In association with the decline of CSA/mass linked to selective type II fiber atrophy, cross-sectional studies in geriatrics (>70 years of age) have also shown that MSC populations are specifically lower in type II muscle fibers relative to type I fibers [21].

Furthermore, the decline in overall abundance of MSCs in type II fibers appears to be specific to those fiber types [34,35]. These data suggest that a decline in satellite cell population likely stemming from reduced proliferation contributes to the loss of muscle mass and function characteristic of sarcopenia.

1.4 Oxidative Stress Accompanies Mitochondrial Aging in Sarcopenia

In addition to loss of satellite cells and regenerative capacity, sarcopenic muscles have also been reported to express hallmarks of mitochondrial dysfunction [36,37]. Aging mitochondria have been associated with the dysregulation of mitochondrial fission and fusion proteins in rodent models, coinciding with abnormal mitochondrial structures—including extensive fragmentation [38]. Notably, aged rodents have been shown to have reduced levels of mitochondrial fusion proteins Mfn1/Mfn2 compared to young controls. A similar dysregulation has been noted in rodent muscles undergoing 7 days of disuse, wherein the expression of the fission proteins Mfn2 and Opa1 were decreased relative to rodents whose dorsiflexor muscles were activated by stimulation over a period of 7 days [39]. The accumulation of fragmented mitochondria in aging tissue have been attributed to reduced clearance of damaged mitochondria by impaired mitophagy. Furthermore, a recent study has linked the CRISPR/Cas9-mediated knockout of the RNA-binding protein Pumilio2 (Pum2) to improved mitophagy, to mitochondrial house-keeping through the regulation of pathway-specific proteins downstream of MFF (mitochondrial fusion factor), and to increased lifespan in *C. elegans* [40]. Mitochondrial fission proteins and healthy mitophagy have been shown to accompany the removal of fragmented mitochondria and are stimulated by mild oxidative stress [41]. Although mild oxidative stress produced by mitochondrial metabolism does stimulate the expression of protective antioxidants through reactive oxygen species (ROS) signaling [42], highly elevated levels of ROS have been associated with mitochondrial dysfunction and oxidative damage to cellular structures [43].

Sarcopenic skeletal muscles show increased rates of dysfunction relative to muscle tissue from non-sarcopenic subjects, including elevated levels of reactive oxygen species (ROS) and oxidative damage. Dysregulation of electron transport chain complexes, like cytochrome-c oxidase (COX), occurs more prevalently in sarcopenic muscle [44], and is a major contributor of ROS production in dysregulated mitochondrial metabolism associated with aging [45]. For example, the superoxide reactive species is produced by NADPH oxidases during oxidative phosphorylation [42]. Unless quenched by protective enzymes—in this case, the family of superoxide dismutase (SOD) proteins—superoxide can react to form more free radicals, which can rapidly accumulate and cause widespread oxidative damage to the cellular environment through radical chain reactions. As such, tissues heavily reliant on oxidative phosphorylation transcribe elevated levels of antioxidants and ROS scavengers—such as the glutathione peroxidase family of enzymes responsible for removal of H2O2—to scavenge free radicals and reduce the amount of oxidative damage done to cellular proteins, membranes, and DNA [46].

Important to the context of sarcopenia, elevated oxidative stress levels have frequently been associated with age-related dysfunction in tissues, particularly in skeletal muscle. Previous work from our lab has shown that subcutaneously implanted allopurinol inhibits xanthine oxidase (XO), a source of ROS production during exhaustive exercise and significantly blunts ROS production in the isolated mitochondria of aged mice [47]. Furthermore, this reduction of mitochondrial ROS was accompanied by an attenuation of force production during electrically-stimulated isometric muscle contractions in aged rodents.

Support for the beneficial effects of reducing oxidative stress in promoting muscle function have also been evidenced by the application of the dietary antioxidants (vitamins E & C) [48] and green tea extract (GTE), whose active ingredient is the polyphenol epigallocatechin-3-gallate (EGCG) [49]. Interestingly, though both models attenuated oxidative damage to the muscles, the profiles of protection differed between dietary supplements. Oxidative stress was shown to be

attenuated in GTE-treated mice through the reduction of protein carbonyls and prostaglandins—markers of oxidative stress—and attenuated muscle wasting from disuse. These mice, however, did not show changes in SOD levels, which accompanied oxidative stress changes in both the previous XO-inhibited mice [47] and vitamin E&C treated mice [48]. Vitamin-fed mice also experienced an increase in muscle work after treatment in aged mice. These data suggest that oxidative stress negatively effects muscle throughout widespread pathways.

1.5 SIRT1 Protein Protects against Cell Death under Stress

Aside from directly affecting muscle function, oxidative stress can activate pro-apoptotic pathways and induce cellular death. One pro-apoptotic protein shown to be activated by oxidative stress and elevated ROS abundance is the tumor suppressor p53. The p53 protein is known to regulate a wide array of cellular processes, most notably cell cycle arrest and the induction of apoptosis. Upregulation of p53 is a multifaceted process dependent on multiple stress-response pathways, but can be initiated by ROS through the initiation of the DNA-damage repair pathway [50]. Although the elevation of ROS levels alone is not strongly linked to increased cell mortality, elevated ROS levels have been linked to increased cell mortality when co-induced with p53 in H1299 lung carcinoma cells [51]. Interestingly, elevated p53 abundance is linked to aging through the DNA-repair pathway, as the accrual of DNA damage is shown to correlate with age across multiple tissue types in human models [52]. In skeletal muscle, p53 abundance is also shown to be elevated in the muscles of C57BL/6-background murine models undergoing prolonged immobilization, linking p53 abundance to disuse-induced muscle atrophy. Indeed, the transgenic knockdown of p53 in these immobilized mouse models significantly attenuates muscle atrophy [53].

One protein that is associated with both oxidative fibers and with inhibiting p53-induced cellular death is sirtuin 1 (SIRT1) (Fig 1-2). SIRT1 is an NAD+-dependent histone deacetylase. Aside from histone deacetylation, SIRT1 has also been shown to directly deacetylate non-histone

proteins to promote or inhibit their function; indeed, one of the first non-histone proteins shown to be targeted for direct deacetylation by SIRT1 is p53 [54]. Targeted knockdown of SIRT1 in yeast has been shown to increase the acetylation of p53 and reduce yeast lifespan, whereas overexpression of SIRT1 decreases p53 acetylation and improves lifespan. Conversely to protecting cellular integrity under oxidative stress, the transgenic knockdown of SIRT1 in leukemia cells has been established as a method to promote p53's downstream apoptotic pathways in order to inhibit chronic myelogenous leukemia tumorigenesis [55].

The protective effects of SIRT1 against muscle atrophy have also been shown through its inhibition of the forehead box O (FOXO) family of proteins. FOXO proteins are a family of transcription factors known to promote apoptosis through the transcription of FasL, the ligand for the Fas-dependent cell-death pathway, and through activation of the pro-apoptotic Bim (Bcl2-like protein 11), a member of the Bcl-2 (B-cell lymphoma 2) family of proteins [56]. The Bcl-2 family of proteins inhibit apoptosis by negative regulation of pro-apoptotic Bax and Bak proteins [57]. Subsequent to the loss of Bcl-2 inhibition, Bax or Bak will generate an apoptotic pore in the mitochondrial outer membrane [58]. This apoptotic pore facilitates the leak of inner mitochondrial substances; one such substance is cytochrome-c, a heme protein important to cytochrome c oxidase (complex IV of the mitochondrial electron transport chain). Immediately after release, cytochrome-c will bind to the apoptotic protease activating factor 1 (Apaf1) protein, initiating the formation of the apoptosome and initiating cellular destruction through the proteolytic caspase cascade [59]. Therefore, inhibition of FOXO proteins by SIRT1 promotes cellular survival by inhibiting apoptosis. Co-immunoprecipitation of FOXO3 and SIRT1 at endogenous levels in 293T HEK (human embryonic kidney) cells has indicated a direct binding of the two proteins, while the isolation and purification of acetylated FOXO3 was shown to be deacetylated by recombinant SIRT1 *in vitro*, and only in the presence of NAD+ [60].

Of particular interest to the context of sarcopenic muscle wasting, SIRT1 levels in type II muscle fibers have been reported to drop significantly after 48 hours of fasting, coinciding with the increased abundance of the atrophy-associated ubiquitin ligases, atrogin1 and Murf1, and increased muscle atrophy [61]. Electroporation of these fasted muscles to increase SIRT1 expression rescues and protects these muscle fibers from atrophy, with the CSA of electroporated fibers out-sizing non-treated fibers by over 48%.

1.6 SIRT1 Protein Association with Type I Fibers

The cumulative protective effects SIRT1 exhibits in preventing muscle wasting from atrophy and apoptosis, especially SIRT1's attenuation of the significant atrophy in type I fibers undergoing long-term caloric deficit, suggest the possibility of a role for SIRT1 in preserving muscles fiber with aging, particularly type I fibers. The rationale underlying this possible role come from the strong association of SIRT1 with mitochondrial metabolism (as high mitochondrial abundance is characteristic of type I fibers). As mentioned previously, NAD+ (nicotinamide adenine dinucleotide) is required for the functional activity of the SIRT1 protein [62,63]. NAD can exist in either the oxidized (NAD+) or reduced (NADH) form, with the reduced NADH acting as an electron carrier to NADH dehydrogenase, complex I of the mitochondrial electron transport chain in oxidative metabolism [64]. During low-energy cellular states, such as after prolonged muscle contraction or during caloric restriction, the ratio of NAD+/NADH increases, promoting SIRT1 activity [65,66].

Beyond its dependence on a key substrate of mitochondrial respiration for activation, SIRT1 is also a known regulator of the peroxisome proliferator-activated receptor γ coactivator-1α, PGC-1α [67]. Co-immunoprecipitation assays of liver extracts were used to identify direct protein-protein interaction between SIRT1 and PGC-1α, while an in vitro deacetylase assay provided evidence that SIRT1 deacetylated PGC-1α upon the addition of NAD+ as a substrate. Previous to this study, PGC-1α induction had been reported as inducing gluconeogenesis [68]

and fatty acid oxidation [69,70] in the liver, a metabolic shift that closely resembled the hepatic response to short-term fasting [71]. Indeed, caloric restriction activates SIRT1 activity through alterations in the NAD+/NADH ratio [72]; furthermore, overexpression of the SIRT1 protein by a transgenic knock-in of SIRT1 through the cre-loxP system, driven by the β-actin promoter, was reported to produce a calorically restricted phenotype in the mouse model [73]. This mouse model was shown to have significantly less bodyweight, fewer circulating free fatty acids and cholesterol, and improved glucose homeostasis, despite taking in more calories than control mice standardized to body weight—hallmarks of increased adipose/mitochondrial metabolism. Recent evidence has also indicated that loss of SIRT1 abundance and activity in muscle satellite cells is a key step during the metabolic shift of MSCs from oxidative to glycolytic metabolism, a process necessary for MSC differentiation [74].

In addition to promoting an oxidative-like metabolic profile, SIRT1 abundance may be related to fiber type distribution in mouse models. Gain-of-function models of SIRT1 activity, with transgenic overexpression induced through the muscle creatine-kinase (MCK) promoter, have been reported to induce fiber type switching in mouse models of Duchenne's muscular dystrophy [75]. However, SIRT1-knockout mice in the same study, also driven by the MCK promoter, did not show a decrease in type I fibers. In a Sprague Dawley rat models of aging, groups of 3 month old and 12 month old rats that underwent swimming training for 40 min/day (5 days/week, 4-12 weeks) showed increased abundance of SIRT1 in the predominantly-type I soleus muscles in exercised rats relative to age-matched sedentary controls, though no difference in SIRT1 abundance was detected between exercised and sedentary groups in isolated gastrocnemius (predominantly type II) muscles [76]. Similar results have also been reported for male Wistar rats subject to endurance exercise interventions of low (20 m/min, 90 min/day) and high (30 m/min, 60 min/day) intensity [77]. Both low and high intensity exercise increased SIRT1 abundance in soleus muscle of trained rats relative to untrained controls, but only high intensity increased SIRT1

expression in the predominantly type II plantaris muscle relative to untrained control rats. Additionally, SIRT1 abundance was shown to be significantly greater in the soleus muscle of these rats when compared to the abundance in plantaris muscles for basal and trained muscles.

Despite the oxidative/caloric restriction phenotype seemingly promoted by SIRT1, some models of SIRT1 overexpression in rodents have been reported to show no benefits to glucose or fatty acid metabolism. MCK-driven, SIRT1 muscle overexpressor mice fed either control or high-fat diets did not report increases in glucose clearance and fatty acid metabolism, metabolic profiles associated with the caloric restriction phenotype [78]. Similar effects were reported in a male Wistar rat model of SIRT1 overexpression, with SIRT1 abundance increased *in vivo* through muscle electroporation of an active or inactive copy of SIRT1 into the tibialis cranialis muscle [79]. Overexpression of SIRT1 over a one-week span did not improve glucose tolerance, nor did it protect against insulin resistance—metabolically protective effects observed in other caloric restriction models. One explanation is that the increased abundance of SIRT1 does not always equate to an increase in the enzymatic activity of SIRT1. However, several activators of SIRT1 have been shown to more consistently induce protective effects against aging.

1.7 SIRT1 Activators Attenuate Effects of Aging

1.71 Caloric Restriction Improves Lifespan

Research on caloric restriction (CR) has been ongoing since the initial report in 1935 that reducing caloric intake could extend the lifespan of rats relative to controls with ad libitum access to food [80]. However, modern research investigating SIRT1's role in linking longevity to CR began with the discovery that overexpression of the yeast protein Sir2 (silent information regulator 2), could increase the lifespan of yeast by up to 50% [81]. Two follow-up studies in Saccharomyces cerevisiae (S. cerevisiae) and in the Caenorhabditis elegans (C. elegans) further established two important facts: (1) caloric restriction activated Sir2 activity in yeast by reducing the availability of

NADH, which acts as a competitive inhibitor to NAD+, to induce Sir2's activity [72], and (2) a family of proteins with high homology to Sir2 were active in C. elegans, and that overexpression of the protein with the highest homology to Sir2, Sir2.1 (31% identity to yeast Sir2), could also extend lifespan of C. elegans by approximately 50% [66]. These proteins with high homology to Sir2 eventually became known as the Sir2-like, and later sirtuin (sir- "two"-in), family of proteins. These investigations laid the groundwork for future studies by establishing solid evidence that CR could activate SIRT1, and that organisms with greater complexity could also benefit from improved longevity via the overexpression of their respective Sir2 homologues.

More complex model organisms have also been shown to benefit from longer lifespan through caloric restriction. In most cases, these models limit calories by 25%-50%, though studies in mammals tend to restrict calories by around 30%. A Drosophila melanogaster (D. melanogaster) model of high (15% yeast, 15% sucrose, 2% agar) and low (5% yeast, 5% sucrose, 5% agar) calorie sucrose cornmeal food (added in equal volume amounts) in transgenic flies expressing normal, elevated, or knocked out levels of Drosophila Sir2 (dSir2) [82]. CR coupled with dSir2 overexpression was shown to increase lifespan by 29% in females and approximately 18% in male flies, though no extension of lifespan was detected for flies endogenous or reduced dSir2 abundance, nor in flies that had already lived long lifespans. Importantly, although these data indicated that dSir2 was important for mediating the extension of lifespan through caloric restriction, they did also reveal that CR alone does not guarantee an extension in lifespan for CR models. Despite these findings, CR alone has been able to induce lifespan extension in rodents by up to 50% and has improved the lifespan of primate models [83].

One recent study investigating CR on grey mouse lemur populations reported that a 30% CR intervention administered at 3 years of age could extend the lifespan of these small primates by close to 50%, from a median of 6.4 years to a median of 9.4 years old, with 7 of the 19 CR-intervention animals surviving up to 13 years (over double the median lifespan of the control

group) [84]. However, the increase in lifespan was also accompanied by the selective atrophy of grey matter in the brain temporal regions, hippocampus, and retrosplenial cortex. Although no cognitive impairment was detected in the CR lemur group, the changes in brain do illustrate that the effects of longevity in longer-lived organisms may not be wholly beneficial, and that the increasing complexity of the organism models may present unexpected outcomes. An important caveat of the grey mouse lemur study is that the lives of the oldest lemurs were extended by 7 years past the medium control group, illustrating an important limitation to these studies: the commitment of extended periods of time to generate data. The impact of CR on primate longevity is still contested due to the resulting lack of a data owed to the longer life-span of these models. Rhesus monkeys, for example, can live for several decades, necessitating long commitments of time and severely slowing the generation of data. As such, current data on rhesus monkeys show conflicting results [85], with research from the University of Maryland and University of Wisconsin reporting a positive association of CR to survival, but the National Institute of Aging reporting no significant association.

1.72 NAD Supplementation Improves Mitochondrial Health

Although CR activates SIRT1 by modulation of the cellular [NAD+]/[NADH] ratio, direct manipulation of [NAD+] can also be used to activate SIRT1. Recent studies have investigated the potential benefits of supplementing the NAD+ precursor, nicotinamide (NAM), to in vivo [86–88] and in vitro [89,90] models. Supplementation of NAM to primary human fibroblasts was shown to reduce mitochondrial mass and improve mitochondrial membrane potential $\Delta\Psi m$ [87]. These effects are accompanied by significant increases in the abundance of the autophagy-related proteins LC3 and ATG-12. Interestingly, the reduction of mitochondrial volume was also observed upon activation of SIRT1 through other activators (resveratrol, SRT1720), but these activators did not affect $\Delta\Psi m$, suggesting the possibility of an alternate mechanism of regulation by NAD+ [88]. NAM supplementation was also reported to reduce the ROS content of primary human fibroblasts,

as detected by detection of the oxidant-sensing probe, DCFH-DA [86]. These data suggest that NAD treatment, through the activation of SIRT1, attenuate mitochondrial markers of aging by inducing mitophagy. Interestingly, NAM-treated fibroblasts were able to divide over 1.5-fold as many times as untreated fibroblasts, indicating an improvement to longevity. However, in C57BL/6 mouse models of high and low-fat dietary intake, oral NAM supplementation improved locomotion functions, glucose tolerance, and reduced liver steatosis—indicating improved metabolic health—but did not increase the lifespan of the mice [90].

A similar profile of metabolic protection was observed in vivo in C57BL/6-backgroun mice chronically fed an alternate NAD+ precursor, nicotinamide riboside (NR) [91]. Interestingly, nicotinamide and nicotinamide riboside are both considered vitamin B3 variants, and both increase the concentration of intracellular NAD+. However, a key difference between these compounds is that NAM supplementation will begin to inhibit SIRT1 activity beyond a certain concentration threshold [86]. Secondly, NR has been shown to activate SIRT3 in addition to SIRT1, suggesting that NAD+ supplementation from NR promotes downstream effects in both the nuclear and mitochondrial compartments [91]. Evidence supporting beneficial effects in the mitochondrial compartment by NR comes from multiple studies of mitochondrial myopathy in Deletor [92] and Sco2 knockout [93] mouse models. These mitochondrial myopathies represent two separate mutations that lead to electron transport chain dysfunction, and share similar characteristics of mitochondrial myopathy (diminished abundance of mitochondrial COX and abnormal mitochondrial structure). In both cases, NR was reported to increase mitochondrial biogenesis in conjunction with an upregulation of the mitochondrial unfolded protein response (UPR). Stimulation of mitochondrial biogenesis was able to provide at least partial rescue for the downregulation of mitochondrial respiration, while mitochondrial structural rescue was attributed to increased protein integrity resulting from greater activation of the mitochondrial UPR. Interestingly, mitochondrial protection is conferred by both NR and NAM with similar downstream

effects, but different activating pathways. For example, abnormal mitochondrial structure is attenuated with treatment by both NAM and NR, but appears to be achieved more through mitophagy in NAM-supplemented models and through the mitochondrial UPR in NR-treated models. These differences illustrate the potential of SIRT1 to promote longevity through multiple pathways depending on the type of activation used to stimulate its activity.

1.7.3 Resveratrol Protects Against Oxidative Stress

After the initial discovery of SIRT1's pro-longevity effects, research began looking into in-depth screenings of small-molecule libraries for potential activators of SIRT1. Of 25 small molecules found to enhance SIRT1 from a pool of over 20,000 molecules, a phenol naturally produced in plants, was found to be the most potent [94]. Supplementation of resveratrol was subsequently shown to increase the lifespan of yeast, mimicking the activation of SIRT1 through caloric restriction [95,96]. Based on reports that resveratrol conferred antioxidant properties that attenuated oxidative stress [97], our lab previously investigated the effects of dietary resveratrol on muscle function and age-related oxidative stress in aged and/or disuse models of rodent muscles. Both short (10 days) [98] and long-term (10 months) [99] supplementation of resveratrol in young and aged mouse models conferred protection against oxidative stress during isometric muscle contractions and in control mice, characterized by decreased muscular H2O2 levels and increased expression of antioxidant and ROS scavenger proteins, including glutathione peroxidase, catalase, and manganese-dependent superoxide dismutase. Despite protecting against oxidative damage, resveratrol did not protect against age-related [99] or disuse atrophy [96], though supplementation of resveratrol promoted the recovery of type II fiber CSA during recovery from disuse atrophy. Interestingly, in elderly human subjects (aged 65-80 years old), resveratrol supplementation coupled with 12 weeks of exercised increased maximum torque production during knee extension exercise and did improve mean muscle fiber CSA [100]. Of particular interest in this study was the increase in type II muscle fiber CSA and satellite cell

population. In conjunction with the improvement of type II fiber CSA after muscle disuse, these data suggest resveratrol activation of SIRT1 may improve muscle regenerative ability after damage by improving the pool of available satellite cells.

1.8 SIRT1 as a Regulator of Muscle Satellite Cells

Although the role of SIRT1 in satellite cells is currently still emerging, increasing evidence suggests SIRT1 activity can influence satellite cell functionality. Short-term (12 weeks) CR has been shown to increase the quantity and proliferation in vivo of satellite cells from young (2 month old) and aged (18 months) C57BL/6-background mice [101]. Interestingly, this study also showed that donor satellite cells engrafted to mice positive for Duchene's muscular dystrophy (mdx) showed a 4-fold greater engraftment of myofibers than satellite cells donated to recipient mdx mice fed a control diet. These findings reflect parabiosis studies, wherein the regenerative of MSCs from aged mouse donors is increased when transplanted into much younger hosts [102]. Taken together, these experiments suggest a high sensitivity for satellite cells to the state of their systemic environments. However, they also suggest that increased MSC proliferative capacity from CR could be promoted through external signaling, rather than through endogenous SIRT1 activity.

Recent literature has reported that aged mice treated with NR show increased regenerative capacity after cardiotoxin (CTX) induced injury, coupled with increased abundance of Pax7-stained MSCs, and were more effective than MSCs from untreated mice in rescuing muscle repair in mdx mice [103]. This regenerative potential was lost with the knockout of SIRT1 in satellite cells induced through the loxP system by a Pax7-cre promotor. These data appear to agree with a separate study that suggest SIRT1 expression acts to maintain quiescence through an oxidative, metabolic switch [74]. This study reports that SIRT1 deacetylase activity and NAD+ levels are elevated in quiescent satellite cells, which present an oxidative metabolic phenotype. Loss of SIRT1 expression in the muscle satellite cells via the same Pax7-cre driven knockout

model results in premature differentiation of satellite cells into muscle dysfunctional myofibers. Down regulation of SIRT1 is shown to occur before satellite cells shift to a glycolytic phenotype, indicating that oxidative metabolism and expression of SIRT1 are majorly important in maintaining quiescent satellite cell populations.

Finally, studies looking at autophagy in stem cells have shown consensus that reduced autophagy and mitophagy contribute to aging by permitting the accumulation of damaged proteins and organelles, leading to senescence [104]. MSCs from murine satellite cell knockout models of Atg7 (autophagy-related protein 7) showed reduced regenerative capability and prematurely expressed cyclin-dependent kinase inhibitors p16, p21, and p15, indicating early cell cycle arrest and stem cell senescence. Mitochondria in MSCs with dysfunctional mitophagy also showed altered mitochondria, which displayed reduced ΔΨm, and were accompanied by a marked increase in cellular ROS. In support of this data, another study has shown that the knockout out of SIRT1 (SIRT1-/-) activity in embryonic stem cells (ESC) reduces autophagic cell death under oxidative stress [105]. When stressed by H2O2-mediated ROS damage, SIRT1-/- ESCs showed reduced abundance of LC3 and decreased ΔΨm, similar hallmarks to the loss of autophagy in the MSC knockout model of Atg-7. The loss of autophagy-associated clearance in both muscle and embryonic stem cells with the loss of SIRT1 suggests a vital role for SIRT1 in MSCs.

1.9 Conclusion

Several factors support the idea that SIRT1's longevity-promoting activity may also be induced to spare skeletal muscle from age-associated wasting. SIRT1 has been shown to directly bind and deacetylate both the pro-apoptotic proteins FOXO3 and p53. Interestingly, SIRT1 seems to act like a stopgap in muscle atrophy; SIRT1 is upregulated during mild caloric restriction and will inhibit FOXO3 to preserve muscle fibers, but eventually abundance of SIRT1 will diminish during prolonged starvation. Some evidence suggests SIRT1 plays a similar role with its inhibition of p53. Although p53 is pro-apoptotic, it has also recently been shown to upregulate DNA repair

machinery in cells. The pro-DNA repair effects of p53 are thought to be mediated through its ability to arrest the cell cycle—thereby granting DNA repair machinery the chance to repair DNA damage before promoting apoptosis. One explanation for SIRT1's pro-longevity effects could be that it only partially inhibits p53 expression—stopping it just enough so that p53 can express it's cell-cycle arrest properties without inducing apoptosis.

Interestingly, some studies have shown that SIRT1 leans more toward muscle repair than increasing satellite cell activity; specifically, the experiments involving resveratrol. Resveratrol has been shown to reduce oxidative damage and improve type II fibers' CSA after reloading injury after muscle disuse and in humans after exercise training. In these studies, SIRT1 activation did not appear to protect muscle fibers from sarcopenic wasting. Instead, beneficial effects were seen after injury—somewhat supporting the idea that SIRT1 could be interacting with p53 to inhibit apoptosis and promote DNA repair. Notably, the studies that seemed to best promote satellite cell activity were those experiments that supplemented satellite cells with nicotinamide riboside, a strong activator of SIRT1 and a cellular marker of SIRT1. This is especially interesting given the data that Ryall et al. show with their satellite cell knockout model of SIRT1; that SIRT1 acts a metabolic regulator to maintain satellite cell quiescence and is downregulated prior to MSC activation, wherein the MSCs shift toward a glycolytic profile. Combined with the fact that satellites cells are highly sensitive to their environment, it is possible that NR supplementation is promoting a calorie-restriction like response across all tissues *in vivo*, thereby promoting SIRT1 and the oxidative metabolic profile both endogenously and exogenously in satellite cells and reinforcing the proliferative phenotype by keeping SIRT1 active in the MSCs.

Regardless, the literature has made a strong case for SIRT1 to be a potential preserver of muscle with age: (1) it protects muscle fibers by inhibiting pro-apoptotic proteins, (2) it promotes mitochondrial biogenesis and mitophagy, processes that help attenuate mitochondrial aging, and (3) it has been shown to promote muscle repair after injury.

References

1. FastStats [Internet]. 21 Mar 2019 [cited 1 Apr 2019]. Available: https://www.cdc.gov/nchs/fastats/life-expectancy.htm

2. Cook J. Nathan Shock, Pioneer on Aging. The New York Times. 15 Nov 1989. Available: https://www.nytimes.com/1989/11/15/obituaries/nathan-shock-pioneer-on-aging.html. Accessed 1 Apr 2019.

3. Rosenberg IH. Sarcopenia: Origins and Clinical Relevance. J Nutr. 1997;127: 990S-991S.

4. Baumgartner RN, Koehler KM, Gallagher D, Romero L, Heymsfield SB, Ross RR, et al. Epidemiology of Sarcopenia among the Elderly in New Mexico. Am J Epidemiol. 1998;147: 755–763. doi:10.1093/oxfordjournals.aje.a009520

5. Baumgartner RN, Wayne SJ, Waters DL, Janssen I, Gallagher D, Morley JE. Sarcopenic Obesity Predicts Instrumental Activities of Daily Living Disability in the Elderly. Obesity Research. 2004;12: 1995–2004. doi:10.1038/oby.2004.250

6. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, Landi F, et al. Sarcopenia: European consensus on definition and diagnosis. Age Ageing. 2010;39: 412–423. doi:10.1093/ageing/afq034

7. Mitchell WK, Williams J, Atherton P, Larvin M, Lund J, Narici M. Sarcopenia, Dynapenia, and the Impact of Advancing Age on Human Skeletal Muscle Size and Strength; a Quantitative Review. Front Physiol. 2012;3. doi:10.3389/fphys.2012.00260

8. Delmonico MJ, Harris TB, Lee J-S, Visser M, Nevitt M, Kritchevsky SB, et al. Alternative Definitions of Sarcopenia, Lower Extremity Performance, and Functional Impairment with Aging in Older Men and Women. Journal of the American Geriatrics Society. 2007;55: 769–774. doi:10.1111/j.1532-5415.2007.01140.x

9. Delmonico MJ, Harris TB, Visser M, Park SW, Conroy MB, Velasquez-Mieyer P, et al. Longitudinal study of muscle strength, quality, and adipose tissue infiltration. Am J Clin Nutr. 2009;90: 1579–1585. doi:10.3945/ajcn.2009.28047

10. von Haehling S, Morley JE, Anker SD. An overview of sarcopenia: facts and numbers on prevalence and clinical impact. J Cachexia Sarcopenia Muscle. 2010;1: 129–133. doi:10.1007/s13539-010-0014-2

11. Beaudart C, Zaaria M, Pasleau F, Reginster J-Y, Bruyère O. Health Outcomes of Sarcopenia: A Systematic Review and Meta-Analysis. PLoS One. 2017;12. doi:10.1371/journal.pone.0169548

12. Hartholt KA, Beeck EF van, Polinder S, Velde N van der, Lieshout EMM van, Panneman MJM, et al. Societal Consequences of Falls in the Older Population: Injuries, Healthcare Costs, and Long-Term Reduced Quality of Life. The Journal of Trauma: Injury, Infection, and Critical Care. 2011;71: 748–753. doi:10.1097/TA.0b013e3181f6f5e5

13. Parry SM, Puthucheary ZA. The impact of extended bed rest on the musculoskeletal system in the critical care environment. Extrem Physiol Med. 2015;4. doi:10.1186/s13728-015-0036-7

14. Oliveira JRS, Mohamed JS, Myers MJ, Brooks MJ, Alway SE. Effects of hindlimb suspension and reloading on gastrocnemius and soleus muscle mass and function in geriatric mice. Experimental Gerontology. 2019;115: 19–31. doi:10.1016/j.exger.2018.11.011

15. Dirks ML, Wall BT, Valk B van de, Holloway TM, Holloway GP, Chabowski A, et al. One Week of Bed Rest Leads to Substantial Muscle Atrophy and Induces Whole-Body Insulin Resistance in the Absence of Skeletal Muscle Lipid Accumulation. Diabetes. 2016;65: 2862–2875. doi:10.2337/db15-1661

16. Hida T, Ishiguro N, Shimokata H, Sakai Y, Matsui Y, Takemura M, et al. High prevalence of sarcopenia and reduced leg muscle mass in Japanese patients immediately after a hip fracture. Geriatrics & Gerontology International. 2013;13: 413–420. doi:10.1111/j.1447-0594.2012.00918.x

17. Sipers WMWH, de Blois W, Schols JMGA, van Loon LJC, Verdijk LB. Sarcopenia is Related to Mortality in the Acutely Hospitalized Geriatric Patient. J Nutr Health Aging. 2019;23: 128–137. doi:10.1007/s12603-018-1134-1

18. Kim YK, Yi SR, Lee YH, Kwon J, Jang SI, Park SH. Effect of Sarcopenia on Postoperative Mortality in Osteoporotic Hip Fracture Patients. J Bone Metab. 2018;25: 227–233. doi:10.11005/jbm.2018.25.4.227

19. Janssen I, Shepard DS, Katzmarzyk PT, Roubenoff R. The healthcare costs of sarcopenia in the United States. J Am Geriatr Soc. 2004;52: 80–85.

20. Schiaffino S, Reggiani C. Fiber Types in Mammalian Skeletal Muscles. Physiological Reviews. 2011;91: 1447–1531. doi:10.1152/physrev.00031.2010

21. Verdijk LB, Snijders T, Beelen M, Savelberg HHCM, Meijer K, Kuipers H, et al. Characteristics of Muscle Fiber Type Are Predictive of Skeletal Muscle Mass and Strength in Elderly Men. Journal of the American Geriatrics Society. 2010;58: 2069–2075. doi:10.1111/j.1532-5415.2010.03150.x

22. Nilwik R, Snijders T, Leenders M, Groen BBL, van Kranenburg J, Verdijk LB, et al. The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. Experimental Gerontology. 2013;48: 492–498. doi:10.1016/j.exger.2013.02.012

23. Kramer IF, Snijders T, Smeets JSJ, Leenders M, van Kranenburg J, den Hoed M, et al. Extensive Type II Muscle Fiber Atrophy in Elderly Female Hip Fracture Patients. J Gerontol A Biol Sci Med Sci. 2017;72: 1369–1375. doi:10.1093/gerona/glw253

24. Yin H, Price F, Rudnicki MA. Satellite Cells and the Muscle Stem Cell Niche. Physiological Reviews. 2013;93: 23–67. doi:10.1152/physrev.00043.2011

25. Grounds MD, Garrett KL, Lai MC, Wright WE, Beilharz MW. Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. Cell & Tissue Research. 1992;267: 99–104. doi:10.1007/BF00318695

26. Fu X, Wang H, Hu P. Stem cell activation in skeletal muscle regeneration. Cell Mol Life Sci. 2015;72: 1663–1677. doi:10.1007/s00018-014-1819-5

27. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 Is Required for the Specification of Myogenic Satellite Cells. Cell. 2000;102: 777–786. doi:10.1016/S0092-8674(00)00066-0

28. von Maltzahn J, Jones AE, Parks RJ, Rudnicki MA. Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc Natl Acad Sci U S A. 2013;110: 16474–16479. doi:10.1073/pnas.1307680110

29. Sambasivan R, Yao R, Kissenpfennig A, Wittenberghe LV, Paldi A, Gayraud-Morel B, et al. Pax7-expressing satellite cells are indispensable for adult skeletal muscle regeneration. Development. 2011;138: 3647–3656. doi:10.1242/dev.067587

30. Kuang S, Kuroda K, Le Grand F, Rudnicki MA. Asymmetric Self-Renewal and Commitment of Satellite Stem Cells in Muscle. Cell. 2007;129: 999–1010. doi:10.1016/j.cell.2007.03.044

31. Wen Y, Bi P, Liu W, Asakura A, Keller C, Kuang S. Constitutive Notch Activation Upregulates Pax7 and Promotes the Self-Renewal of Skeletal Muscle Satellite Cells. Molecular and Cellular Biology. 2012;32: 2300–2311. doi:10.1128/MCB.06753-11

32. Kadi F, Charifi N, Denis C, Lexell J. Satellite cells and myonuclei in young and elderly women and men. Muscle Nerve. 2004;29: 120–127. doi:10.1002/mus.10510

33. Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z. Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Dev Biol. 2006;294: 50–66. doi:10.1016/j.ydbio.2006.02.022

34. Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HHCM, Loon LJC van. Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. American Journal of Physiology - Endocrinology and Metabolism. 2007;292: E151–E157. doi:10.1152/ajpendo.00278.2006

35. Verdijk LB, Snijders T, Drost M, Delhaas T, Kadi F, van Loon LJC. Satellite cells in human skeletal muscle; from birth to old age. Age (Dordr). 2014;36: 545–557. doi:10.1007/s11357-013-9583-2

36. Coen PM, Musci RV, Hinkley JM, Miller BF. Mitochondria as a Target for Mitigating Sarcopenia. Front Physiol. 2019;9. doi:10.3389/fphys.2018.01883

37. Alway SE, Mohamed JS, Myers MJ. Mitochondria Initiate and Regulate Sarcopenia. Exerc Sport Sci Rev. 2017;45: 58–69. doi:10.1249/JES.0000000000000101

38. Joseph A-M, Adhihetty PJ, Wawrzyniak NR, Wohlgemuth SE, Picca A, Kujoth GC, et al. Dysregulation of Mitochondrial Quality Control Processes Contribute to Sarcopenia in a Mouse Model of Premature Aging. PLoS One. 2013;8. doi:10.1371/journal.pone.0069327

39. Iqbal S, Ostojic O, Singh K, Joseph A-M, Hood DA. Expression of mitochondrial fission and fusion regulatory proteins in skeletal muscle during chronic use and disuse. Muscle & Nerve. 2013;48: 963–970. doi:10.1002/mus.23838

40. D'Amico D, Mottis A, Potenza F, Sorrentino V, Li H, Romani M, et al. The RNA-Binding Protein PUM2 Impairs Mitochondrial Dynamics and Mitophagy During Aging. Molecular Cell. 2019;73: 775-787.e10. doi:10.1016/j.molcel.2018.11.034

41. Frank M, Duvezin-Caubet S, Koob S, Occhipinti A, Jagasia R, Petcherski A, et al. Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2012;1823: 2297–2310. doi:10.1016/j.bbamcr.2012.08.007

42. Schieber M, Chandel NS. ROS Function in Redox Signaling and Oxidative Stress. Curr Biol. 2014;24: R453–R462. doi:10.1016/j.cub.2014.03.034

43. Egea J, Fabregat I, Frapart YM, Ghezzi P, Görlach A, Kietzmann T, et al. European contribution to the study of ROS: A summary of the findings and prospects for the future from the COST action BM1203 (EU-ROS). Redox Biol. 2017;13: 94–162. doi:10.1016/j.redox.2017.05.007

44. Bua EA, McKiernan SH, Wanagat J, McKenzie D, Aiken JM. Mitochondrial abnormalities are more frequent in muscles undergoing sarcopenia. Journal of Applied Physiology. 2002;92: 2617–2624. doi:10.1152/japplphysiol.01102.2001

45. Ji LL, Leeuwenburgh C, Leichtweis S, Gore M, Fiebig R, Hollander J, et al. Oxidative Stress and Aging: Role of Exercise and Its Influences on Antioxidant Systems. Annals of the New York Academy of Sciences. 1998;854: 102–117. doi:10.1111/j.1749-6632.1998.tb09896.x

46. Mikhed Y, Daiber A, Steven S. Mitochondrial Oxidative Stress, Mitochondrial DNA Damage and Their Role in Age-Related Vascular Dysfunction. International Journal of Molecular Sciences. 2015;16: 15918–15953. doi:10.3390/ijms160715918

47. Ryan MJ, Jackson JR, Hao Y, Leonard SS, Alway SE. Inhibition of xanthine oxidase reduces oxidative stress and improves skeletal muscle function in response to electrically stimulated isometric contractions in aged mice. Free Radic Biol Med. 2011;51: 38–52. doi:10.1016/j.freeradbiomed.2011.04.002

48. Ryan MJ, Dudash HJ, Docherty M, Geronilla KB, Baker BA, Haff GG, et al. Vitamin E and C supplementation reduces oxidative stress, improves antioxidant enzymes and positive muscle work in chronically loaded muscles of aged rats. Exp Gerontol. 2010;45: 882–895. doi:10.1016/j.exger.2010.08.002

49. Alway SE, Bennett BT, Wilson JC, Sperringer J, Mohamed JS, Edens NK, et al. Green tea extract attenuates muscle loss and improves muscle function during disuse, but fails to improve muscle recovery following unloading in aged rats. J Appl Physiol (1985). 2015;118: 319–330. doi:10.1152/japplphysiol.00674.2014

50. Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol. 2002;192: 1–15. doi:10.1002/jcp.10119

51. Jiang L, Hickman JH, Wang S-J, Gu W. Dynamic roles of p53-mediated metabolic activities in ROS-induced stress responses. Cell Cycle. 2015;14: 2881–2885. doi:10.1080/15384101.2015.1068479

52. Soares JP, Cortinhas A, Bento T, Leitão JC, Collins AR, Gaivã I, et al. Aging and DNA damage in humans: a meta-analysis study. Aging (Albany NY). 2014;6: 432–439.

53. Fox DK, Ebert SM, Bongers KS, Dyle MC, Bullard SA, Dierdorff JM, et al. p53 and ATF4 mediate distinct and additive pathways to skeletal muscle atrophy during limb immobilization. Am J Physiol Endocrinol Metab. 2014;307: E245–E261. doi:10.1152/ajpendo.00010.2014

54. Vaziri H, Dessain SK, Eaton EN, Imai S-I, Frye RA, Pandita TK, et al. hSIR2SIRT1 Functions as an NAD-Dependent p53 Deacetylase. Cell. 2001;107: 149–159. doi:10.1016/S0092-8674(01)00527-X

55. Li L, Wang L, Li L, Wang Z, Ho Y, McDonald T, et al. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell. 2012;21: 266–281. doi:10.1016/j.ccr.2011.12.020

56. Carter ME, Brunet A. FOXO transcription factors. Current Biology. 2007;17: R113–R114. doi:10.1016/j.cub.2007.01.008

57. Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? Genes to Cells. 1998;3: 697–707. doi:10.1046/j.1365-2443.1998.00223.x

58. Westphal D, Dewson G, Czabotar PE, Kluck RM. Molecular biology of Bax and Bak activation and action. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2011;1813: 521–531. doi:10.1016/j.bbamcr.2010.12.019

59. Cytochrome C - an overview | ScienceDirect Topics [Internet]. [cited 3 Apr 2019]. Available: https://www-sciencedirect-com.www.libproxy.wvu.edu/topics/neuroscience/cytochrome-c

60. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. Stress-Dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase. Science. 2004;303: 2011–2015. doi:10.1126/science.1094637

61. Lee D, Goldberg AL. SIRT1 Protein, by Blocking the Activities of Transcription Factors FoxO1 and FoxO3, Inhibits Muscle Atrophy and Promotes Muscle Growth. J Biol Chem. 2013;288: 30515–30526. doi:10.1074/jbc.M113.489716

62. Landry J, Sutton A, Tafrov ST, Heller RC, Stebbins J, Pillus L, et al. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc Natl Acad Sci U S A. 2000;97: 5807–5811.

63. Landry J, Slama JT, Sternglanz R. Role of NAD+ in the Deacetylase Activity of the SIR2-like Proteins. Biochemical and Biophysical Research Communications. 2000;278: 685–690. doi:10.1006/bbrc.2000.3854

64. Cantó C, Auwerx J. NAD+ as a signaling molecule modulating metabolism. Cold Spring Harb Symp Quant Biol. 2011;76: 291–298. doi:10.1101/sqb.2012.76.010439

65. Cantó C, Auwerx J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Curr Opin Lipidol. 2009;20: 98–105. doi:10.1097/MOL.0b013e328328d0a4

66. Tissenbaum HA, Guarente L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature. 2001;410: 227–230. doi:10.1038/35065638

67. Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. Nature. 2005;434: 113–118. doi:10.1038/nature03354

68. Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, et al. Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature. 2001;413: 131–138. doi:10.1038/35093050

69. Rhee J, Inoue Y, Yoon JC, Puigserver P, Fan M, Gonzalez FJ, et al. Regulation of hepatic fasting response by PPARγ coactivator-1α (PGC-1): Requirement for hepatocyte nuclear factor 4α in gluconeogenesis. Proc Natl Acad Sci U S A. 2003;100: 4012–4017. doi:10.1073/pnas.0730870100

70. Gulick T, Cresci S, Caira T, Moore DD, Kelly DP. The peroxisome proliferator-activated receptor regulates mitochondrial fatty acid oxidative enzyme gene expression. Proc Natl Acad Sci U S A. 1994;91: 11012–11016.

71. Dhahbi JM, Mote PL, Wingo J, Tillman JB, Walford RL, Spindler SR. Calories and aging alter gene expression for gluconeogenic, glycolytic, and nitrogen-metabolizing enzymes. American Journal of Physiology-Endocrinology and Metabolism. 1999;277: E352–E360. doi:10.1152/ajpendo.1999.277.2.E352

72. Lin S-J, Ford E, Haigis M, Liszt G, Guarente L. Calorie restriction extends yeast life span by lowering the level of NADH. Genes Dev. 2004;18: 12–16. doi:10.1101/gad.1164804

73. Bordone L, Cohen D, Robinson A, Motta MC, Van Veen E, Czopik A, et al. SIRT1 transgenic mice show phenotypes resembling calorie restriction. Aging Cell. 2007;6: 759–767. doi:10.1111/j.1474-9726.2007.00335.x

74. Ryall JG, Dell'Orso S, Derfoul A, Juan A, Zare H, Feng X, et al. The NAD+-Dependent SIRT1 Deacetylase Translates a Metabolic Switch into Regulatory Epigenetics in Skeletal Muscle Stem Cells. Cell Stem Cell. 2015;16: 171–183. doi:10.1016/j.stem.2014.12.004

75. Chalkiadaki A, Igarashi M, Nasamu AS, Knezevic J, Guarente L. Muscle-Specific SIRT1 Gain-of-Function Increases Slow-Twitch Fibers and Ameliorates Pathophysiology in a Mouse Model of Duchenne Muscular Dystrophy. PLOS Genetics. 2014;10: e1004490. doi:10.1371/journal.pgen.1004490

76. Huang C-C, Wang T, Tung Y-T, Lin W-T. Effect of Exercise Training on Skeletal Muscle SIRT1 and PGC-1α Expression Levels in Rats of Different Age. International Journal of Medical Sciences. 2016;13: 260–270. doi:10.7150/ijms.14586

77. Suwa M, Nakano H, Radak Z, Kumagai S. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor γ coactivator-1α protein expressions in rat skeletal muscle. Metabolism. 2008;57: 986–998. doi:10.1016/j.metabol.2008.02.017

78. White AT, Philp A, Fridolfsson HN, Schilling JM, Murphy AN, Hamilton DL, et al. High-fat diet-induced impairment of skeletal muscle insulin sensitivity is not prevented by SIRT1 overexpression. American

Journal of Physiology-Endocrinology and Metabolism. 2014;307: E764–E772. doi:10.1152/ajpendo.00001.2014

79. Brandon AE, Tid-Ang J, Wright LE, Stuart E, Suryana E, Bentley N, et al. Overexpression of SIRT1 in Rat Skeletal Muscle Does Not Alter Glucose Induced Insulin Resistance. PLOS ONE. 2015;10: e0121959. doi:10.1371/journal.pone.0121959

80. Cantó C, Auwerx J. Caloric restriction, SIRT1 and longevity. Trends in Endocrinology & Metabolism. 2009;20: 325–331. doi:10.1016/j.tem.2009.03.008

81. Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev. 1999;13: 2570–2580.

82. Rogina B, Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proc Natl Acad Sci U S A. 2004;101: 15998–16003. doi:10.1073/pnas.0404184101

83. Koubova J, Guarente L. How does calorie restriction work? Genes Dev. 2003;17: 313–321. doi:10.1101/gad.1052903

84. Pifferi F, Terrien J, Marchal J, Dal-Pan A, Djelti F, Hardy I, et al. Caloric restriction increases lifespan but affects brain integrity in grey mouse lemur primates. Communications Biology. 2018;1: 30. doi:10.1038/s42003-018-0024-8

85. Mattison JA, Colman RJ, Beasley TM, Allison DB, Kemnitz JW, Roth GS, et al. Caloric restriction improves health and survival of rhesus monkeys. Nature Communications. 2017;8: 14063. doi:10.1038/ncomms14063

86. Kang HT, Lee HI, Hwang ES. Nicotinamide extends replicative lifespan of human cells. Aging Cell. 2006;5: 423–436. doi:10.1111/j.1474-9726.2006.00234.x

87. Kang HT, Hwang ES. Nicotinamide enhances mitochondria quality through autophagy activation in human cells. Aging Cell. 2009;8: 426–438. doi:10.1111/j.1474-9726.2009.00487.x

88. Jang S, Kang HT, Hwang ES. Nicotinamide-induced Mitophagy EVENT MEDIATED BY HIGH NAD+/NADH RATIO AND SIRT1 PROTEIN ACTIVATION. J Biol Chem. 2012;287: 19304–19314. doi:10.1074/jbc.M112.363747

89. Katsyuba E, Mottis A, Zietak M, Franco FD, Velpen V van der, Gariani K, et al. De novo NAD + synthesis enhances mitochondrial function and improves health. Nature. 2018;563: 354. doi:10.1038/s41586-018-0645-6

90. Mitchell SJ, Bernier M, Aon MA, Cortassa S, Kim EY, Fang EF, et al. Nicotinamide improves aspects of healthspan but not lifespan in mice. Cell Metab. 2018;27: 667-676.e4. doi:10.1016/j.cmet.2018.02.001

91. Cantó C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, et al. The NAD+ precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet induced obesity. Cell Metab. 2012;15: 838–847. doi:10.1016/j.cmet.2012.04.022

92. Khan NA, Auranen M, Paetau I, Pirinen E, Euro L, Forsström S, et al. Effective treatment of mitochondrial myopathy by nicotinamide riboside, a vitamin B3. EMBO Mol Med. 2014;6: 721–731. doi:10.1002/emmm.201403943

93. Cerutti R, Pirinen E, Lamperti C, Marchet S, Sauve AA, Li W, et al. NAD+-Dependent Activation of Sirt1 Corrects the Phenotype in a Mouse Model of Mitochondrial Disease. Cell Metabolism. 2014;19: 1042–1049. doi:10.1016/j.cmet.2014.04.001

94. Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature. 2006;444: 337–342. doi:10.1038/nature05354

95. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG, et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature. 2003;425: 191. doi:10.1038/nature01960

96. Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, et al. Calorie restriction-like effects of 30 days of Resveratrol (resVida^{TM}) supplementation on energy metabolism and metabolic profile in obese humans. Cell Metab. 2011;14. doi:10.1016/j.cmet.2011.10.002

97. Bisht K, Wagner K-H, Bulmer AC. Curcumin, resveratrol and flavonoids as anti-inflammatory, cyto- and DNA-protective dietary compounds. Toxicology. 2010;278: 88–100. doi:10.1016/j.tox.2009.11.008

98. Ryan MJ, Jackson JR, Hao Y, Williamson CL, Dabkowski ER, Hollander JM, et al. Suppression of Oxidative Stress by Resveratrol After Isometric Contractions in Gastrocnemius Muscles of Aged Mice. J Gerontol A Biol Sci Med Sci. 2010;65A: 815–831. doi:10.1093/gerona/glq080

99. Jackson JR, Ryan MJ, Alway SE. Long-Term Supplementation With Resveratrol Alleviates Oxidative Stress but Does Not Attenuate Sarcopenia in Aged Mice. J Gerontol A Biol Sci Med Sci. 2011;66A: 751–764. doi:10.1093/gerona/glr047

100. Alway SE, McCrory JL, Kearcher K, Vickers A, Frear B, Gilleland DL, et al. Resveratrol Enhances Exercise-Induced Cellular and Functional Adaptations of Skeletal Muscle in Older Men and Women. J Gerontol A Biol Sci Med Sci. 2017;72: 1595–1606. doi:10.1093/gerona/glx089

101. Cerletti M, Jang YC, Finley LWS, Haigis MC, Wagers AJ. Short-term calorie restriction enhances skeletal muscle stem cell function. Cell Stem Cell. 2012;10: 515–519. doi:10.1016/j.stem.2012.04.002

102. Conboy IM, Conboy MJ, Wagers AJ, Girma ER, Weissman IL, Rando TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. Nature. 2005;433: 760–764. doi:10.1038/nature03260

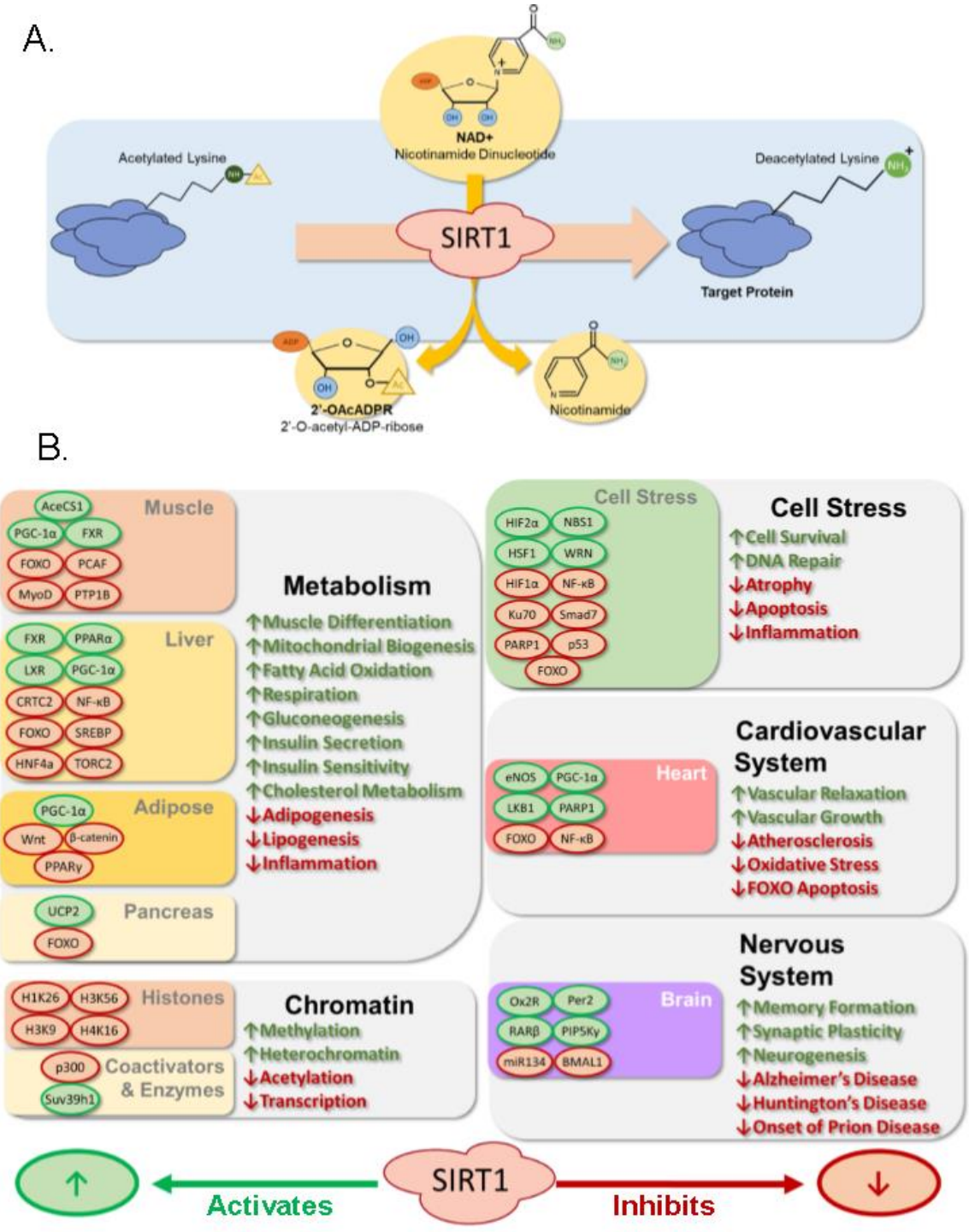
103. Zhang H, Ryu D, Wu Y, Gariani K, Wang X, Luan P, et al. NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice. Science. 2016;352: 1436–1443. doi:10.1126/science.aaf2693

104. García-Prat L, Martínez-Vicente M, Perdiguero E, Ortet L, Rodríguez-Ubreva J, Rebollo E, et al. Autophagy maintains stemness by preventing senescence. Nature. 2016;529: 37–42. doi:10.1038/nature16187

105. Ou X, Lee MR, Huang X, Messina-Graham S, Broxmeyer HE. SIRT1 positively regulates autophagy and mitochondria function in embryonic stem cells under oxidative stress. Stem Cells. 2014;32: 1183–1194. doi:10.1002/stem.1641

1.11 Figure Legends

1.11.1 Figure 1



1.11.2 Figure 1. The biochemical function and cellular roles of SIRT1. (**A**) SIRT1 requires the substrate NAD+ to initiate deacetylation of lysine residues on target proteins. Upon binding of the target acetylated lysine group to the active site, SIRT1 catalyzes the hydrolytic cleavage of nicotinamide from the ADP-ribose base. During this process, the acetyl group is transferred to the ribose, forming 2'-O-acetyl-ADP-ribose. (**B**). The regulatory roles of SIRT1 in various cell types. Proteins in green are positively regulated by SIRT1 deacetylase activity, while proteins in red are negatively regulated.

Chapter 2:

The role of SIRT1 in skeletal muscle function and repair of older mice

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2.1 Abstract

Background. Sirtuin 1 (SIRT1), is a NAD+ sensitive deacetylase, that has been linked to longevity and has been suggested to confer beneficial effects that counter aging-associated deterioration. Muscle repair is dependent upon satellite cell function, which is reported to be reduced with aging; however, it is not known if this is linked to aging-suppression of SIRT1. This study tested the hypothesis that Sirtuin 1 (SIRT1) overexpression would increase the extent of muscle repair and muscle function in older mice.

Methods. We examined satellite cell dependent repair in tibialis anterior, gastrocnemius, and soleus muscles of 13 young wild type mice (20-30 weeks) and 49 older (80+ weeks) mice that were controls (n=13), overexpressed SIRT1 in skeletal muscle (n=14), had a skeletal muscle SIRT1 knockout (n=12), or a satellite cell SIRT1 knockout (n=10). Acute muscle injury was induced by injection of cardiotoxin (CTX) and phosphate buffered saline was used as a vector control. Plantar flexor muscle force and fatigue were evaluated before or 21 days after CTX injection. Satellite cell proliferation and mitochondrial function were also evaluated in undamaged muscles.

Results. Maximal muscle force was significantly lower in control muscles of older satellite cell knockout SIRT1 mice compared to young adult wild type (YWT) mice ($P < 0.001$). Mean contraction force at 40Hz stimulation was significantly greater after recovery from CTX injury in older mice that overexpressed muscle SIRT1 than age-matched SIRT1 knockout mice ($P < 0.05$). SIRT1 muscle knockout models ($P < 0.05$) had greater levels of p53 ($P < 0.05$ MKO, $P < 0.001$ OE) in CTX-damaged tissues as compared to YWT CTX mice. SIRT1 overexpression with co-expression of p53 was associated with increased fatigue resistance and increased force potentiation during repeated contractions as compared to wild-type or SIRT1 knockout models ($P < 0.001$). Muscle structure and mitochondrial function were not different between the groups, but proliferation of satellite cells was significantly greater in older mice with SIRT1 muscle knockout

($P < 0.05$), but not older SIRT1 satellite cell knockout models, *in vitro*, although this effect was attenuated *in vivo* after 21 days of recovery.

Conclusions. The data suggest skeletal muscle structure, function, and recovery after CTX-induced injury are not significantly influenced by gain or loss of SIRT1 abundance alone in skeletal muscle; however, muscle function is impaired by ablation of SIRT1 in satellite cells. SIRT1 appears to interact with p53 to improve muscle fatigue resistance after repair from muscle injury.

Key words. Muscle atrophy; Skeletal muscle; Mitochondrial function; Satellite cells; Muscle force; Fatigue; Sarcopenia

2.2 Introduction

Sarcopenia is the age-associated wasting of skeletal muscle which results in a loss of function [1]. Sarcopenia is accompanied by an increased risk for physical disabilities, fall-induced injuries, hospitalization/ institutionalization, and mortality [2], and it is exacerbated by obesity and metabolic disorders [3]. Mitochondria regulate muscle metabolism and mitochondrial dysfunction may play a role in sarcopenia [4]. Indeed, altered size and granularity have been observed in aging mitochondria [5,6], as well as in the mitochondria of subjects diagnosed with type 2 diabetes mellitus [7]. Concordantly, loss of mitochondrial function also occurs with aging and metabolic disorders [8], although some research suggests age-associated alterations in mitochondrial morphology are independent of lifespan [9]. It is interesting that the highly-oxidative type I muscle fiber, which has ~6% mitochondria volume [10] are generally thought to be more resistant to sarcopenia-related muscle wasting than type II fibers [11] which have ~3% mitochondria volume density [10] . This might be due in part to a greater sensitivity of mitochondria in type II fibers to oxidative stress [12]. Preserving mitochondrial function is therefore a potential strategy for reducing sarcopenia in both fiber types.

Sirtuin 1 (SIRT1) is a class III histone deacetylase [13] which is dependent on NADH to deacetylate its targets, making SIRT1 highly sensitive to metabolism. SIRT1 has been identified as a link between caloric restriction and longevity, with the overexpression of SIRT1 linked to increased lifespans for several organism models [14]. Furthermore, SIRT1 has also been shown to inhibit type I fiber atrophy during intermittent fasting, by deacetylating and inhibiting the transcription activity of FoxO1 and FoxO3 [15]. In addition, activating SIRT1 in muscle cells prevents the reduction in slower fiber type myosin heavy chain gene expression and prevents myotube wasting after glucose [16,17]. Importantly, SIRT1 has been identified as a regulator of protein peroxisome proliferator activated receptor gamma coactivator 1α (PGC1α) [18], which is a well-established regulator of mitochondrial biogenesis and glycolytic-to-oxidative fiber type

switching in mammalian skeletal muscle [19]. PGC-1α activation (i.e. deacetylation) occurs via AMPK activation of the deacetylase SIRT1 [20]. In fact, SIRT1 deacetylation of PGC-1α increased the expression of key glucose and lipid metabolism genes [21]. However, mitochondrial biogenesis in response to exercise is not impaired under SIRT1 knock out conditions and therefore SIRT1 deacetylation is thought to be via the control of acetyltransferase GCN5 [22].

Satellite cell dysfunction is another potential contributor to muscle loss in sarcopenia [23]. Satellite cells are mononucleated muscle stem cells that are responsible for the majority of muscle regeneration after injury [24,25]. Both the expression of SIRT1 and satellite cell populations have been shown to decrease with aging [26], inviting the possibility that increasing SIRT1 expression in older muscle models might act as a countermeasure to preserve muscle regenerative capacity and therefore improve muscle function after muscle injury in aging. Consistent with this idea, resistance exercise coupled with consuming resveratrol, a known activator of SIRT1, increased satellite cell proliferation and improved muscle fiber size and function in muscles of older humans to a greater extent than resistance exercise alone [27]. Furthermore, resveratrol also prevents cell death and increases differentiation of myotubes [28], whereas SIRT1 silencing evokes greater cell death and reduced differentiation [28]. In addition, SIRT1 was reported to maintain pools of satellite cells in the state of quiescence and SIRT1 loss-of-function was reported to invoke premature differentiation of muscle satellite cells [29]. Strengthening this argument, mild endurance exercise which increased SIRT1 was shown to rescue an aging-induced reduction in satellite cell numbers in rats [26,30]. However, other studies have shown differential patterns of SIRT1 expression according to age and tissue type [31], calling into question whether aging has universal effects on SIRT1. Thus, there is a need to further study the role of SIRT1 on mitochondrial function and satellite cell regulation of muscle repair in the context of muscle sarcopenia.

The purpose of this study was to investigate the role of SIRT1 in repairing muscle and restoring skeletal muscle function after acute muscle damage in older mice. Our hypothesis was that muscle overexpression of SIRT1 improves mitochondrial function and satellite cell activation, which improves muscle repair and function after acute injury, and in contrast, loss of SIRT1 in muscle or satellite cells of older mice reduces muscle repair and function after injury by suppressing satellite cell activation and reducing mitochondrial function. Contrary to our expectation, we found that muscle performance and the recovery of skeletal muscle after injury are largely unaffected by the abundance of SIRT1 in skeletal muscle, but ablation of SIRT1 in satellite cells impairs muscle repair and function. Nevertheless, SIRT1 abundance may work synergistically with p53 to reduce muscle fatigue after repair following muscle injury.

2.3 Materials and Methods

2.3.1 Research design.

Two separate experiments were conducted on the five groups of mice. Three to five mice were examined in each age group for each experimental comparison. In the first experiment the plantarflexor muscles (gastrocnemius and soleus muscles) received an acute cardiotoxin (CXT)-induced injury. Plantarflexor force and fatigue were measured before and then again after 21 days of recovery following CTX injury to assess the degree that manipulation of SIRT1 protein abundance had on muscle function and recovery/repair from muscle damage. In a separate study, the plantar flexor muscles and the dorsiflexor (tibialis anterior) muscles from mice in each of 5 groups received a CTX injection to induce acute muscle injury. These muscles were used to assess the role of SIRT1 on muscle structure solely. As there was the potential that electrical stimulation to induce evoked contractions could affect muscle fiber size or structure acutely, we did not assess muscle function in this second study.

2.3.2 Animal Models.

Five mouse models were examined. These included: (i) young wild-type (YWT) mice that were 20-30 weeks of age, (ii) older wild-type mice that were 80-120 weeks of age (WT-80), (iii) SIRT1 overexpressor older mice that were 80-120 weeks of age (OE-80), (iv) SIRT1 older adult skeletal-muscle knockout mice that were 80-120 weeks of age (MKO-80), and (v) SIRT1 satellite cell knockout mice that were 80-120 weeks of age (SKO-80). As ~50% of the animals died before they were 120 weeks of age, we chose to include older animals that were 80 weeks of age in our oldest group because, this is at a point where muscle signaling changes and muscle atrophy and function begins to deteriorate [32,33]. While these animals could not yet be considered to be old, this group had mice that ranged in age from 80-120 weeks, and the oldest animals in this group would be considered old. Therefore, we have chosen to describe this combined group as “older”. All wild-type mice were generated from C57BL/6J background mice purchased from Jackson Laboratories, USA. MKO-80 and SKO-80 mice were generated using the cre-lox system by crossing B6;129-Sirt1^{tm1Ygu}/J mice containing *loxP* sites directing the removal of exon 4 of SIRT1 with MCK-cre expressing B6.FVB (129S4)-Tg(Ckmm-cre)5Khn/J and Pax7-cre expressing Pax7$^{tm1(cre)Mrc}$/J mice, respectively. Mice that overexpressed SIRT1 were generated from B6.Cg-Tg(SIRT1)ASrn/J mice containing a wild-type mouse SIRT1 gene promoted by an endogenous BAC transgene.

2.3.3 CTX-induced acute muscle injury.

Acute muscle injury was induced by injecting the gastrocnemius, soleus, and tibialis anterior (TA) muscles with 10 μM cardiotoxin (CTX; *Naja mossambica;* Sigma, St. Louis MO, USA) [34] in 150 μL of phosphate-buffered saline (PBS). The contralateral limb was injected with 150 μL of PBS as a vehicle control. Cage control recovery occurred for 21 days post-injection. During recovery, mice were given water containing 0.8mg/mL 5-bromo-2’-deoxyuridine (BrdU) (MP Biomedicals) to identify satellite cells that achieved DNA synthesis over the recovery period

as previously used in our lab [24]. Post-recovery, the experimental groups were euthanized by myocardial extraction under heavy anesthesia. The muscles were then immediately collected and frozen in 2-methylbutane (ThermoFisher Scientific, Pittsburgh PA, USA) chilled by liquid nitrogen. Frozen tissue sections were mounted on corkboard with Tissue Tek O.C.T. compound (ThermoFisher Scientific, Pittsburgh PA, USA).

2.3.4 BrdU Immunohistochemistry.

Frozen tissue sections of control and post-CTX injured muscles were obtained with a Leica model CM3050S cryostat and the tissue was mounted on charged glass slides (ThermoFisher Scientific, Pittsburgh PA, USA). The frequency of muscle nuclei that were BrdU positive were measured as previously described by our lab . Briefly, the slides were fixed at room temperature with 1:1 methanol:acetone (ThermoFisher Scientific, Pittsburgh PA, USA) and then permeabilized with phosphate buffered saline in 0.4% Triton X-100. Slides were then washed with PBS and allowed to incubate in 2M hydrochloric acid (ThermoFisher Scientific, Pittsburgh PA, USA) for 1 hour at room temperature, followed by neutralization in 0.1M Borate Buffer (pH 8.5). The tissue sections were blocked with 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and incubated overnight in primary antibodies targeting BrdU (1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Secondary antibodies targeting IgG1 (AlexaFluor-488, Invitrogen-ThermoFisher Scientific, Carlsbad CA, USA) were used for fluorescent imaging of BrdU+ nuclei. Images were captured with a Nikon E800 microscope. BrdU+ cells were quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland). BrdU+ labeled nuclei were quantified if they could be clearly associated with either the periphery or interior of a muscle fiber. All of the nuclei from six nonoverlapping fields were quantified with light microscopy at an objective magnification of 40X. There were two repeats for each biological section and the data averaged for each section. The BrdU labeling index was expressed as a percent of the total nuclei and determined by: (the number of BrdU-positive nuclei associated with muscle fibers)/(labeled

unlabeled nuclei associated with muscle fibers). Data are presented as mean ± standard deviation.

2.3.5 Fiber-Type Immunohistochemistry.

Muscle fiber types were identified using antibodies to myosin heavy chains (MHC) as previously reported by Bloemberg and Quadrilatero [35], with slight modifications as reported by our lab [24]. Briefly, isolated muscles were flash frozen in 2-methylbutane chilled by liquid nitrogen and mounted on corkboard with Tissue Tek O.C.T. compound (ThermoFisher Scientific, Pittsburgh PA, USA). Muscle tissue cross sections were obtained at -20°C with a Leica model CM3050S cryostat. Tissue sections were blocked with normal goat serum (Vector Labs, Burlingame, CA, USA) and stained with primary antibodies targeting MHC I (BA-F8S), MHC IIa (SC-71), and MHC IIb (BF-F3), at a 1:100 dilution overnight at 4°C in a humidified chamber. Primary antibodies were obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA USA. After washing with PBS, the slides were incubated in secondary antibodies at a 1:500 dilution for 2 hours at room temperature. Secondary antibodies targeting IgG2b (AlexaFluor-647), IgGM (AlexaFluor-546), and IgG1 (AlexaFluor-488), were obtained from Invitrogen-ThermoFisher Scientific, Carlsbad CA, USA, and were used for fluorescent imaging. Images were captured with a Nikon E800 microscope. Fiber cross sectional areas were determined by planimetry from a minimum of 500 fibers taken from 8-12 randomly selected fields from the tissue cross sections. All the fibers encompassed in a field were traced. Mean fiber area was calculated using ImageJ software (National Institutes of Health, Bethesda, Maryland). The cross-sectional images were captured with a Nikon E800 microscope. Whole muscle CSA was determined by tracing the entire circumference of the muscle section if the section could be captured as a single image, or otherwise as the summation of total muscle area traced from a composite picture. Type I fiber CSA was determined by individually tracing all fluorescently labeled fibers within a given section, with (n=3) muscle sections per group.

2.3.6 Satellite Cell Isolation.

To evaluate the role of SIRT1 on the potential for repair during aging, mononuclear muscle cells were isolated from the combined muscle mass of control non-CTX injured mouse TA and quadriceps muscles from both hindlimbs using the protocol of Pasut, and colleagues [36] with some modifications. Briefly, muscle samples were cut into small sections and incubated in 1500 U collagenase type II (Worthington Biochemical, USA) in DMEM + 10% horse serum, followed by incubation in 300 U collagenase type II with 6U dispase (Gibco, Invitrogen, Gaithersburg, MD, USA) in DMEM + 10% horse serum. After trituration, muscle supernatants were collected and filtered through 40 μm cell strainers (ThermoFisher Scientific, Pittsburgh PA, USA). Aliquots of isolated mononuclear cells were then washed in PBS-azide and blocked in 10% normal goat serum (Vector Labs, Burlingame, CA, USA). Satellite cells were separated from the muscle mononuclear cells by labeling the cells with fluorophore-conjugated primary antibodies as modified from methods described by Liu et al. [37]. Briefly, isolated mononuclear cells were labeled with fluorophore-conjugated primary antibodies for the following cell surface markers: CD31 (-APC), Sca-1 (-PacBlue), CD45 (-PE-Cy7), and VCAM-1 (-Biotin) (BioLegend, San Diego, CA, USA). A second incubation was used to bind Rhodamine-conjugated avidin (Vector Labs, Burlingame, CA, USA) to the biotin-conjugated antibody. After washing, the fluorophore-conjugated cells were resuspended in fetal bovine serum (FBS) and $VCAM^{+}/CD31^{-}/CD45^{-}/Sca\text{-}1^{-}$ satellite cells were isolated by a FACS Calibur flow cytometer equipped with a 15-MW 488nm argon laser and 633 nm red diode laser (Becton and Dickinson). All flow cytometric measurements were performed in conjunction with the West Virginia University Flow Cytometry Core Facility.

2.3.7 Protein immunoprecipitation Assays.

Immunoprecipitation of selected proteins from cell lysates was conducted as previously reported [38] Briefly, muscle cells were fractionated by the method described by the Rothaermel

et al. [39]. Protein isolates from skeletal muscles were incubated for 1h at 4°C with 25μL Pierce Protein A/G magnetic beads (ThermoFisher Scientific, Pittsburgh PA, USA) to reduce non-specific binding. Clean antigen solutions were then incubated for 2h at 4°C with an anti-PGC1α primary antibody (Santa Cruz Biotech. Sant Cruz, CA, USA). Pre-cleaned Protein A/G beads were next added to the antigen/antibody mixture and allowed to incubate overnight at 4°C. After the incubation, the protein A/G beads were collected with a magnetic apparatus and the supernatant was discarded. After washing, the magnetic beads were again collected, and the precipitated proteins were eluted with a 1x NuPAGE LDS sample buffer (ThermoFisher Scientific, Pittsburgh PA, USA) for 20min at room temperature. Protein concentrations of samples were determined by the Lowry method, and the purity of each fraction was confirmed as reported previously [40]. Forty micrograms of protein were loaded into each well of a 4–12% gradient polyacrylamide gel (Invitrogen, Carlsbad, CA) and separated by routine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 h at 120 V. The proteins were transferred to a nitrocellulose membrane for 1.5 h at 25 V. Nonspecific protein binding was blocked by incubating the membranes in 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 [24,41,42].

2.3.8 Mitochondrial Isolation.

To evaluate the effect of SIRT1 on mitochondria, right and left non-CTX injured hindlimb plantar flexor muscles were collected from YWT and older SIRT1 variant-expression models, with both hindlimb flexor groups were pooled into a single sample. Collected tissues were washed in phosphate-buffered saline (pH 7.4). Subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) were isolated as previously described [43,44]. Mitochondrial pellets were resuspended in KME buffer (100mM KCl, 50mM MOPS, and 0.5mM EDTA; pH 7.4) for analysis of mitochondrial enzymatic activity [45,46].

2.3.9 Mitochondrial Size, Internal Complexity, and Membrane Potential.

The size, internal complexity/granulation, and membrane potential of isolated mitochondria were analyzed with a FACSCalibur flow cytometer equipped with a 15-MW 488nm argon laser and 633nm red diode laser (Becton and Dickinson, Franklin Lakes, NJ, USA), as previously described [43,45]. The ratiometric dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes, Carlsbad, CA) was used to selectively probe and gate for respiring mitochondria [47] using 488 nm excitation with 530 nm and 590 nm bandpass emission filters. A greater concentration of JC-1 aggregates form as a result of greater mitochondrial uptake, which increases the orange fluorescent emission signal, as opposed to the green fluorescent monomer of JC-1. The resultant ratio of orange fluorescence (polarized mitochondrial membrane) to green fluorescence (depolarized mitochondrial membrane) was used to characterize membrane potential for isolated mitochondrial subpopulations.

Size and internal complexity/granularity were determined for mitochondrial populations with forward-scatter (FSC; absolute particle size) and side-scatter (SSC; refracted light proportional to mitochondrial granularity) detectors, respectively. All flow cytometric measurements were performed in conjunction with the West Virginia University Flow Cytometry Core Facility.

2.3.10 Mitochondrial electron transport chain complex activities.

The activities of mitochondrial complex I, III, and IV and ATP synthase were determined as previously described [43]. Complex I and ATP synthase activities were determined separately by measuring the oxidation of NADH at 340 nm, while complex III and complex IV activities were determined separately by measuring the reduction and oxidation of cytochrome *c* at 550nm,

respectively. Protein content was determined with the Bradford method and all complex activities were expressed in nanomoles of oxygen consumed per minute per milligram protein.

2.3.11 Muscle force and fatigue.

Young wild-type **(**YWT) and older SIRT1 variant-expression mouse models underwent measures to determine plantar flexor force and fatigability to assess the role of SIRT1 on muscle recovery post CTX injection. Functional measures were obtained via indirect electric stimulation of the plantar flexor muscles through the tibial nerve and measured with a custom-built dynamometer (Aurora Scientific, Aurora, Ontario, Canada) as previously described [24,38] with the mice anesthetized under 2-3% isoflurane.

Electrically evoked contractions (200μs pulse width) were measured with the mice on a heated plate to maintain body temperature under anesthesia. Hindlimb ankles were positioned at 90° flexion. The functional assessment began with a three electrically evoked muscle twitches, followed by a 120s rest. A second stimulation of 75Hz, was then applied to induce tetanus followed by a 300s rest. This was repeated three times. Finally, a single series of 180 contractions were performed at 40Hz (330ms activation followed by 670 ms of rest) over a span of 180s. All stimulations were applied to the common peroneal nerve with subcutaneous platinum electrons with 200 μs pulse width, via commercially available stimulator and controlling software provided by Aurora Scientific (Aurora, Ontario, Canada). Mice were checked continually for vitality and responsiveness. After the functional evaluation, the right hindlimb gastrocnemius and soleus muscles were each injected with 100μL of 10μM CTX (*Naja mossambica*) to induce muscle damage [34] as described above. An equal volume of PBS was injected into the contralateral leg as a vector control. Muscle recovery/repair occurred for 21 days after CTX injection and then the plantar flexors were examined using the same physiological evaluation. Immediately following the functional measures, the mice were euthanized by myocardial extraction under heavy anesthesia and the hindlimb muscles were collected for analysis.

2.3.12 Proliferation Analysis.

The proliferation potential of isolated populations of satellite cells was quantified from CTX-injected and control TA muscles using Click-iT EdU Alexa Fluor 488 (Invitrogen-ThermoFisher Scientific, Carlsbad CA, USA). Briefly, satellite cells were isolated from TA muscles as described above. The isolated cells were then resuspended in growth medium Dulbecco's modified Eagle's medium (DMEM; high glucose, L-glutamine) with 20% fetal bovine serum (FBS), 10% horse serum (HS), and 1% antibiotic/antimycotic (Gibco, Invitrogen, Gaithersburg, MD, USA) and plated overnight on glass coverslips coated in 0.2% gelatin. Plated satellite cells were then incubated for 1 hour in 10μM EdU in DMEM. After incubation, the cells were fixed in 1mL of 3.7% formaldehyde in PBS for 15 minutes at room temperature, then washed twice with 1mL of 3% bovine serum albumin (BSA) in PBS. The cells were then permeabilized in 1mL of 0.5% Triton X-100 in PBS for 20min. After a second wash in PBS +3% BSA, cells were treated with 500μL of reaction buffer, consisting of copper sulfate, the Alexa Fluor-conjugated azide, and sodium ascorbate, then allowed to incubate for 30 min at room temperature, protected from light. The cells then underwent a final wash and were transferred to microscope slides for analysis with a Nikon E800 microscope. Coefficients of proliferation were calculated as the number of illuminated cells counted on 9 separate fields divided by the number of cells plated. Experiments were run in triplicate and are presented as the mean ± standard deviation.

2.3.13 Western Blot Analysis.

Skeletal muscle lysates were analyzed with Western immunoblots as previously described [24,38,41,42], with slight modifications. SDS-PAGE was run on 4-12% gradient NuPAGE Bis-Tris gels (ThermoFisher Scientific, Pittsburgh PA, USA) at 120V for 2h in NuPAGE MES SDS buffer (ThermoFisher Scientific, Pittsburgh PA, USA). Proteins were transferred to a nitrocellulose membrane for 1h at 15V with a Trans-Blot SD semi-dry transfer cell (Bio-Rad). All proteins were probed at the same time from the same samples, requiring only GADPH loading control.

Membranes were then carefully cut to separate the proteins based on protein molecular weight (MW) to allow for the simultaneous probing of multiple different proteins for each treatment group. The MW for the proteins were: FoxO3a (80kDA), p53 (53kDA) and GAPDH (35.8kDA). After blocking with TBST + 5% nonfat dry milk, membranes were incubated overnight at 4°C in 1:1000 dilutions of primary antibodies for Sirt1 (Millipore), PGC1α (Santa Cruz), GAPDH (Cell Signaling, Danvers, MA, USA), p53 (Cell Signaling, Danvers, MA, USA), Acetyl-Lysine (Cell Signaling, Danvers, MA, USA), and FOXO3a (Cell Signaling, Danvers, MA, USA). Membranes were then incubated in HRP-linked anti-mouse and anti-rabbit secondary antibodies at 1:5000 dilutions (Cell Signaling, Danvers, MA, USA). HRP signals were developed with an enhanced chemiluminescent substrate (ThermoFisher Scientific, Pittsburgh PA, USA) with a G:Box Bioimaging System (Syngene, Fredrick, MD, USA). Band intensity was normalized to GAPDH.

2.3.14 Verification of antibody specificity.

The antibodies which were used in our experiments were validated prior to utilization. We obtained antibodies that were supplied from vendors which were able to provide validation, maintenance testing and production validation to ensure that lots of the antibodies remained constant over the study duration. In addition, validation of the antibodies included testing on appropriate positive protein controls or mouse models (deficient in gene/protein) by immunoblot and immunostaining. Western blots were conducted to validate band sizes, and immunohistochemistry on each antibody to check for non-specific staining in tissue sections. Several different antibodies to the same protein were used to confirm staining patterns and/or location of the protein band on a western blot. Negative controls were conducted without the primary antibody or without the secondary antibody to confirm that there was no inappropriate cross-labeling by the antibodies.

2.3.15 Statistical Analysis.

Quantitative analysis was performed on experiments done at least in triplicate. Statistically significant differences between groups were determined using GraphPad Prism 6 software (La Jolla, CA, USA) performing a Two-way ANOVA (age group x condition) with corrections made for multiple comparisons against a baseline control group by the Dunnett's test. Fatigue analysis was performed using Two-way Repeated ANOVA analysis with corrections for multiple comparisons against the baseline control. Statistical differences between right and left hindlimb pre-treatment groups for each genotype were compared using paired t-tests for muscle force, indicating the difference between hindlimbs was not significant from zero. Multiple unpaired t-tests for contractile force as a function of the number of muscle contractions was also performed between the left and right hindlimb pre-treatment groups for each genotype, also indicating no significant difference between hindlimb function within the individual models. As such, data for the left hindlimb pre-treatment groups were expressed as baseline data for each model. Unpaired t-tests were also used, when appropriate, to determine statistical significance between two groups, such as treatment and control groups within each genotype, with significant differences noted when present. Effect sizes were reported using the Cohen's d statistic and were calculated as the difference in means over the pooled standard deviation. The results were expressed as mean ± standard deviation (SD), ($P < 0.05$).

2.4 Results

2.4.1 Animal Model Characteristics.

Mouse models were categorized into five groups, based on age and SIRT1 expression. Young wild-type (YWT) mice were 20-30 weeks old, while older mice were 80-120 weeks of age. The older mice included: wild-type mice (WT-80), SIRT1 overexpressor mice (OE-80), SIRT1 adult skeletal-muscle knockout mice (MKO-80), and SIRT1 satellite cell knockout mice (SKO-80).

Each group included both male and female mice. Although the older mouse groups showed no significant differences in body weight between males and females, YWT females were significantly smaller than corresponding YWT males ($P \leq 0.05$) (**Table 1**).

2.4.2 SIRT 1 protein abundance in the mouse models.

To characterize differences in SIRT1 protein abundance between animal groups, the hindlimb muscles were collected from young wild-type (YWT, *n*=3) and older (80+ weeks) wild-type (WT-80, *n*=3), SIRT1 overexpressor (OE-80, *n*=3), SIRT1 skeletal muscle knockout (MKO-80, *n*=3), and SIRT1 satellite cell knockout (SKO-80, *n*=3) mice, 21 days after phosphate buffered saline (PBS) or cardiotoxin (CTX) injection. The mice were examined prior to and 2 hours after exercise (-ex) because exercise is a known inducer of SIRT1 [48]. As expected, SIRT1 was greater in the OE muscles and lowest in the muscles from SKO and MKO mice (**Fig. 1B**). These data indicate that SIRT1 protein levels were as anticipated based on the crosses of the respective animal models. SIRT1 was not absent in SKO-80 mice muscles, although the SIRT1 protein abundance was significantly lower in PBS-injected, but not CTX-injected hindlimb muscles from SKO-80 relative to YWT mice. Interestingly, ANOVA analyses showed significant differences between the OE-80 PBS and SKO-80 PBS groups as compared to wild-type controls, but not the MKO-80 PBS groups. However, the OE-80, SKO-80, and MKO-80 muscle SIRT1 abundances were significantly different from the wild-type controls when compared with individual t-tests ($P < 0.05$, data not shown). Only the muscles from SKO-80 mice showed a significant increase in SIRT1 abundance in CTX-injected muscles relative to their own PBS group ($P < 0.05$).

We next measured FoxO3a and p53 protein abundance because they are downstream targets of SIRT1 (**Fig. 1C**). Protein abundance was not markedly different for FoxO3a among the groups. Abundance of p53 followed a similar distribution to the SIRT1 protein expression, although a significant difference in p53 protein abundance was not detected between the OE-80 groups and the YWT PBS control (**Fig. 1D**). Interestingly, while a significant increase in p53

protein abundance was not detected between injury-recovered muscles and muscles treated with the saline control for either the OE-80 or MKO-80 groups, the relative abundance of p53 for those groups was significantly greater than the p53 abundance detected in muscles from the YWT CTX-injected mice (comparison denoted by #). Even though the muscle protein abundance of p53 in the YWT CTX mice differed significantly from the protein abundance levels in muscles from the OE-80 CTX and the MKO-80 CTX groups, p53 protein abundance in muscles from YWT CTX-injected mice did not differ significantly from the levels found in muscles from the WT-80 CTX and the SKO-80 CTX groups (**Fig. 2C-H**).

We also evaluated the acetylation status of PGC1α, another downstream target of SIRT1 to assess SIRT1 activity (**Fig. 1E**). Although we expected to see decreased acetylation of PGC1α in muscles from the SIRT1 overexpressor mouse model and increased acetylation in the muscles from SIRT1 knockout mice, qualitative analysis of immunoblots showed no apparent change in acetylation state, regardless of apparent PGC1α expression in the various mouse genotypes (**Fig. 1F**).

2.4.3 Skeletal Muscle Function.

2.4.3.1 Muscle Force.

We tested whether SIRT1 protein abundance had a role in regulating skeletal muscle function in muscles of older mice after acute injury induced by cardiotoxin (CTX). Before treatment, average, evoked maximal plantar flexion contractile force at 75 Hz stimulation was significantly lower in the older SKO-80 mice as compared to YWT PRE, indicating a lower basal contraction force in older mice deficit of SIRT1 in satellite cell (**Fig. 2A**). Next, contractile force was assessed in each group following a 21-day recovery from acute injury by CTX or treatment with PBS to determine if SIRT1 abundance in skeletal muscle had an effect on the recovery of contractile function. Mean maximal contractile force in YWT mice generated at a stimulation

frequency of 75Hz had recovered to control levels by 21 days after CTX-treatment. The maximal contractile force was significantly lower in the PBS-injected, vector control hindlimb for WT-80 compared to the WT-80 PRE baseline, suggesting that the older muscles in the WT-80 animals may have been susceptible to injection- injury (**Fig. 2A**). As expected, aging per se resulted in a lower plantar flexion maximal force production in wild-type mice, as seen by the lower force production in the muscles of the WT-80 PBS group relative to the WT-80 PRE group.

We next compared the percent recovery of contractile force in hindlimb muscles 21 days post-recovery from CTX injection (**Fig. 2B**). The maximal post PBS- or CTX-injection plantar flexion force was normalized to the respective pre-treatment force output for each animal. The aged mice were expected to show decreased force recovery in PBS control hindlimbs relative to the young wild-type mice. However, despite the WT-80 having a force recovery under 1-fold, no older group showed significantly altered, basal force recovery in control PBS injected hindlimbs relative to the YWT PBS group. This suggests that aging did not impair recovery after injury. Contrary to our original expectations, only the youngest group of mice (YWT) showed decreased recovery in maximal hindlimb force from muscles that had been acutely injured by CTX as compared to PBS treated muscles. Although the overall plantar flexion force output for SKO-80 hindlimb muscles was significantly lower than YWT mice, the SKO-80 mice appeared to recover to pre-treatment levels after 21 days of recovery. The relative force produced 21 days after CTX-induced injury was not diminished in the older SKO-80 animals as compared to young animals although the absolute levels of force was also depressed in the SKO-80 animals as compared to other groups.

2.4.3.2 Muscle fatigue.

Muscle fatigability was examined in hindlimb plantar flexor muscles from young and older mice by inducing a series of 180 electrically evoke contractions at 40 Hz over a period of 3 minutes. While the mean force output over time shows the amount of fatigue occurring in a

contracting muscle group, it does not necessarily mimic resistance to fatigue for those muscles. Therefore, we examined fatigue resistance by looking at the depletion of muscle contractile force as a function of consecutive electrically evoked contractions by normalizing the force of each 40 Hz contraction to the force of the initial 40Hz contraction (**Fig. 2C-H**). The baseline fatigability of the young, pre-treatment wild-type mice (YWT PRE) mice did not differ significantly from the fatigability of the WT-80 PRE, OE-80 PRE, MKO-80 PRE, or SKO-80 PRE-groups (**Fig. 2D, H)**. Neither acute muscle injury from CTX, nor injection with the PBS vector control, had significant effects on muscle fatigability in YWT, WT-80, or SKO-80 mice post-recovery (**Fig. 2D, E, H**). Surprisingly, muscles that underwent repair after CTX injury had greater fatigue resistance in both the aged SIRT1 overexpression and skeletal muscle knockout models of SIRT1 (**Fig. 2F, G**). The OE-80 CTX group showed the greatest magnitude and most consistent attenuation of fatigue following recovery from acute muscle injury, and the OE-80 CTX was also the only group to show signs of post-activation potentiation, so that muscle contractile force increases with time rather than diminishing. Although the effect was smaller for the MKO-80 CTX group compared to the OE-80 CTX mice, protection from fatigue was present only in muscles that had recovered from acute CTX injury (**Fig. 2E-G**), which would have required activation of satellite cells to induce repair. Interestingly, when compared to the fatigability of YWT CTX mice, the OE-80 CTX and MKO-80 CTX groups still showed significant protective fatigue resistance, albeit to a lesser degree, and were the only groups to do so. Furthermore, the fatigue resistance seen in the OE-80 CTX and MKO-80 CTX models coincides with the increased abundance of p53 after repair from CTX as seen in Figure 1C.

2.4.4 Fiber cross sectional area.

Given the greater susceptibility of type II fibers to aging-induced atrophy [49] and the general resistance of type I fibers to sarcopenic changes [50], we decided to investigate the effect of SIRT1 expression on these two primary muscle fiber types in the gastrocnemius muscle of

older mice. Although the exact fiber type distribution of the mouse gastrocnemius can vary between strains, the gastrocnemius muscle typically contains a mixture of type I and type II muscle fibers although it is predominantly composed of type II fibers [51]. To determine the effects of SIRT1 expression on gastrocnemius muscle structure after acute injury, we used immunofluorescence techniques to determine the cross-sectional area (CSA) of whole muscles, and type I and type II fibers from PBS- and CTX-injected gastrocnemius muscles of the YWT and older groups (**Fig. 3F-H**). As expected, the CSA for the gastrocnemius muscle was lower in older mice 21 days after PBS- or CTX-injection CSA relative to muscles in YWT mice 21 days after PBS (**Fig. 3F**). However, we did not find a significant difference between any older group and the YWT control. Given that the Cohen's d test shows effect sizes greater than 2 standard deviations for two groups (WT-80 PBS and WT-80 CTX), it is likely that the absence of significance may have been due to a low sample size.

Similarly, the CSA in the soleus muscle (**Fig. 3G**), had a significant F statistical main effect from One-Way ANOVA with corrections for multiple comparisons, however, no groups had significant differences were found between the YWT PBS control group and other groups. Groups of mice showing a large effect size (>1 standard deviation) were YWT CTX, WT-80 PBS, OE-80 CTX, and SKO-80 CTX groups. Next, we calculated mean type I fiber CSA from each gastrocnemius section (**Fig. 3A-E**), from which we calculated the percent type I CSA standardized to the total CSA of the gastrocnemius muscle (**Fig. 3H**). Despite the apparent difference of the percent of type I fibers, this did not reach a statistically significant difference when represented as a percentage of the entire gastrocnemius muscle CSA or the specific comparisons of the treatment groups to the YWT PBS control.

2.4.5 Regenerative Capacity

Loss of function in aging skeletal muscle is characterized by reduced capacity for muscle satellite cells to regenerate damaged tissue [52]. Previously, our lab has used 5-bromo-2'-

deoxyuridine (BrdU) administered in water supplied *ad libitum* to gauge satellite cell proliferation to an experimental intervention [24]. Therefore, to examine satellite cell division in response to skeletal muscle regeneration, we injected the tibialis anterior (TA) muscles of older WT, OE, MKO, and SKO mice with CTX or PBS as a control and supplied the recovering mice with 0.8mg/mL BrdU for 21 days post-injury. After recovery, the mice were sacrificed and BrdU expression was identified in the TA or gastrocnemius muscles by immunohistochemically. The number of BrdU+ nuclei in CTX-recovery hindlimb muscles were then counted (**Fig. 4A-D**) and normalized to the CSA of their respective muscles. In contrast to the plantar flexor muscle group, the TAs showed no significant difference between PBS and CTX-recovery groups for mean muscle CSA or effect size (**Fig. 4E**). No significant difference was seen between the MKO-80 and WT-80 groups, while a significantly lower number of BrdU+ nuclei/μm^2 was found for the OE-80 and SKO-80 CTX-recovery groups (**Fig. 4F**). Nevertheless, all animal groups showed effect sizes larger than 1 standard deviation ($P \leq 0.05$).

Next, to determine the regenerative capacity of satellite cells from the older SIRT1 expression variable mouse models, we isolated satellite cells by FACS and used them in proliferation analysis. Isolated satellite cells were diluted so that 10,000 cells could be plated in triplicate onto a gelatin-coated coverslip in a 24 well plate. Cells were allowed to recover overnight before treatment with 5-ethynyl-2'-deoxyuridine (EdU) prior to fixation and permeabilization. Similar to BrdU, EdU is incorporated into DNA from proliferating cells. Thereafter, images from 9 fields from each coverslip were captured and the total number of EdU+ cells were counted (**Fig. 5A-D**). The proliferation factor was calculated and expressed as the number of EdU+ cells in 9 image fields over the number of cells initially plated. Satellite cells that were isolated from muscles of MKO-80 mice showed a significant increase in proliferation relative to WT-80 mice, with an effect size greater than 3 standard deviations (**Fig. 5E**).

2.4.6 Mitochondrial Structure and Function.

There is likely a link between mitochondrial dysfunction and aging [53] including sarcopenia [4]. As SIRT1 is a known regulator of PGC1α which in turn regulates mitochondria biogenesis, we next decided to investigate the effects that SIRT1 expression levels had on mitochondrial activity in the skeletal muscle of older mice. To determine changes in mitochondrial size and complexity/granularity, freshly isolated interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM) subpopulations were analyzed in control mice by forward-scattering (FSC) and side-scattering (SSC) light, respectively [44,54]. Mitochondria were isolated from the same mice that were used for satellite cell isolation experiments and therefore it was not possible to evaluate mitochondria after CTX experiments in these mice. Older mice had greater mitochondrial size for both SSM and IFM relative to YWT control mice for both SIRT-1 overexpressor and knockout groups (**Fig. 6A, D**). No significant difference was found for the internal complexities of IFM or SSM; however, a significant F ratio was found for the SSM internal complexity, despite having no significant differences among the groups relative to the YWT control (**Fig. 6B, E**).

Changes in mitochondrial membrane potential (ΔΨm) typically occur in tangent with diseases like diabetes [45,54] and also in aging [55]. Isolated mitochondria were incubated with 5,5',6,6'-etrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1). A shift from JC-1 green to red fluorescence indicated an increase in the ΔΨm. Previous studies have shown mitochondria from diabetic animals or humans had a greater orange/green ratio as compared to control mitochondria [45,54]. A significantly lower ΔΨm was found in SSM populations for OE-80 mice relative to YWT control mice as reflected by a lower orange/green fluorescent ratio (**Fig. 6C**). A similar decrease in ΔΨm was seen for the IFM mitochondria in OE-80 mice, although statistical significance was not achieved (**Fig. 6F**). While significant differences for ΔΨm were not found between groups, a One-way ANOVA with corrections for multiple variances with the

Dunnett's test (p = 0.05), the global analyses revealed a statistical difference between the group means. Given the large effect sizes for some of the groups (>1 SD), it is possible that the marginally non-significant pairwise contrasts in (**Fig. 6F**) may have produced a significant difference if there the experimental sample size had been larger.

2.4.7 Mitochondrial Complex Activity and Muscle Repair.

To further examine the effects of variant SIRT1 expression coupled with aging on mitochondria, we next assessed the relative activity of the mitochondrial electron transport chain complexes in control muscles. These same mice were used for satellite cell isolation experiments and therefore it was not possible to evaluate mitochondria following CTX injury in these mice. Significant differences were found for activities of the SSM complexes; specifically, a greater complex III activity for WT-80 mice (**Fig. 6H**), and a reduced ATP-synthase activity for OE-80 mice (**Fig. 6J**). No significant change was seen for SSM complex I activity (**Fig. 6G**) or SSM complex IV activity (**Fig. 6I**), although the One-Way ANOVA global test did suggest there was a significance in differences between the means. No significant group differences were found for IFM complex I activity (**Fig. 6K**), complex III activity (**Fig. 6L**) or complex IV activity (**Fig. 6M**), however, the global analysis did show that a significant difference existed for the IFM ATP-synthase activity (**Fig. 6N**).

2.5 Discussion

In skeletal muscle, SIRT1 has been established as a regulator of PGC1α and consequently, mitochondria [21,56], which are important for generating ATP during muscle repair. SIRT1 is generally high in type I fibers and promoted by endurance exercise [48], and because type I oxidative fibers are generally though to resist aging-related atrophy, SIRT1 has become a target of interest in studies of sarcopenia with a potential role in preventing muscle wasting. Support for this idea came from studies that show overexpression of SIRT1 prevented type I fiber atrophy

under conditions of long-term caloric deficit [15]. Furthermore, SIRT1 overexpression also improved the pathophysiology of muscle wasting including Duchenne muscular dystrophy [50]. The data in the present study show that SIRT1 is important for proper muscle function in older mice, with loss-of-function most impactful to muscle satellite cells. This suggests that SIRT1 may have a role in satellite cell regulated muscle repair. Unexpectedly, both our SIRT1 overexpressing model (OE-80) and skeletal muscle-specific SIRT1 knockout model (MKO-80), demonstrated increased resistance to muscle fatigue after recovering from acute CTX-induced muscle damage. Although knocking out SIRT1 in satellite cells impairs muscle contraction in aged mice, ablation of SIRT1 from skeletal muscle promotes satellite cell proliferation and therefore the muscle's regenerative capacity, suggesting that the most beneficial or detrimental effects of SIRT1 may be determined by its site of localization within muscle cells.

2.5.1 Muscle and satellite cell loss of SIRT1.

Interestingly, the MKO and SKO models showed differential effects for SIRT1 knockout. The MKO mice had a greater resistance to fatigue after repair following CTX injury but a similar level of regenerative capacity as compared to the older models. In contrast to the SKO model, the cre protein in the MKO model is induced by the MCK promoter, so SIRT1 it is only expressed in mature skeletal muscle. In contrast, the SKO model's cre protein is promoted by PAX7 so that SIRT1 is inactive in only satellite cells, but not in skeletal muscle when PAX7 is downregulated.

2.5.2 Satellite cell but not muscle loss of SIRT1 reduces maximal force production.

Muscles from older MKO mice did not have a loss of muscle force production when compared to age-matched or 20-week-old, wild-type control mice. This suggests that loss or gain of SIRT1 does not affect the muscle's ability to generate maximal muscle force (presumably by not affecting the number or general function of the myosin cross bridges). In contrast, SKO mice that have a satellite cell specific loss of SIRT1 had lower overall plantarflexion force than young

or older wildtype or SIRT1 overexpressor mice, although gastrocnemius and soleus muscles were not markedly smaller in this group. This suggests that loss of SIRT1 in satellite cells did not change overall cross bridge number in the muscle's CSA, but interestingly it did affect muscle function. While it is possible that SIRT1 could affect force per cross bridge, we think a more likely possibility is that the lower force output in muscles from SKO mice may indicate a neural problem which resulted in an incomplete activation of muscle in the old mice. This could potentially occur at the level of the neuromuscular junction perhaps as an accelerated aging loss of dystrophin [57]. However, this study was not designed to evaluate the role of SIRT1 in neural regulation of force.

2.5.3 SIRT1 expression may improve but is not necessary for recovery of muscle function after injury.

Muscle repair is dependent upon satellite cell activation and satellite cell function thought to be depressed with aging [23,52]. In elderly humans, SIRT1 activation by resveratrol was shown to be associated with increased satellite cell activation in response to loading exercise [27]. Although the MKO-80 and OE-80 aged models in our study vary with respect to their levels of SIRT1 expression in skeletal muscle, both groups showed some improvement in fatigue resistance in response to repeated contractions of muscles in hindlimbs previously injured by CTX as compared to uninjured control muscles. This fatigue resistance was not reproduced in CTX-injured hindlimbs for the YWT, WT-80, or SKO-80 models after a 21-day recovery period that was observed in the other groups. Furthermore, none of the 5 animal groups experienced a loss of function during the fatigue testing in hindlimbs injured by CTX or injected with the PBS control, indicating that functional recovery of skeletal muscle is not influenced by loss or gain of SIRT1 abundance in skeletal muscle. The gain of function after injury in both SIRT1 overexpressor and SIRT1 knockout models suggests a common repair-induced modification in muscle function may exist as long as some SIRT1 is present in satellite cells (even if it does not exist in the muscle cells per se).

2.5.4 SIRT1 loss in muscle increases acute, but not long-term proliferation of satellite cell.

Ryall and colleagues reported that knocking SIRT1 out of satellite cells caused premature differentiation of satellite cells [29], and therefore we expected SIRT1 loss or overexpression would induce different satellite cell proliferation responses after CTX injury.

In vitro analysis of cell proliferation within 24 hours of culturing isolated satellite cells showed that a muscle knockout of SIRT1 had the greatest potential for satellite cell proliferation among the experimental groups. Of the five experimental groups, only the MKO model lacked expression of SIRT1 in skeletal muscle (despite PAX7 not being expressed in skeletal muscle, Western blots showed reduced or ablated bands for SKO mice, which should only lack SIRT1 in satellite cells). However, because the SKO muscles showed a comparatively blunted level of proliferation compared to the MKO model, muscle loss of SIRT1 while maintaining satellite cell SIRT1 appeared to increase satellite cell proliferation. However, this potential was not realized *in vivo* in response to repair following CTX injury.

In vivo proliferation was assessed 21 days after acute CTX injury in muscles of older mice and was reflected by the frequency of BrdU+ nuclei present in muscle sections post-repair. As 21 days represents a point where recovery from acute injury should be complete, this experiment reflected long-term satellite cell proliferation following an acute injury and not the rate of cell proliferation. After 21 days, the MKO model no longer had the greatest proliferation capacity, but instead was the only SIRT1-expressor variant that did not differ significantly in the number of BrdU+ nuclei quantified in the older, WT-80 group. Both OE-80 and SKO-80 showed significantly fewer BrdU+ cells relative to the WT-80 and MKO-80 models. This indicates that factors outside of the satellite cells (potentially cross-talk between muscle and satellite cells) *in vivo* limited satellite cell proliferation in muscles of old animals, and one of those could be muscle SIRT1.

2.5.5 Mitochondrial function and SIRT1 in muscle.

As SIRT1 has a role in regulating oxidative metabolism and connecting global metabolism to longevity and regeneration, we also examined mitochondrial subpopulations to identify potential differences in these subpopulations resulting from variant SIRT1 expression. Mitochondrial dysfunction may be a fundamental component that regulates aging responses along several pathways [27,58]. Previous work has suggested that interfibrillar mitochondria (IFM) and subsarcolemmal mitochondria (SSM) subpopulations are affected differently in cardiac muscle by diabetes [54]. Furthermore, SIRT1 is a regulator of PGC1-α which moderates mitochondrial proliferation [59].

As a reduced mitochondrial membrane potential has been characterized as an effect of aging and correlates with increased ROS production [55,60] we had expected that mitochondrial membrane potential ($\Delta\Psi m$) would be improved when SIRT1 was overexpressed in muscles of older mice, but this was not the case. Interestingly, we saw that muscles from older mice overexpressing SIRT1 had a significantly lower $\Delta\Psi m$ and ATP-synthase activity in the SSM subpopulation. While, we found a significant F ratio for IFM $\Delta\Psi m$ by One-way ANOVA, there was no significance difference in the mitochondrial membrane potential as an indicator of mitochondrial function between animal groups (denoted in our figures by *F**), for IFM-$\Delta\Psi m$ and IFM ATP-synthase activity. The IFM results may have been a function of significant differences in activity that were masked by the low sample sizes of the experiments. While it is possible that the decrease in $\Delta\Psi m$ in muscle mitochondria from SIRT1 overexpressor mice, may extend to the IFM subpopulation as well, we cannot verify this in the current study.

Despite loss of SIRT1 function, there was no significant differences in muscle mitochondrial characteristics among MKO-80, SKO-80 and YWT mice aside from significantly increased size in the older mice (perhaps as a function of decreased mitochondrial fission or increased fusion). Because analysis of these mitochondria was performed on isolated skeletal

muscle and not isolated satellite cells, some differences in mitochondrial characteristics may be present during the repair process in satellite cells that could contribute to a reduced maximal contraction force in muscles of SKO-80 mice, or the increase in proliferation of satellite cells seen in MKO-80 mice. However, the similarities between the experimental models for the majority of the mitochondrial factors closely reflects the physiological data that showed little change between muscle fatigability between pre-treatment and saline-injected control groups. Although the reduced ΔΨm and ATP synthase activity in SSM from OE-80 mice, coupled with the significantly reduced number of proliferating BrdU+ cells detected after injury, support the idea that mitochondria dysfunction may impair the recovery of muscle injury in aging [61]. The force and fatigue data collected before and after injury suggest that alterations in mitochondria characteristics did not impair muscle function after repair from CTX-induced injury.

One explanation for the increased satellite cell proliferation seen in MKO-80 mice after injury and the blunted satellite cell response seen in the OE-80 model might be related to the mitochondrial related reactive oxygen species (ROS) concentration in the relative cellular environments. For example, attenuation of muscle regeneration in satellite cells from old mice was shown to decrease when aged satellite cells were paired to young circulatory environments [23], indicating that repair can be influenced by the local cellular environment. Several studies have suggested that excess ROS generated from mitochondrial respiration can damage cellular structures and may contribute to cellular aging [62]. It is possible, although speculative, that skeletal muscle containing high levels of SIRT1 may release more mitochondrial-associated ROS than SIRT1 knockout models during injury and repair, and thereby impair the proliferation capacity in satellite cells during muscle repair. Although our study was not designed to measure ROS levels, we did see a reduction in SSM- ΔΨm and in ATP synthase activity for the OE-80 model. Reduced NAD+ and SIRT1 activity has been observed in aged tissues, concurrent with reduced mitochondrial respiratory chain activity and decreased expression of proteins that protect against

oxidative damage [63]. Conversely, treatment with nicotinamide (NAM), the precursor to the SIRT1 cofactor nicotinamide adenine dinucleotide (NAD+), has been shown to increase mitochondrial membrane potential and decrease ROS concentration through pathways utilizing [64], and pathways independent of SIRT1 [65]. It is possible that the OE-80 model, which has increased expression of SIRT1 and a decreased SSM- ΔΨm, is depleting NAD+ more than wild-type or knockout models. If this was the case, NAM abundance would be decreased and ROS abundance would be increased, which in turn would be expected to reduce satellite cell proliferation as a result of greater oxidative damage.

As resveratrol-mediated SIRT1 activation was shown to be protective against ROS-induced mitochondrial-mediated apoptotic signaling in muscle [41], and SIRT1 increases antioxidant production in muscle [27,32] we had expected that mitochondrial function would be improved in muscles that overexpressed SIRT1after repair following CTX injury mice. However, muscles from OE-80 animals had the lowest SSM-ΔΨm and SSM ATP-synthase activity. As SSM lie close to satellite cells, we would have expected this mitochondrial subpopulation to have the greatest impact on proliferating satellite cells if mitochondrial were important for this process. Nevertheless, the abundance of BrdU positive satellite cell nuclei were not different in muscles among OE-80, MKO-80 or SKO animals after repair from CTX-injury. Thus, the potential for SIRT1 abundance to alter mitochondria did not appear to affect the potential for satellite cell-proliferation during repair after CTX injury.

2.5.6 p53 protein abundance is increased in SIRT1 variant models that gained improved muscle function after recovery from CTX-induced injury.

Tumor suppressor p53 is a well-characterized downstream target of SIRT1. While SIRT1 deacetylation usually inhibits p53 activity, p53 can be upregulated through several pathways, especially in response to cellular stress or damage [66]. Indeed, p53 was only found to be significantly increased in the CTX-injured muscle of the OE-80 and MKO-80 models, but only

when compared to basal levels of p53 in control muscles that have also recovered from CTX injury. Because one of the main functions of p53 is to repair DNA in response to injury, the presence of p53 in the CTX-injured muscles provides a plausible explanation as to why our OE-80 and MKO-80 mouse models that have opposite protein abundances of SIRT1 could paradoxically both benefit from CTX-induced injury. Furthermore, p53 has recently begun to emerge as an important factor in exercise metabolism, as genetic knockouts of p53 reduce IFM and SSM mitochondrial yield, inhibit mitochondrial respiration, and reduce exercise capacity during fatiguing exercises in rodent models [67]. Interestingly, although the expression of p53 coincides with improved fatigue resistance in the SIRT1 muscle knockout, the improvement in muscle function after CTX-recovery is markedly greater in the OE-80 model, indicating high synergy between the abundances of the SIRT1 and the p53 proteins during muscle recovery.

2.5.7 Expression of p53 may underlie the attenuation of MKO-induced satellite cell proliferation.

Previous work has established that SIRT1 expression in satellite cells helps to maintain the quiescence of satellite cells, and that eliminating SIRT1 in satellite cells causes premature proliferation [29]. Normal downregulation of SIRT1 in satellite cells precedes a metabolic shift from fatty acid metabolism to glycolysis, which is a necessary step for the transition between quiescence and proliferation. Because the metabolic profile of stem cells also acts to regulate proliferation and differentiation [68], it is possible that the increased satellite cell proliferation seen in the MKO mouse model is due to the absence of SIRT1 in muscle that disrupts the necessary metabolic change required to end satellite cell proliferation. This possibility is strengthened by evidence showing that there is an increased abundance of p53 in the CTX-injected hindlimb muscles of the MKO mouse model and that there is an attenuation of satellite proliferation that was identified by BrdU+ 21 days post CTX injury. This is because overload-induced muscle growth increases nuclear p53 [69] and ablating p53 activity reduces myoblast differentiation [70].

A likely scenario is that in the absence of SIRT1 in MKO muscle causes satellite cells to undergo increased proliferation (a reduced proliferation “off-signal”), while the overexpression of SIRT1 would inhibit proliferation, which corresponds to a lower number of BrdU+ cells in the OE-80 model when compared to WT-80. Ironically, CTX-induced muscle damage that should promote satellite cell proliferation to stimulate repair also increased p53 expression, which would be expected to inhibit proliferation and promote differentiation in satellite cells of the repairing muscles in MKO-80 mice and potentially lead to muscle growth/repair. This is consistent with the observation that cell proliferation was attenuated in isolated satellite cells from MKO-80 mice *in vitro* after 24 hours by EdU analysis.

2.5.8 The synergistic effects of SIRT1 and p53 that improve fatigue resistance after injury may also protect type I fibers from sarcopenic wasting.

Although the expression of p53 in the CTX-recovery hindlimbs of the MKO-80 mice provide a possible explanation as to why the MKO-80 mice have attenuated satellite cell proliferation after CTX injury and why injury may induce a protective effect against fatigue, the inhibitory effect that SIRT1 exerts on p53 suggests that co-expression in muscle fibers should ablate the protective effects of p53 on fatigability. To the contrary, our data show that the OE-80 mice gain a strong protective effect against fatigue when p53 is upregulated in hindlimbs following CTX injury and even show signs of improving muscle function following injury through post-activation potentiation [71,72]. One possible explanation is that the inhibition of p53 by SIRT1 only inhibits p53 activity detrimental to oxidative fibers, but not mitochondrial function or DNA repair activity crucial to preventing metabolic ROS damage. Furthermore, p53 is an established upregulator of immobilization-induced skeletal muscle atrophy [73]. Selective inhibition of this activity could explain why type I muscle fibers are resistant to sarcopenic wasting while type II fibers selectively undergo atrophy. It will be interesting to determine in future studies if co-expression of SIRT1 and p53 selectively inhibit atrophy-related activity but not the DNA repair

functions, thereby ensuring that p53 cannot promote atrophy in oxidative fibers, where SIRT1 is highly expressed.

2.5.9 Study limitations.

The loss of older mice which died at old ages prior to conducting these studies, and removal of mice due to deteriorating health conditions at old ages limited the number of animals that were available to complete the experiments and therefore reduced the power of the study. The low number of older animals available for this study prevent us from obtaining mitochondrial measures in muscles after repair from CTX. The lower than ideal number of animals may also have contributed to several experiments which had significant differences in their global One-way ANOVA analyses but failed to detect significant between group differences. Due to the large effect sizes for some of the values calculated by the respective Cohen's d statistics, the likelihood that some differences were masked by the sample size numbers is high. Increasing the power of these experiments and expanding the scope of the mitochondrial aspects of the study to include ROS production and mitochondrial respiration could potentially elucidate a secondary, external role of SIRT1 in modifying satellite cell viability through oxidative damage and/or mitochondrial dysfunction.

2.5.10 Conclusions.

The expression of SIRT1 in satellite cells is important for the functional development of skeletal muscle both initially and for full muscle regeneration. However, despite its functional significance in immature skeletal muscle, SIRT1 shows modest benefit on muscle mass or muscle function when it is expressed in the older skeletal muscle of C57BL6/j-background mice after acute injury. Our data suggest that there is little benefit for the physiological force or fatigue responses by overexpression of SIRT1 *in vivo* in the repaired older skeletal muscle of mice in the absence of any other interventions. However, SIRT1 may synergistically improve the effect of

beneficial proteins like p53 after injury, improving muscle adaptability and functionality. Ablation of basal SIRT1 expression in satellite cells, however, critically impacts future/mature muscle function, resulting in lower force production. The MCK-driven knockout of SIRT1 in the skeletal muscle of older mice showed an improved satellite cell dependent muscle regeneration potential, but this regeneration is possibly attenuated by the expression of p53 after muscle injury. Future work is needed to identify the synergistic effects between SIRT1 and p53 in muscle repair. We speculate that the beneficial effects produced by co-expression of SIRT1 and p53 may also underlie important mechanisms related to longevity, potentially including the mechanism behind type I fiber preservation during sarcopenia.

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2.7 Conflict of Interest

Matthew J. Myers, Danielle L. Shepherd, Andrya J. Durr, David S. Stanton, Junaith S. Mohamed, John M. Hollander, and Stephen E. Alway declare that they have no conflict of interest.

2.8 REFERENCES

1 Evans, W. J. What is sarcopenia? *J Gerontol A Biol Sci Med Sci* **50 Spec No**, 5-8 (1995).

2 Cruz-Jentoft, A. J. *et al.* Prevalence of and interventions for sarcopenia in ageing adults: a systematic review. Report of the International Sarcopenia Initiative (EWGSOP and IWGS). *Age Ageing* (2014).

3 Stenholm, S. *et al.* Sarcopenic obesity: definition, cause and consequences. *Curr Opin Clin Nutr Metab Care* **11**, 693-700, doi:10.1097/MCO.0b013e328312c37d (2008).

4 Alway, S. E., Mohamed, J. S. & Myers, M. J. Mitochondria Initiate and Regulate Sarcopenia. *Exerc Sport Sci Rev* **45**, 58-69 (2017).

5 Bua, E. A., McKiernan, S. H., Wanagat, J., McKenzie, D. & Aiken, J. M. Mitochondrial abnormalities are more frequent in muscles undergoing sarcopenia. *J Appl Physiol (1985)* **92**, 2617-2624, doi:10.1152/japplphysiol.01102.2001 (2002).

6 Thomsen, K. *et al.* Initial brain aging: heterogeneity of mitochondrial size is associated with decline in complex I-linked respiration in cortex and hippocampus. *Neurobiol Aging* **61**, 215-224, doi:10.1016/j.neurobiolaging.2017.08.004 (2018).

7 Jarosz, J. *et al.* Changes in mitochondrial morphology and organization can enhance energy supply from mitochondrial oxidative phosphorylation in diabetic cardiomyopathy. *Am J Physiol Cell Physiol* **312**, C190-C197, doi:10.1152/ajpcell.00298.2016 (2017).

8 Marzetti, E. *et al.* Mitochondrial dysfunction and sarcopenia of aging: from signaling pathways to clinical trials. *Int J Biochem.Cell Biol.* **45**, 2288-2301 (2013).

9 Regmi, S. G., Rolland, S. G. & Conradt, B. Age-dependent changes in mitochondrial morphology and volume are not predictors of lifespan. *Aging (Albany NY)* **6**, 118-130, doi:10.18632/aging.100639 (2014).

10 Alway, S. E., MacDougall, J. D., Sale, D. G., Sutton, J. R. & McComas, A. J. Functional and structural adaptations in skeletal muscle of trained athletes. *J Appl Physiol (1985)* **64**, 1114-1120 (1988).

11 Demontis, F., Piccirillo, R., Goldberg, A. L. & Perrimon, N. Mechanisms of skeletal muscle aging: insights from Drosophila and mammalian models. *Dis Model Mech* **6**, 1339-1352, doi:10.1242/dmm.012559 (2013).

12 Lustgarten, M. S. *et al.* MnSOD deficiency results in elevated oxidative stress and decreased mitochondrial function but does not lead to muscle atrophy during aging. *Aging Cell* **10**, 493-505, doi:10.1111/j.1474-9726.2011.00695.x (2011).

13 Haigis, M. C. & Sinclair, D. A. Mammalian sirtuins: biological insights and disease relevance. *Annu.Rev.Pathol.* **5**, 253-295 (2010).

14 Houtkooper, R. H., Pirinen, E. & Auwerx, J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* **13**, 225-238, doi:10.1038/nrm3293 (2012).

15 Lee, D. & Goldberg, A. L. SIRT1 protein, by blocking the activities of transcription factors FoxO1 and FoxO3, inhibits muscle atrophy and promotes muscle growth. *J Biol.Chem.* **288**, 30515-30526 (2013).

16 Brandon, A. E. *et al.* Overexpression of SIRT1 in rat skeletal muscle does not alter glucose induced insulin resistance. *PLoS One* **10**, e0121959, doi:10.1371/journal.pone.0121959 (2015).

17 Dugdale, H. F. *et al.* The role of resveratrol on skeletal muscle cell differentiation and myotube hypertrophy during glucose restriction. *Mol Cell Biochem* **444**, 109-123, doi:10.1007/s11010-017-3236-1 (2018).

18 Purushotham, A. *et al.* Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab* **9**, 327-338, doi:10.1016/j.cmet.2009.02.006 (2009).

19 Rasbach, K. A. *et al.* PGC-1alpha regulates a HIF2alpha-dependent switch in skeletal muscle fiber types. *Proc Natl Acad Sci U S A* **107**, 21866-21871, doi:10.1073/pnas.1016089107 (2010).

20 Canto, C. *et al.* AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature* **458**, 1056-1060 (2009).

21 Nemoto, S., Fergusson, M. M. & Finkel, T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. *J Biol.Chem.* **280**, 16456-16460 (2005).

22 Philp, A. *et al.* Sirtuin 1 (SIRT1) deacetylase activity is not required for mitochondrial biogenesis or peroxisome proliferator activated receptor-{gamma} coactivator-1{alpha} (PGC-1{alpha}) deacetylation following endurance exercise. *J Biol.Chem.* **286**, 30561-30570 (2011).

23 Conboy, I. M. & Rando, T. A. Aging, stem cells and tissue regeneration: lessons from muscle. *Cell Cycle* **4**, 407-410 (2005).

24 Brooks, M. J., Hajira, A., Mohamed, J. S. & Alway, S. E. Voluntary wheel running increases satellite cell abundance and improves recovery from disuse in gastrocnemius muscles from mice. *J Appl Physiol (1985)* **124**, 1616-1628, doi:10.1152/japplphysiol.00451.2017 (2018).

25 Hwang, A. B. & Brack, A. S. Muscle Stem Cells and Aging. *Curr Top Dev Biol* **126**, 299-322, doi:10.1016/bs.ctdb.2017.08.008 (2018).

26 Day, K., Shefer, G., Shearer, A. & Yablonka-Reuveni, Z. The depletion of skeletal muscle satellite cells with age is concomitant with reduced capacity of single progenitors to produce reserve progeny. *Dev Biol* **340**, 330-343 (2010).

27 Alway, S. E. *et al.* Resveratrol enhances exercise-induced cellular and functional adaptations of skeletal muscle in older men and women. *J Gerontol A Biol.Sci Med Sci* **72**, 1595-1606, doi:10.1093/gerona/glx089 (2017).

28 Saini, A., Al-Shanti, N., Sharples, A. P. & Stewart, C. E. Sirtuin 1 regulates skeletal myoblast survival and enhances differentiation in the presence of resveratrol. *Exp Physiol* **97**, 400-418, doi:10.1113/expphysiol.2011.061028 (2012).

29 Ryall, J. G. *et al.* The NAD(+)-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. *Cell Stem Cell* **16**, 171-183, doi:10.1016/j.stem.2014.12.004 (2015).

30 Shefer, G., Rauner, G., Yablonka-Reuveni, Z. & Benayahu, D. Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. *PLoS.One.* **5**, e13307 (2010).

31 Kwon, Y., Kim, J., Lee, C. Y. & Kim, H. Expression of SIRT1 and SIRT3 varies according to age in mice. *Anat Cell Biol* **48**, 54-61, doi:10.5115/acb.2015.48.1.54 (2015).

32 Jackson, J. R., Ryan, M. J. & Alway, S. E. Long-term supplementation with resveratrol alleviates oxidative stress but does not attenuate sarcopenia in aged mice. *J Gerontol A Biol Sci Med Sci* **66**, 751-764 (2011).

33 Del Campo, A. *et al.* Muscle function decline and mitochondria changes in middle age precede sarcopenia in mice. *Aging (Albany NY)* **10**, 34-55, doi:10.18632/aging.101358 (2018).

34 Ohno, Y. *et al.* Suppression of Myostatin Stimulates Regenerative Potential of Injured Antigravitational Soleus Muscle in Mice under Unloading Condition. *Int J Med Sci* **13**, 680-685, doi:10.7150/ijms.16267 (2016).

35 Bloemberg, D. & Quadrilatero, J. Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. *PLoS One* **7**, e35273, doi:10.1371/journal.pone.0035273 (2012).

36 Pasut, A. *et al.* Notch Signaling Rescues Loss of Satellite Cells Lacking Pax7 and Promotes Brown Adipogenic Differentiation. *Cell Rep* **16**, 333-343, doi:10.1016/j.celrep.2016.06.001 (2016).

37 Liu, L., Cheung, T. H., Charville, G. W. & Rando, T. A. Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. *Nat Protoc* **10**, 1612-1624, doi:10.1038/nprot.2015.110 (2015).

38 Mohamed, J. S., Wilson, J. C., Myers, M. J., Sisson, K. J. & Alway, S. E. Dysregulation of SIRT-1 in aging mice increases skeletal muscle fatigue by a PARP-1-dependent mechanism. *Aging (Albany NY)* **10**, 820-834 (2014).

39 Rothermel, B. *et al.* A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. *J Biol Chem* **275**, 8719-8725 (2000).

40 Siu, P. M., Pistilli, E. E., Butler, D. C. & Alway, S. E. Aging influences cellular and molecular responses of apoptosis to skeletal muscle unloading. *Am J Physiol Cell Physiol* **288**, C338-C349 (2005).

41 Haramizu, S. *et al.* Dietary resveratrol confers apoptotic resistance to oxidative stress in myoblasts. *J Nutr Biochem* **50**, 103-115, doi:10.1016/j.jnutbio.2017.08.008 (2017).

42 Takahashi, H. *et al.* Epigallocatechin-3-gallate increases autophagy signaling in resting and unloaded plantaris muscles but selectively suppresses autophagy protein abundance in reloaded muscles of aged rats. *Exp Gerontol* **92**, 56-66, doi:10.1016/j.exger.2017.02.075 (2017).

43 Croston, T. L. *et al.* Functional deficiencies of subsarcolemmal mitochondria in the type 2 diabetic human heart. *Am J Physiol Heart Circ Physiol* **307**, H54-65, doi:10.1152/ajpheart.00845.2013 (2014).

44 Dabkowski, E. R. *et al.* Diabetic cardiomyopathy-associated dysfunction in spatially distinct mitochondrial subpopulations. *Am.J.Physiol.Heart.Circ.Physiol.* **296**, H359-H369 (2009).

45 Nichols, C. E. *et al.* Cardiac and mitochondrial dysfunction following acute pulmonary exposure to mountaintop removal mining particulate matter. *Am J Physiol Heart Circ Physiol* **309**, H2017-2030, doi:10.1152/ajpheart.00353.2015 (2015).

46 Ryan, M. J., Jackson, J. R., Hao, Y., Leonard, S. S. & Alway, S. E. Inhibition of xanthine oxidase reduces oxidative stress and improves skeletal muscle function in response to electrically stimulated isometric contractions in aged mice. *Free Radic Biol Med* **51**, 38-52, doi:10.1016/j.freeradbiomed.2011.04.002 (2011).

47 Wang, Y., Mohamed, J. S. & Alway, S. E. M-cadherin-inhibited phosphorylation of B-catenin augments differentiation of mouse myoblasts. *Cell Tissue Res* **351**, 183-200 (2013).

48 Suwa, M., Nakano, H., Radak, Z. & Kumagai, S. Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor gamma coactivator-1alpha protein expressions in rat skeletal muscle. *Metabolism* **57**, 986-998 (2008).

49 Nilwik, R. *et al.* The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. *Exp.Gerontol.* **48**, 492-498 (2013).

50 Chalkiadaki, A., Igarashi, M., Nasamu, A. S., Knezevic, J. & Guarente, L. Muscle-specific SIRT1 gain-of-function increases slow-twitch fibers and ameliorates pathophysiology in a mouse model of duchenne muscular dystrophy. *PLoS.Genet.* **10**, e1004490 (2014).

51 Rodgers, J. T., Lerin, C., Gerhart-Hines, Z. & Puigserver, P. Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. *FEBS Lett.* **582**, 46-53 (2008).

52 Sousa-Victor, P. & Munoz-Canoves, P. Regenerative decline of stem cells in sarcopenia. *Mol Aspects Med* **50**, 109-117 (2016).

53 Herbst, A. *et al.* Accumulation of mitochondrial DNA deletion mutations in aged muscle fibers: evidence for a causal role in muscle fiber loss. *J Gerontol.A Biol.Sci.Med.Sci.* **62**, 235-245 (2007).

54 Williamson, C. L. *et al.* Enhanced apoptotic propensity in diabetic cardiac mitochondria: influence of subcellular spatial location. *Am J Physiol Heart Circ Physiol* **298**, H633-H642 (2010).

55 Parihar, M. S. & Brewer, G. J. Simultaneous age-related depolarization of mitochondrial membrane potential and increased mitochondrial reactive oxygen species production correlate with age-related glutamate excitotoxicity in rat hippocampal neurons. *J Neurosci Res* **85**, 1018-1032, doi:10.1002/jnr.21218 (2007).

56 Canto, C. & Auwerx, J. Caloric restriction, SIRT1 and longevity. *Trends Endocrinol Metab* **20**, 325-331, doi:10.1016/j.tem.2009.03.008 (2009).

57 Hughes, D. C. *et al.* Age-related Differences in Dystrophin: Impact on Force Transfer Proteins, Membrane Integrity, and Neuromuscular Junction Stability. *J Gerontol A Biol Sci Med Sci* **72**, 640-648, doi:10.1093/gerona/glw109 (2017).

58 Picca, A. *et al.* Circulating Mitochondrial DNA at the Crossroads of Mitochondrial Dysfunction and Inflammation During Aging and Muscle Wasting Disorders. *Rejuvenation Res* **21**, 350-359, doi:10.1089/rej.2017.1989 (2018).

59 Amat, R. *et al.* SIRT1 controls the transcription of the peroxisome proliferator-activated receptor-gamma Co-activator-1alpha (PGC-1alpha) gene in skeletal muscle through the PGC-1alpha autoregulatory loop and interaction with MyoD. *J Biol Chem* **284**, 21872-21880, doi:10.1074/jbc.M109.022749 (2009).

60 Marzetti, E. *et al.* Role of mitochondrial dysfunction and altered autophagy in cardiovascular aging and disease: from mechanisms to therapeutics. *Am.J Physiol Heart Circ.Physiol* **305**, H459-H476 (2013).

61 Sun, N., Youle, R. J. & Finkel, T. The Mitochondrial Basis of Aging. *Mol Cell* **61**, 654-666, doi:10.1016/j.molcel.2016.01.028 (2016).

62 Seo, D. Y. *et al.* Age-related changes in skeletal muscle mitochondria: the role of exercise. *Integr Med Res* **5**, 182-186, doi:10.1016/j.imr.2016.07.003 (2016).

63 Braidy, N. *et al.* Age related changes in NAD+ metabolism oxidative stress and Sirt1 activity in wistar rats. *PLoS One* **6**, e19194, doi:10.1371/journal.pone.0019194 (2011).

64 Jang, S. Y., Kang, H. T. & Hwang, E. S. Nicotinamide-induced mitophagy: event mediated by high NAD+/NADH ratio and SIRT1 protein activation. *J Biol Chem* **287**, 19304-19314, doi:10.1074/jbc.M112.363747 (2012).

65 Song, S. B. *et al.* Modulation of Mitochondrial Membrane Potential and ROS Generation by Nicotinamide in a Manner Independent of SIRT1 and Mitophagy. *Mol Cells* **40**, 503-514, doi:10.14348/molcells.2017.0081 (2017).

66 Williams, A. B. & Schumacher, B. p53 in the DNA-Damage-Repair Process. *Cold Spring Harb Perspect Med* **6**, doi:10.1101/cshperspect.a026070 (2016).

67 Bartlett, J. D., Close, G. L., Drust, B. & Morton, J. P. The emerging role of p53 in exercise metabolism. *Sports Med* **44**, 303-309, doi:10.1007/s40279-013-0127-9 (2014).

68 Pala, F. *et al.* Distinct metabolic states govern skeletal muscle stem cell fates during prenatal and postnatal myogenesis. *J Cell Sci* **131**, doi:10.1242/jcs.212977 (2018).

69 Siu, P. M. & Alway, S. E. Subcellular responses of p53 and Id2 in fast and slow skeletal muscle in response to stretch-induced overload. *J Appl Physiol (1985)* **99**, 1897-1904, doi:10.1152/japplphysiol.00374.2005 (2005).

70 Porrello, A. *et al.* p53 regulates myogenesis by triggering the differentiation activity of pRb. *J Cell Biol* **151**, 1295-1304 (2000).

71 Alway, S. E., Hughson, R. L., Green, H. J., Patla, A. E. & Frank, J. S. Twitch potentiation after fatiguing exercise in man. *Eur J Appl Physiol* **56**, 461-466 (1987).

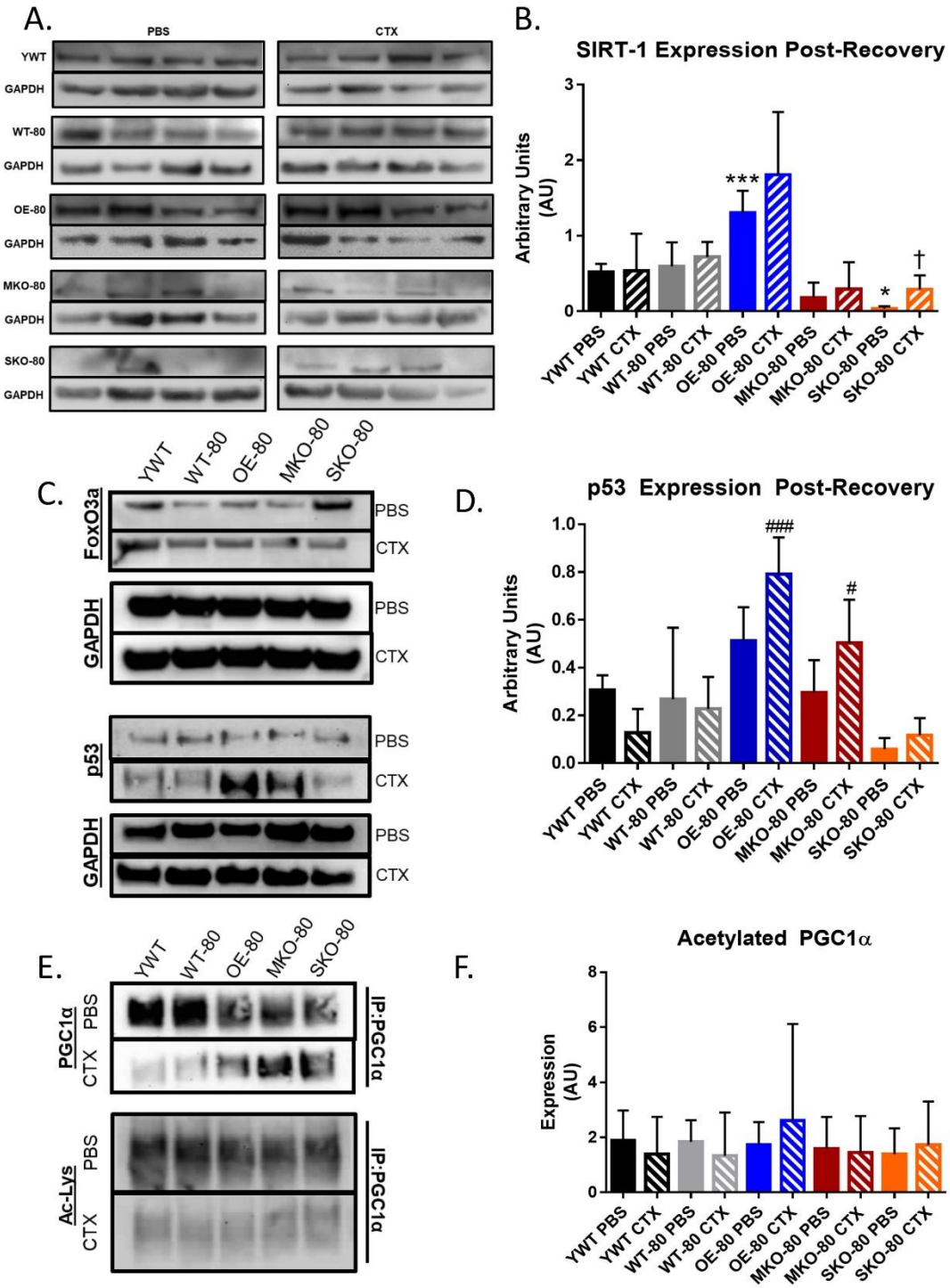
72 Lorenz, D. Postactivation potentiation: an introduction. *Int J Sports Phys Ther* **6**, 234-240 (2011).

73 Siu, P. M. & Alway, S. E. Id2 and p53 participate in apoptosis during unloading-induced muscle atrophy. *Am J Physiol Cell Physiol* **288**, C1058-C1073 (2005).

74 von Haehling, S., Morley, J. E., Coats, A. J. S. & Anker, S. D. Ethical guidelines for publishing in the journal of cachexia, sarcopenia and muscle: update 2017. *J Cachexia Sarcopenia Muscle* **8**, 1081-1083, doi:10.1002/jcsm.12261 (2017).

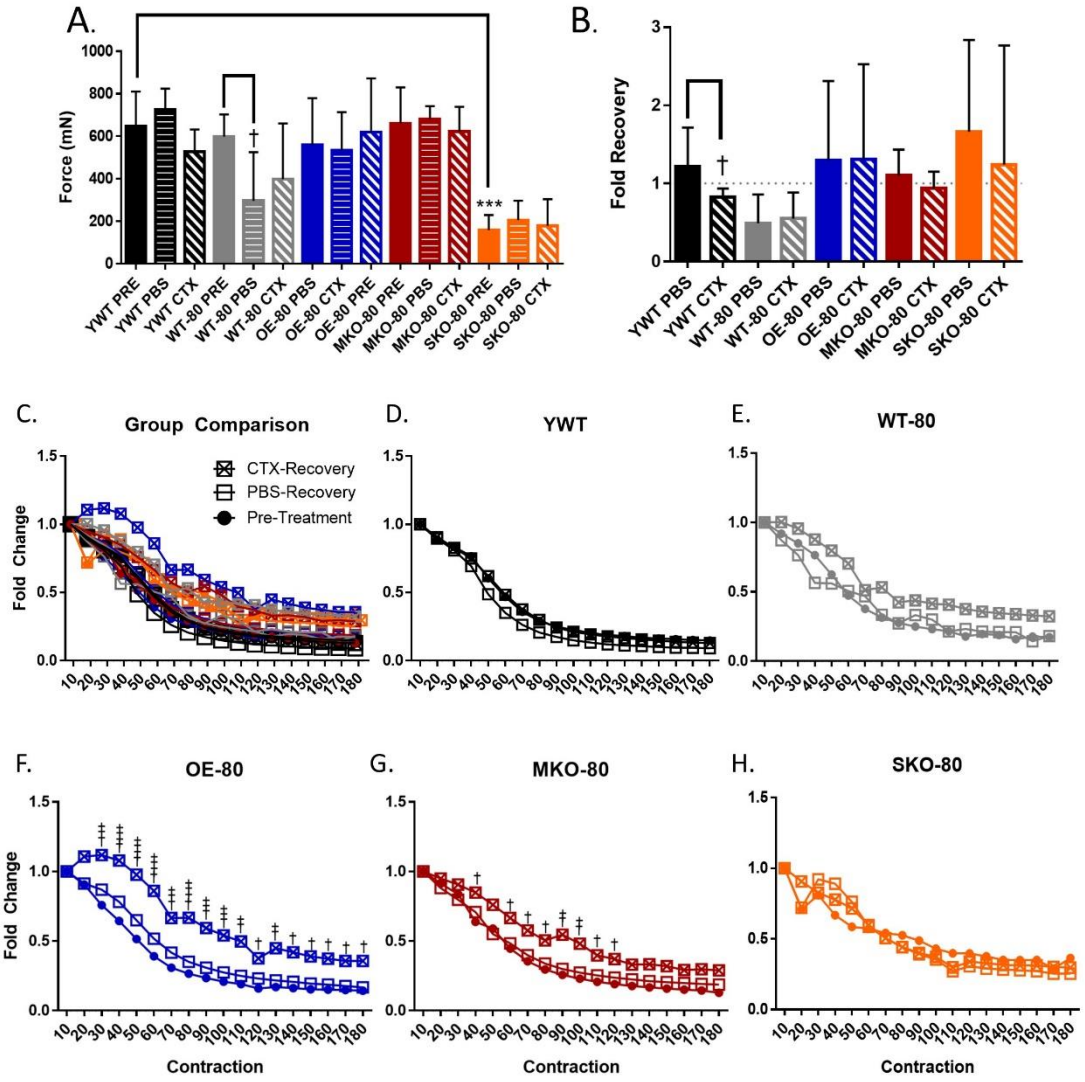
2.9 Figure Legends

2.9.1 Figure 1



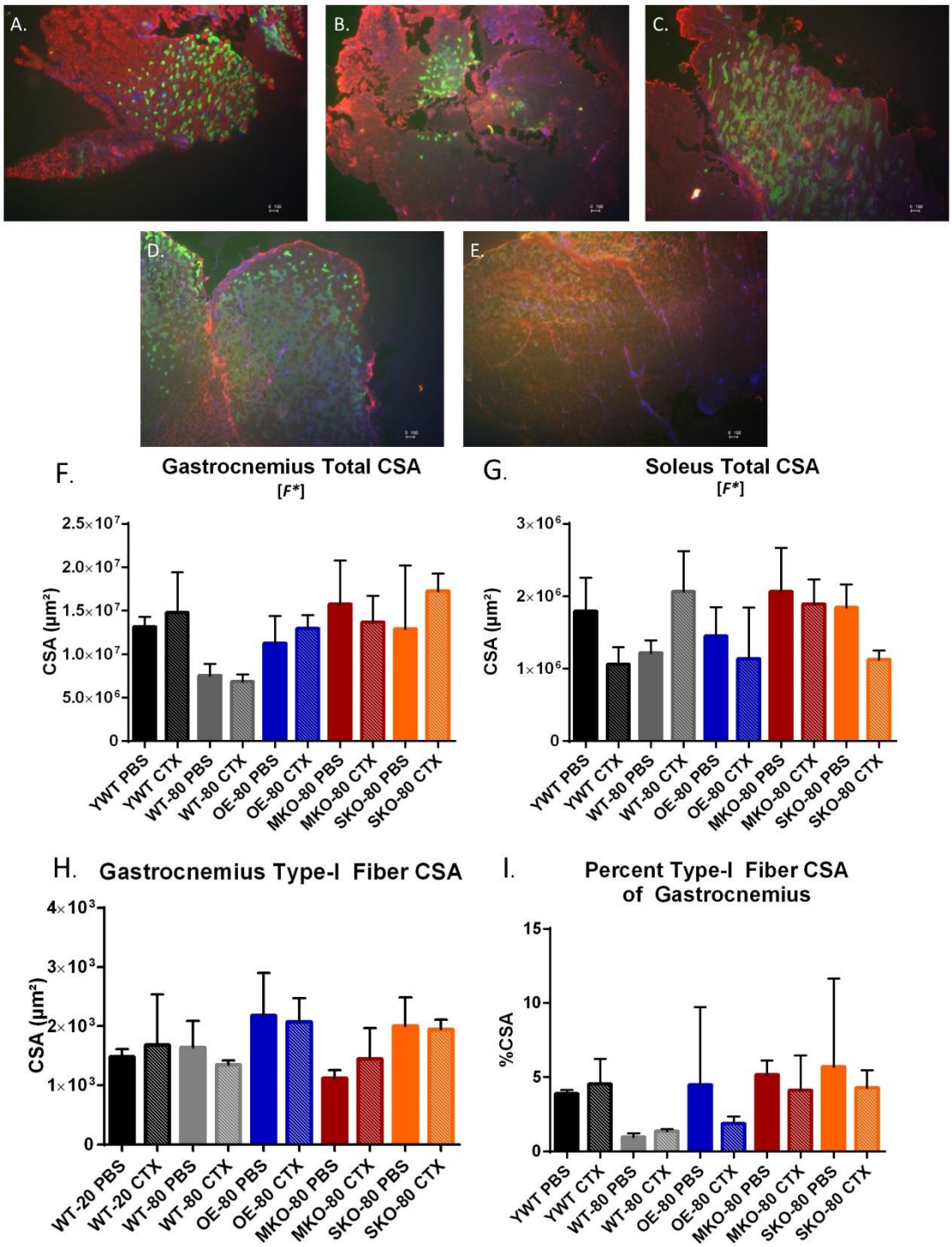
2.9.2 Figure 1. Protein expression in young wild-type and older **variant SIRT1 expressing mice.** (**A**). SIRT1 protein expression collected from 12-week-old wild-type (WT), overexpressor (OE), satellite cell knockout (SKO) and skeletal muscle knockout (MKO) mice prior to and 2 hours after exercise (-ex). (**B**). Densitometric and immunoblot analysis of SIRT1 protein expression measured in young wild-type (YWT, *n*=3) and older (80+ weeks) wild-type (WT-80, *n*=3), SIRT1 overexpressor (OE-80, *n*=3), SIRT1 skeletal muscle knockout (MKO-80, *n*=3), and SIRT1 satellite cell knockout (SKO-80, *n*=3) mice hind limbs 21 days after phosphate buffered saline (PBS) or cardiotoxin (CTX) injection. Basal SKO-80 SIRT1 expression was significantly reduced relative to YWT mice (*P < 0.05), while OE-80 PBS SIRT1 expression was significantly increased (***P < 0.001) relative to YWT PBS. Only SKO-80 mice showed a significant increase in SIRT1 abundance for CTX injected muscles relative to their own PBS injected hind limbs (†P < 0.05). (**C**). Representative immunoblots of SIRT1 downstream targets FoxO3a and p53 and loading control GAPDH for SIRT1 variant expressers after PBS/CTX injection and recovery. Densitometric analysis of the immunoblots revealed no significant differences in protein expression. (**D**). Densitometric analysis of the immunoblots revealed significant differences in the abundance of p53 protein for OE-80 CTX ($^{\#\#\#}$P < 0.001) and MKO-80 CTX ($^{\#}$P < 0.05) groups relative to YWT CTX mice. (**E**). Representative immunoprecipitation immunoblots for SIRT1 downstream target PGC1α and its acetylation state. (**F**). Densitometric analysis of relative PGC1α acetylation. No significant difference was discovered between groups versus the YWT control when using one-way ANOVA with corrections for multiple comparisons.

2.9.3 Figure 2


A.
Force (mN)
1000
800
600
400
200
0
†
***
YWT PRE
YWT PBS
YWT CTX
WT-80 PRE
WT-80 PBS
WT-80 CTX
OE-80 PBS
OE-80 CTX
OE-80 PRE
MKO-80 PRE
MKO-80 PBS
MKO-80 CTX
SKO-80 PRE
SKO-80 PBS
SKO-80 CTX
B.
Fold Recovery
3
2
1
0
†
YWT PBS
YWT CTX
WT-80 PBS
WT-80 CTX
OE-80 PBS
OE-80 CTX
MKO-80 PBS
MKO-80 CTX
SKO-80 PBS
SKO-80 CTX
C.
Group Comparison
CTX-Recovery
PBS-Recovery
Pre-Treatment
Fold Change
D.
YWT
E.
WT-80
F.
OE-80
G.
MKO-80
H.
SKO-80
Contraction

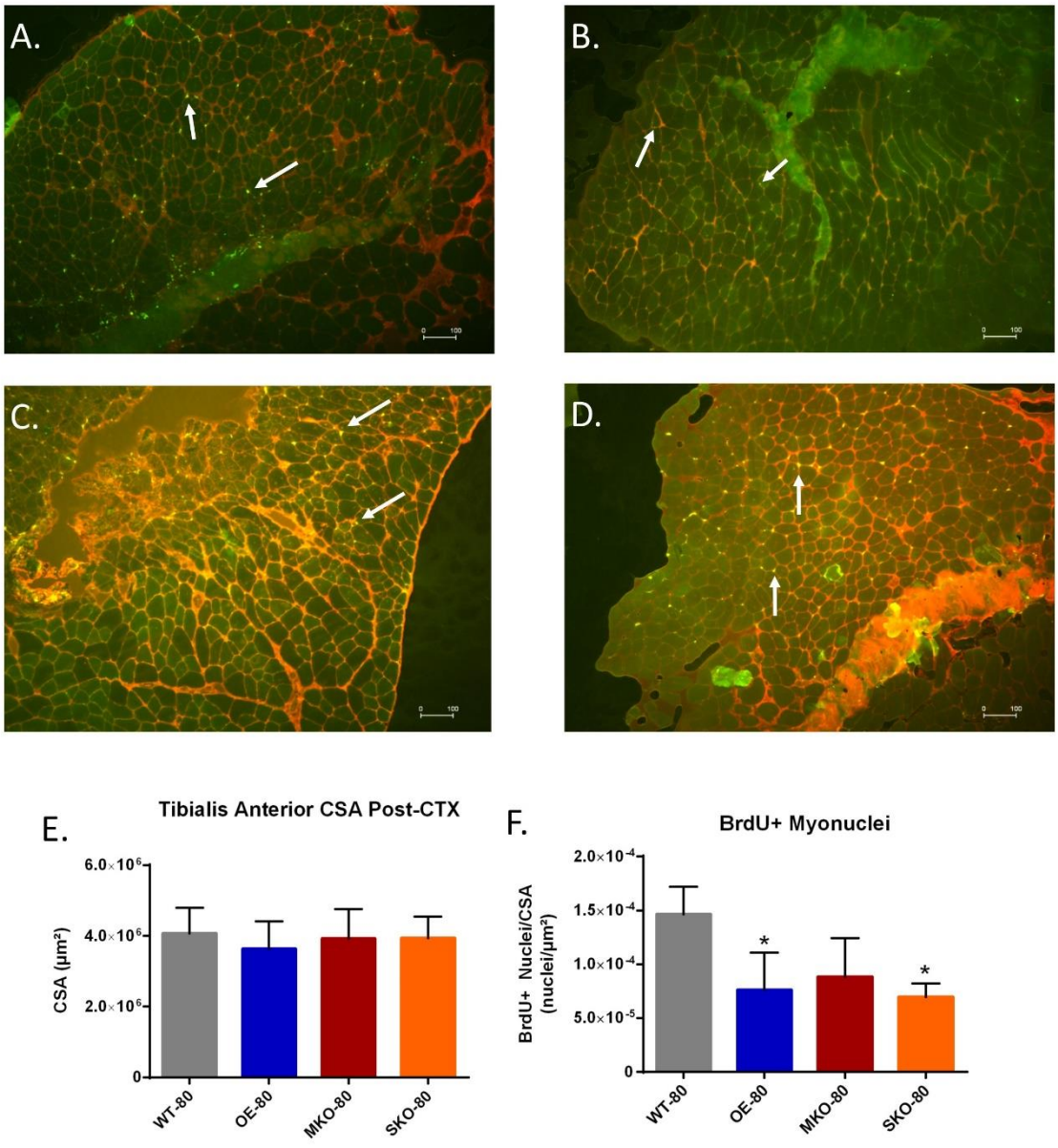
2.9.4 Figure 2. The effects of SIRT1 expression on hindlimb skeletal muscle function in young wild-type and older mice. Young wild-type (YWT, 20-30 weeks, *n*=8) and older (80+ weeks) wild-type (WT-80, *n*=8), SIRT1 overexpressor (OE-80, *n*=9), SIRT1 skeletal muscle knockout (MKO-80, *n*=7), and SIRT1 satellite cell knockout (SKO-80, *n*=4) mice were anesthetized with 2-3% isoflurane and electrically stimulated once at 75Hz (**A, B**) and 180 times at 40Hz (**C-P**) to produce muscle contractions (pre-treatment, PRE) prior to injection with snake cardiotoxin (CTX) and vehicle control (PBS). 21 days post-injection, muscle force was reexamined. Data are presented as the mean force at 75Hz ± SD (***P<0.01; †P < 0.05) (* denotes a significant difference from the YWT control, † denotes a significant difference between a genotype's control and treatment). **B**. Force recovery was measured by normalizing PBS/CTX data to the same limb pre-treatment. Data presented as fold change ± SD. **C.** Comparison of all and individual (**C-H**) fatigue profiles for experimental groups normalized to earliest maximum force. Data are presented as fold change (†P < 0.05, ††P<0.01, †††P<0.01, ††††P<0.001).

2.9.5 Figure 3


A.
B.
C.
D.
E.
F.
Gastrocnemius Total CSA
[F*]
CSA (µm²)
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
YWT PBS
YWT CTX
WT-80 PBS
WT-80 CTX
OE-80 PBS
OE-80 CTX
MKO-80 PBS
MKO-80 CTX
SKO-80 PBS
SKO-80 CTX
G.
Soleus Total CSA
[F*]
CSA (µm²)
3×10⁶
2×10⁶
1×10⁶
0
YWT PBS
YWT CTX
WT-80 PBS
WT-80 CTX
OE-80 PBS
OE-80 CTX
MKO-80 PBS
MKO-80 CTX
SKO-80 PBS
SKO-80 CTX
H.
Gastrocnemius Type-I Fiber CSA
CSA (µm²)
4×10³
3×10³
2×10³
1×10³
0
WT-20 PBS
WT-20 CTX
WT-80 PBS
WT-80 CTX
OE-80 PBS
OE-80 CTX
MKO-80 PBS
MKO-80 CTX
SKO-80 PBS
SKO-80 CTX
I.
Percent Type-I Fiber CSA
of Gastrocnemius
%CSA
15
10
5
0
YWT PBS
YWT CTX
WT-80 PBS
WT-80 CTX
OE-80 PBS
OE-80 CTX
MKO-80 PBS
MKO-80 CTX
SKO-80 PBS
SKO-80 CTX

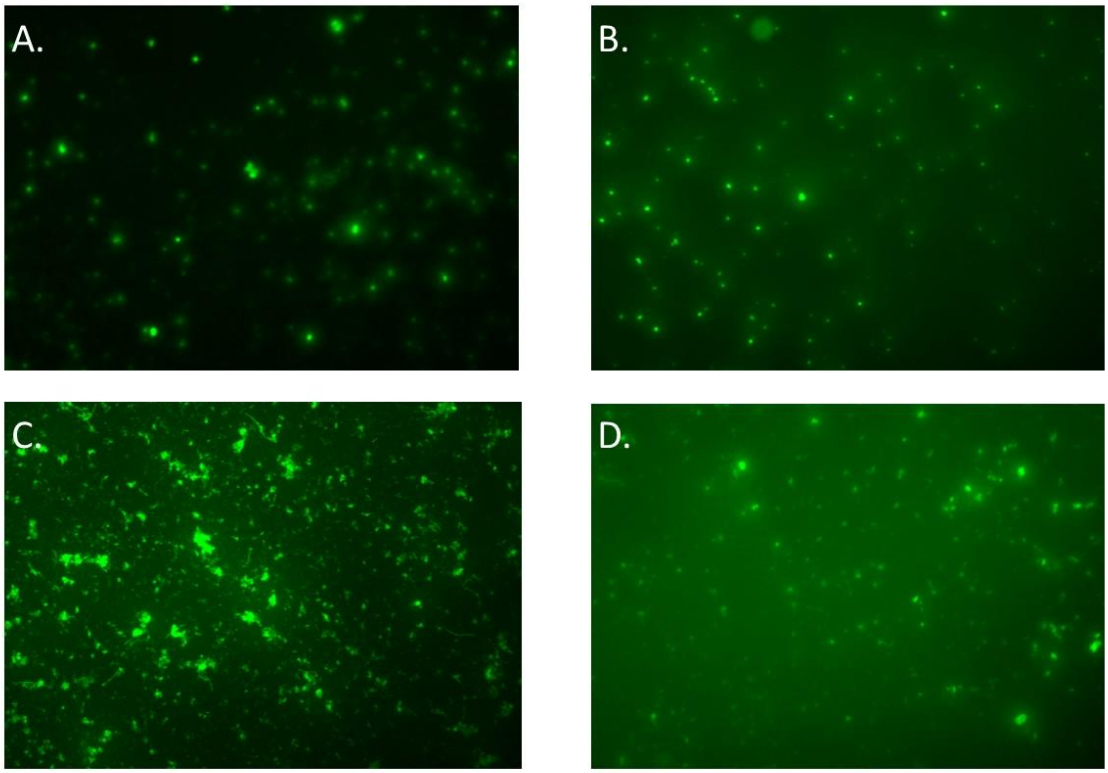
2.9.6 Figure 3. The effects of SIRT1 expression on hindlimb skeletal muscle structure in young wild-type and older **mice (A-E).** Gastrocnemius and soleus muscles for YWT (**A**), WT-80 (**B**), OE-80 (**C**), MKO-80 (**D**), and SKO-80 (**E**), were dissected after physiological evaluation and flash frozen in methyl-butane chilled by liquid nitrogen. Cross sections were cut at 16μm and stained for MHC I (green) or MHC IIa/b and dystrophin (red), then mounted with DAPI hardset (blue). Total cross-sectional area of gastrocnemius cross-sections (**F**), soleus cross sections (**G**), and type I fiber cross sections (**H**), were calculated by fiber tracing with the ImageJ software package on images captured at 4x resolution. Data are presented as mean ± SD compared to YWT PBS, (*n*=3). [*F**] denotes that a significant difference was observed for the global One-way ANOVA tests, but not for specific comparisons. **I.** The gastrocnemius type I percentage was calculated by dividing the summed type I fiber CSA for each section by the total CSA of the respective gastrocnemius (*n*=3). Scale bar = 100 μm.

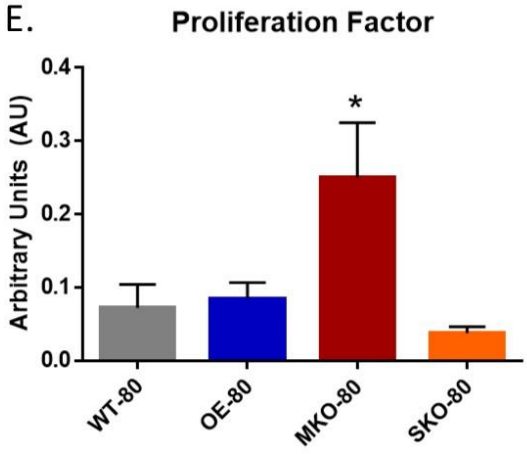
2.9.7 Figure 4


A.
B.
C.
D.
E.
Tibialis Anterior CSA Post-CTX
CSA (µm²)
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
WT-80
OE-80
MKO-80
SKO-80
F.
BrdU+ Myonuclei
BrdU+ Nuclei/CSA (nuclei/µm²)
2.0×10⁻⁴
1.5×10⁻⁴
1.0×10⁻⁴
5.0×10⁻⁵
0
*
*
WT-80
OE-80
MKO-80
SKO-80

2.9.8 Figure 4. The effects of SIRT1 expression on cell replication in older murine skeletal muscle after recovery from injury. A-D. Tibialis anterior (TA) cross-sections were taken for WT-80 (**A**), OE-80 (**B**), MKO-80 (**C**), and SKO-80 (**D**) mice 21 days after CTX injury at 4x resolution. Sections were stained against BrdU (green, indicated by white arrows) and dystrophin (red). **E.** Total CSAs of TA cross-sections were calculated and presented as mean CSA (μm^2) ± SD (*n*=3). **F.** BrdU positive nuclei (**A-D**) were quantified and normalized to their respective TA CSA. Data are presented as mean ± SD (*n*=3); (*P < 0.05). Scale bar = 100 μm

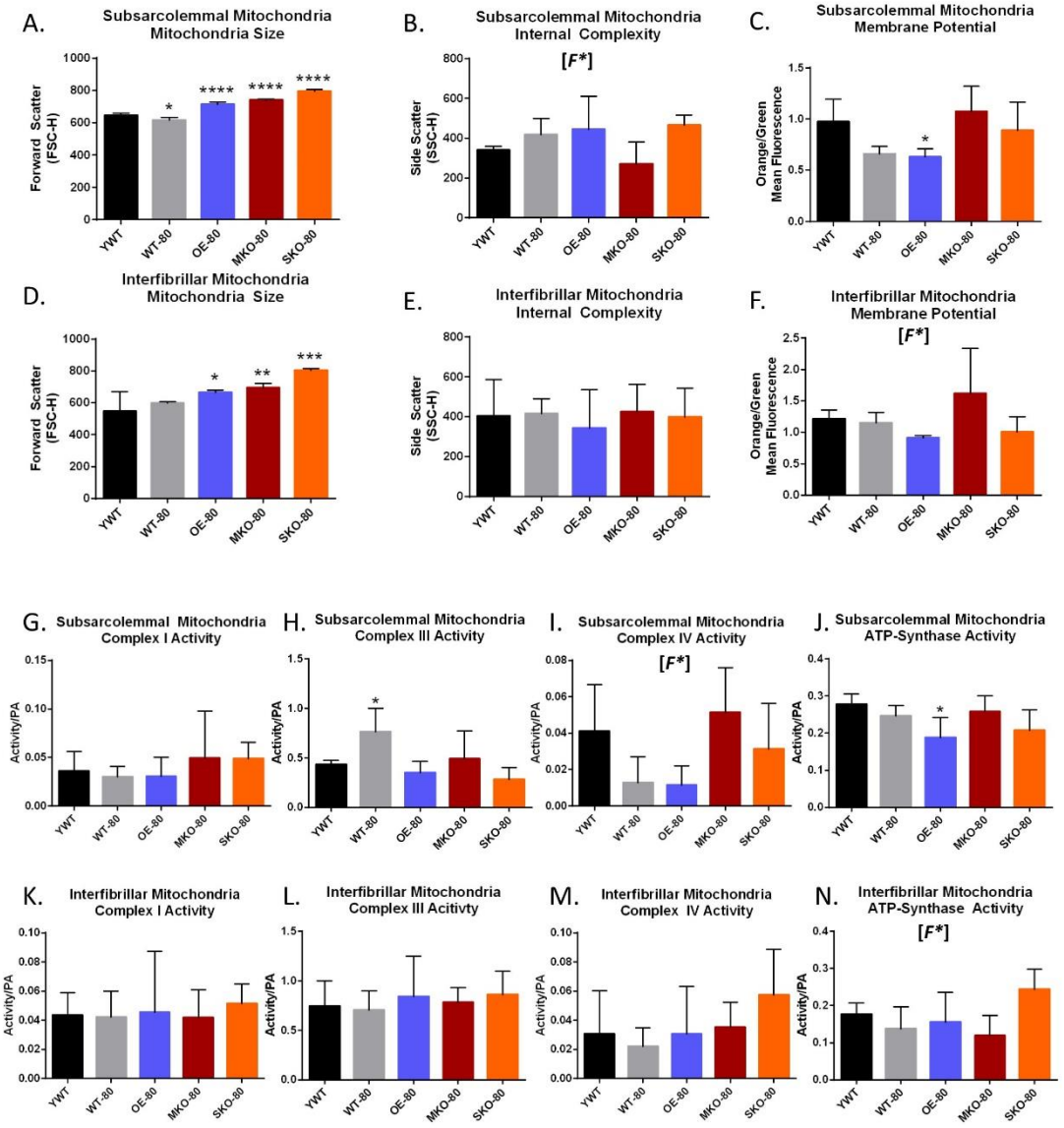
2.9.9 Figure 5


A.
B.
C.
D.


E.
Proliferation Factor
Arbitrary Units (AU)
0.4
0.3
0.2
0.1
0.0
*
WT-80
OE-80
MKO-80
SKO-80

2.9.10 Figure 5. The effects of SIRT1 expression on satellite cell proliferation in older **murine skeletal muscle.** (**A-D**). EdU+ cells were identified from FACS-isolated satellite cell populations from TA muscles of WT-80 (**A**), OE-80 (**B**), MKO-80 (**C**), and SKO-80 (**D**) mice. (**E**)**.** The proliferation factor was determined by the total of EdU+ cells counted from 9 fields, normalized to the total number of cells in the plate. Experiments were repeated in triplicate and data are presented as mean ± SD (*n*=3); (*P < 0.05).

2.9.11 Figure 6



2.9.12 Figure 6. Characteristics of mitochondria in young wild-type and older **SIRT1-expression models of murine skeletal muscle.** Plantar flexor muscle groups (gastrocnemius, soleus, and plantaris) were obtained from YWT and older SIRT1-expressor mice prior to isolation of IFM and SSM subpopulations. Significant differences were found in the sizes of older mitochondria relative to YWT mitochondria for both SSM (**A**) and IFM (**D**). No significant differences were found for specific comparisons of internal complexity in SSM (**B**) or in IFM (**E**). OE-80 showed a significantly lower mitochondrial membrane potential (ΔΨm) than YWT mice mitochondria as reflected in the lower ratio of orange/green mitochondria relative to YWT in SSM (**C**) and IFM (**D**) [*F**]. MKO-80 mice and OE-80 mice show the potential for having inversely affected ΔΨm (**F**). The shift from green to orange occurs as the JC-1 dye forms aggregates upon membrane polarization causing shifts in emitted light from 530 nm (green) to 590 nm (orange). Addition of 200 μM dinitrophenol, which collapses the ΔΨm was used as an assay control. Data are expressed as the mean orange fluorescence divided by the mean green fluorescence of 20,000 mitochondrial events per individual mitochondrial sample. The activity for mitochondrial electron transport chain complexes was calculated for SSM (**G**-**J**) and IFM (**K**-**N**). OWT-80 showed increased complex III activity in SSM (**H**), while OE-80 mice showed decreased ATP synthase activity (**J**). Complex IV activity is depressed in older WT and OE models in SSM, but not SIRT1 knockout models (**I**). Data are presented as mean ± SD (*n*=5). (*P < 0.05, **P < 0.01, ***P < 0.001). [*F**] denotes that a significant difference was observed for the global one-way ANOVA tests, but not for specific comparisons.

Table 1: Characteristics of Study Animals

	YWT	OWT	OE	MKO	SKO
Age (Weeks)	24.8 ± 0.7	83.7 ± 0.5	86.9 ± 1.5	90.1 ± 2.9	84.2 ± 4.2
Mass (g) - Total	28.4 ± 1.4	35.8 ± 2.1	31.7 ± 0.8	34.9 ± 1.5	31.2 ± 1.7
Mass (g) - Males	32.1 ± 1.1*	37.3 ± 2.6	32.7 ± 0.7	34.9 ± 2.0	34.5 ± 2.1
Mass (g) - Female	24.0 ± 0.9*	33.5 ± 3.6	29.6 ± 1.7	34.8 ± 2.5	31.4 ± 2.1
%Male	53.9%	61.6%	66. 7%	58.3%	30%
%Female	46.2%	38.5%	33.3%	41. 7%	70%
n	13	13	14	12	10

Mass of each group was recorded immediately prior to euthanasia. Experimental groups consisted of randomly distributed males and females. Young wild type (YWT) males differed significantly in body mass only from YWT females (*$P < 0.05$). Data are expressed as means ± SD.

Chapter 3:

Discussion

3.1. Summary

The **primary objectives** of this investigation were to (1) elucidate the role of SIRT1 in regulating muscle function and fatigue resistance in regenerating skeletal muscle of aged mice, (2) determine the role of SIRT1 in regulating mitochondrial biogenesis and function in regenerating aging skeletal muscle, and (3) determine if SIRT1 activation can improve proliferation in satellite cells in aging. The **long-term goal** of this study was to determine the physiological relevance of SIRT1 expression in the functionality of aging skeletal muscle and provide insight as to whether SIRT1 is an effective therapeutic target for interventions designed to treat or prevent muscle wasting with age. The **central hypothesis** of this dissertation was that overexpression of the protein SIRT1 would provide protective benefits from functional muscle loss accompanying aging and that SIRT1 expression would act to promote longevity in skeletal muscle by improving mitochondrial health and increasing the proliferation of muscle satellite cell proliferation after injury. Our **rationale** for the proposed research was that SIRT1 has been strongly linked to longevity, that type I skeletal muscle fibers both resist sarcopenic muscle wasting with age and highly express the protein SIRT1, and that SIRT1 is a known up-stream regulator of mitochondria—whose dysfunction has been long linked to aging [1,2]—through the PGC-1α axis [3].

In this dissertation, we demonstrated that in aged, non-sarcopenic mice, hindlimb muscle function is not affected by the abundance of SIRT1 in adult skeletal muscle, although major reductions in contraction force can be seen if SIRT1 is absent from muscle satellite cells in aged mice. Young, wild-type mice showed impaired strength recovery after recovery from CTX-induced injury, though this difference was not seen in aged mice of any genotype. (This finding may be explained in part by the [F*] seen in our data for the CSA of the YWT mice—although we did not show significant differences, the CSA of YWT mice post-CTX recovery indicated that the soleus muscles of young mice did not recover to their full size. Secondly, because it was difficult to detect

significant reductions in muscle cross sectional area for the aged models, the older mice may have possessed enough increased oxidative stress in their muscles associated with aging to promote further muscle repair while not possessing enough stress to impair muscle functionality.) Neither the presence nor absence of SIRT1 affected fatigue resistance in the hindlimbs of un-injured, aged mice. Interestingly, both the muscle knockout and overexpressor models of SIRT1 showed improved fatigue resistance relative to baseline fatigability, with the SIRT1 overexpressor model's force profile suggesting the muscles underwent postactivation potentiation—an increase in muscle force during low frequency activation (for example, during endurance exercise) following a conditioning activity, such that a previous muscle contractions improves the following contraction [4].

Next, we showed that very few differences occurred in the mitochondria between different genotypical models of SIRT1 expression in aged mice compared to the young wild-type controls. Although mitochondria tended to be significantly larger in aged mouse models relative to the young, wild-type controls, the models showed very little variability in number, internal complexity, mitochondrial membrane potential, or mitochondrial complex activity. Although this data was surprising based on our initial expectations, the accrual of recent literature has begun to suggest that the intercellular levels of [NAD+] and its enzymatic regulators are more important to the pro-longevity functions associated with SIRT1 than actual SIRT1 abundance [5–7]. Also surprising was that despite expecting to find an increase in mitochondrial complex activity, mitochondrial membrane potential, and mitochondrial population in the mitochondria from our aged overexpressor model, our data suggested that $\Delta\Psi m$ and the activity of complex IV and ATP-synthase were instead blunted with the overexpression of SIRT1, a visible difference and trend relative to the other transgenic models we investigated.

Finally, we showed that the loss of SIRT1 in adult skeletal muscle, rather than the overexpression of SIRT1, improved early proliferation of satellite cells, although this effect appeared to be attenuated somewhat later in the process of regeneration.

Overall, the importance of SIRT1 in the skeletal muscle of aged mice is largely dependent on its abundance in muscle satellite cells, rather than in adult skeletal muscle. Furthermore, even the large boost in satellite cell proliferation seen in the skeletal muscle knockout model of SIRT1 appears to have very little effect on muscle function. Surprisingly, it seems that a disruption in SIRT1 activity in aged skeletal muscle improves the fatigue resistance of the muscle after recovery from injury, regardless of whether the muscle has an increase or loss of function, indicating that SIRT1 may be a secondary or tertiary regulator for muscle function after injury. Interestingly, the injury-recovery models that showed an improvement in their fatigabilities also showed an increased abundance of the protein p53, a downstream target of SIRT1. Thus, although our current evidence indicates altering SIRT1 abundance should not be a primary focus for interventions against muscle loss associated with aging, the investigation of SIRT1's relationship with p53 in the process of muscle repair is an important next step in understanding the aging of skeletal muscle.

3.2. General Discussion

Despite the many studies examining the expression of SIRT1 in various models of aging [8], few have focused on the translational effects of SIRT1 expression in muscle to functional relevance [9]. Our data show interesting and novel insights regarding SIRT1's effect on hindlimb muscle function in aged and injured mouse models. However, some data seemingly conflict with previous studies, especially in the context of muscle fiber structure and composition. Earlier studies from the Guarente lab have shown that overexpression of SIRT1 could rescue mice from the phenotype of Duchenne's muscular dystrophy and shift the fiber-type distribution toward oxidative fibers [10]. These findings were strengthened by studies showing that overexpression

of PGC-1α, a downstream target of SIRT1 regulation, and resveratrol, a known activator of SIRT1 [11], produced a similar rescue of dystrophic function and fiber-type shift [12,13]. These conflicts may arise from the degree of dysfunction in the dystrophic phenotype, as SIRT1 transgenic mice have also been shown to have distinctly similar muscle characteristics compared to wild-type mice in several studies, including size and weight [7,10,14]. Furthermore, the loss of dystrophin has been shown to affect the structural integrity of satellite cells during regeneration by dysregulating scaffold formation, a contributing phenotypical difference not present in the non-dystrophic C57BL/6J background mice [15].

Although our study only takes a shallow look into the make-up of the gastrocnemius, soleus, and tibialis anterior muscles of the mouse hindlimb, we have shown no significant difference in muscle fiber cross sectional area or fiber-type composition between the SIRT1 models. One explanation for this mismatch is the power of our statistical analysis. Analysis of the cross-sectional areas (CSAs) of fiber types revealed significant differences did exist between groups when assessed by two-way ANOVA with corrections for multiple comparisons against the YWT control, but significance could not be established between specific groups, likely due to a masking effect, as indicated by the large effect sizes calculated for the Cohen's d statistic. Although it is possible to infer from the data that the CSA of gastrocnemius muscles is decreased in aged wild-type mice, but is possibly rescued by altering the expression of SIRT1, these results cannot be confirmed by our current data. However, the total cross-sectional area attributed to type I fibers in the gastrocnemius muscles does stay consistent among the models, indicating that altering SIRT1 expression levels alone does not seem to influence the relative make-up of mixed-fiber muscles.

Along with the small differences detected in the muscle structure, we also reported few differences between the majority of the transgenic models in respect to mitochondrial complexity, population, and complex activity. These findings suggest that SIRT1's role in promoting longevity

or a caloric-restriction phenotype likely requires very little expression of the protein itself, and is more dependent on an outside activator. Recent literature has indicated that SIRT1 overexpression models show significantly different genetic profiles compared to caloric restriction or exercise trained mice, and possess different mitochondrial functionality compared to such models, characterized by lower rates of mitochondrial respiration and lower glucose clearance [7]. These findings are supported by studies showing the vast effectiveness of supplementation of NAD+ conjugates, such as nicotinamide riboside, in promoting cellular longevity [5] and pro-longevity factors, such as increased mitophagy, lower blood glucose levels, and muscle stem cell population [16,17]

In other aspects, however, our data reflect the findings in studies using similar models; for example, literature has reported that the muscle creatine kinase-knockout model of SIRT1 (MKO) shows that loss of SIRT1 activity does not affect oxidative metabolism or myofiber composition in skeletal muscle [10]. Furthermore, their MKO model also showed no difference from WT mice in fatigability under more mild exercise training. An important caveat is that dystrophic mice suffer from structural weakness due to a dysfunctional dystrophin protein; furthermore, affected muscle in these cases are susceptible to contraction-induced rupture of the sarcolemma [12] and often present many fibers with centrally-located nuclei [10], a phenotypical sign of muscle damage. Our data may shed some light on how SIRT1 overexpression rescues muscle function, as our work has shown that after injury, in models where the protein p53 is significantly increased in recovered muscle, resistance to fatigue is improved; moreover, co-expression of the SIRT1 overexpressed protein and p53 show signs of muscle potentiation, improving the force output for fatigue contractions in the early stages of the exercise protocol.

The protein p53 is a highly studied gene, important to cancer research, that encodes a transcription-regulating protein with wide effects on cellular biology, most notably its ability to promote cell cycle arrest and apoptosis [18]. The ability of p53 to induce cell cycle arrest may also

function as a stop-gap in which p53 can act to promote DNA damage repair, as recent evidence continues to link the protein to several DNA-repair pathways [19]. Curiously, our data indicates that p53 shows an increase in abundance in both the MKO and OE aged models after injury, hinting that SIRT1 dysregulation may lead to increased protein expression of p53 through multiple regulatory pathways or by altogether different means. A possible explanation for the MKO model is that in the absence of SIRT1, which promotes the mitochondrial regulatory activity of PGC-1α including stimulating biogenesis and upregulating the antioxidant proteins that manage oxidative phosphorylation, CTX-induced muscle damage coupled with more overall oxidative stress (from the lower abundance of antioxidants) could cause the stimulation of p53 transcription/translation to rise as a compensatory mechanism. Because a loss of SIRT1 would also reduce inhibition of the SIRT1 target NF-κB, an inducer of the inflammatory response and inhibitor of p53, increased NF-κB could be potentially inhibiting the apoptotic-functions of p53 [20,21]. Thus, only the DNA repair pathways mediated by p53 after CTX-injury might be activated and could therefore account for the improved fatigue resistance in MKO mice. Alternatively, our data indicate that the loss of SIRT1 abundance in adult skeletal muscle promotes early proliferation of satellite cells as determined by EdU staining *in vitro*, with an apparent attenuation of activity after 21 days as determined by BrdU staining *in vivo.* These assays also indicate the attenuation of satellite cell proliferation in the OE-80 and SKO-80 models relative to proliferation in WT-80 mice, which strongly suggests the upregulation of SIRT1 during the process of skeletal muscle differentiation inhibits satellite cell proliferation. This data is supported by Ryall et al. [22], who showed that SIRT1 protein expression correlating with the oxidative metabolic state of quiescent satellite cells acts to inhibit their activation—whereas downregulation of SIRT1 in satellite cells promotes a metabolic switch to glycolysis and subsequent activation of these cells. Thus, the down-regulation of SIRT1 prior to satellite cell activation and up-regulation of SIRT1 during differentiation may be part of a metabolic signal within the muscle that controls satellite cell activity. This would indicate a pathway possibly independent of p53, wherein the MKO-80 model's improved muscle

performance after hindlimb injury and repair stems from improved muscle satellite cell proliferation.

Conversely in the OE models, p53 recruited after injury may be more effectively enhanced by SIRT1 overexpression, which acts to directly bind and deacetylate p53 to inhibit its apoptotic functions by blockings its translocation to the nucleus [22]. Blocking p53's access to the nucleus may therefore promote translocation of p53 to mitochondria, where it can act to form mitochondrial DNA complexes [23]. Because previous studies have shown that p53 expression can improve endurance exercise capacity [24] it may likely be through this mechanism that p53 upregulation correlates with improved endurance exercise capability in the OE model after injury. Research published by Zhao et al. have shown that prior to apoptosis, p53 translocates to the mitochondria where it targets the manganese-dependent superoxide dismutase (MnSOD) [25]. Subsequently, the loss of MnSOD's superoxide scavenging activity results in an increase of oxidative stress in the mitochondria was associated with a drop in $\Delta\Psi m$. These findings reflect the data we reported for the mitochondrial membrane potential and complex activities, wherein the $\Delta\Psi m$ and activities were similar for most of the SIRT1 transgenic models, except for the OE-80 model which showed a trend for decreased $\Delta\Psi m$ and complex IV and ATP synthase activity. Furthermore, our data suggest a masked significant decrease for the mitochondrial abundance of the OE-80 model relative to the control model (**Fig. 1**). Taken together, these findings indicate that increased SIRT1 abundance from overexpression may be interacting with increased levels of p53 stimulated by muscle injury to redirect p53 from the nucleus to the mitochondria. In cases of cellular stress, SIRT1 has been shown to promote cellular protection by inhibiting the apoptotic capabilities of the FOXO proteins, which share somewhat similar functions with p53, while improving their DNA repair capability in cells chemically stressed by H_2O_2 [26]. This process likely results from partial deacetylation (and therefore, only partial deactivation) of the FOXO/p53 transcription factors; indeed, p53 is known to be a target of both the SIRT1 and PID/HDAC1 deacetylases [22] that can

alter its translocation targets. This possibility is supported by recent literature reporting that p53 localization to the mitochondria helps to adapt the mitochondria to endurance exercise training, an effect which is attenuated in muscle knockout models of SIRT1 [14]. Furthermore, these models indicated p53 possesses some sort of regulatory function for mitochondrial autophagy—in the absence of p53, autophagy signaling was increased, but clearance of autophagosomes was impaired. Taken altogether, a possible explanation for our data is that the abundance of SIRT1 in the aged overexpressor model interacts with p53 after muscle injury to promote mitochondrial health by inducing translocation of p53 to the mitochondria, where it promotes mitochondrial housekeeping functions.

3.3. Future Directions

Although the idea behind this mechanism comes from several established studies, further investigation is required to test and verify the veracity of the beneficial effects of SIRT1 and p53 co-expression in skeletal muscle. Future studies could focus on models with alternate levels of p53 expression, much like the various SIRT1 mouse models employed in this dissertation. Alternatively, experiments focusing on the overexpression of SIRT1 and p53 both independently and under co-expression in mouse models treated with exercise and/or injury would be a first step in deriving a mechanism by which these proteins interact.

Another point to expand upon for future studies is examining how metabolic interventions affect SIRT1's and/or p53's roles in muscle repair after injury. Caloric restriction has long been linked to SIRT1 and is often employed as an activator of SIRT1 activity [8]. It is possibly that the muscle structure for our SIRT1 OE mice did not show significant differences from WT mice because the SIRT1 was not entirely activated, despite being overexpressed. Our initial experimental design included a component to include caloric intake as a variable, but budgetary and physical restraints related to the housing cost of animals and the ability to accurately assess obese mouse hindlimb function (obese mice were too large to fit in our dynamometer set-up and

thus their muscle function could not be accurately assessed) prohibited the inclusion of these variables. Thus, examining the physiological effects of caloric restriction or diet-induced obesity on the muscle function in these variant SIRT1 expressing mouse models could further elucidate the role of SIRT1 in muscle function. These data could prove interesting, given that SIRT1 overexpression appeared to work synergistically with p53 expression to promote increased fatigue resistance in muscle recovered from CTX-induced injury despite the absence of a secondary intervention designed to activate SIRT1 activity.

Finally, another set of future experiments could focus on understanding the role of SIRT1 in muscle fiber development during the formation of new fibers from muscle satellite cells. Although a paper published from the Sartorelli lab by Ryall et al [27] suggest that the lack of SIRT1 in early muscle satellite cells causes premature differentiation of the satellite cells into dysfunctional and deformed muscle fibers, some discrepancies within the model still exist. Specifically, both our data and data published by the Ryall study seem to indicate that in the Pax7-promoter driven muscle satellite cell knockout of SIRT1, SIRT1 expression is still ablated in adult skeletal muscle. However, Pax7 is only expressed in quiescent satellite cells, so SIRT1 should theoretically be expressed at or near the level of SIRT1 in of wild-type mice in skeletal muscle where the Pax7 promoter is no longer active. Establishing the role of SIRT1 and its importance in the various steps of the metabolic regulation of muscle satellite cells during proliferation and/or differentiation could further elucidate the results seen in our data and help to establish a timeline for the importance of SIRT1 expression in the regulation of satellite cell differentiation and nascent fiber formation. These studies could be further supported with a complementary genetic conditional knockout model combining the MKO-cre and Pax7-cre mice (crossed with SIRT1-floxed mice) to create a SIRT1 knockout model with continuous ablation of SIRT1 from the satellite cell to the fully formed muscle fiber. This proposed study would be important in determining where

SIRT1 shifts from being necessary for normal function to being non-critical, but beneficial to muscle repair.

3.4 References

1. 1. Calvani R, Joseph A-M, Adhihetty PJ, Miccheli A, Bossola M, Leeuwenburgh C, et al. Mitochondrial pathways in sarcopenia of aging and disuse muscle atrophy. Biol Chem. 2013;394: 393–414. doi:10.1515/hsz-2012-0247

2. Carter HN, Chen CCW, Hood DA. Mitochondria, Muscle Health, and Exercise with Advancing Age. Physiology. 2015;30: 208–223. doi:10.1152/physiol.00039.2014

3. Cantó C, Auwerx J. PGC-1alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure. Curr Opin Lipidol. 2009;20: 98–105. doi:10.1097/MOL.0b013e328328d0a4

4. Lorenz D. POSTACTIVATION POTENTIATION: AN INTRODUCTION. Int J Sports Phys Ther. 2011;6: 234–240.

5. Ho C, van der Veer E, Akawi O, Pickering JG. SIRT1 markedly extends replicative lifespan if the NAD+ salvage pathway is enhanced. FEBS Letters. 2009;583: 3081–3085. doi:10.1016/j.febslet.2009.08.031

6. Cerutti R, Pirinen E, Lamperti C, Marchet S, Sauve AA, Li W, et al. NAD+-Dependent Activation of Sirt1 Corrects the Phenotype in a Mouse Model of Mitochondrial Disease. Cell Metabolism. 2014;19: 1042–1049. doi:10.1016/j.cmet.2014.04.001

7. Boutant M, Kulkarni SS, Joffraud M, Raymond F, Métairon S, Descombes P, et al. SIRT1 Gain of Function Does Not Mimic or Enhance the Adaptations to Intermittent Fasting. Cell Reports. 2016;14: 2068–2075. doi:10.1016/j.celrep.2016.02.007

8. Cantó C, Auwerx J. Caloric restriction, SIRT1 and longevity. Trends in Endocrinology & Metabolism. 2009;20: 325–331. doi:10.1016/j.tem.2009.03.008

9. Pardo PS, Boriek AM. The physiological roles of Sirt1 in skeletal muscle. Aging (Albany NY). 2011;3: 430–437.

10. Chalkiadaki A, Igarashi M, Nasamu AS, Knezevic J, Guarente L. Muscle-Specific SIRT1 Gain-of-Function Increases Slow-Twitch Fibers and Ameliorates Pathophysiology in a Mouse Model of Duchenne Muscular Dystrophy. PLOS Genetics. 2014;10: e1004490. doi:10.1371/journal.pgen.1004490

11. Ghosh S, Liu B, Zhou Z. Resveratrol activates SIRT1 in a Lamin A-dependent manner. Cell Cycle. 2013;12: 872–876. doi:10.4161/cc.24061

12. Selsby JT, Morine KJ, Pendrak K, Barton ER, Sweeney HL. Rescue of Dystrophic Skeletal Muscle by PGC-1α Involves a Fast to Slow Fiber Type Shift in the mdx Mouse. PLOS ONE. 2012;7: e30063. doi:10.1371/journal.pone.0030063

13. Hori YS, Kuno A, Hosoda R, Tanno M, Miura T, Shimamoto K, et al. Resveratrol Ameliorates Muscular Pathology in the Dystrophic mdx Mouse, a Model for Duchenne Muscular Dystrophy. J Pharmacol Exp Ther. 2011;338: 784–794. doi:10.1124/jpet.111.183210

14. Beyfuss K, Erlich AT, Triolo M, Hood DA. The Role of p53 in Determining Mitochondrial Adaptations to Endurance Training in Skeletal Muscle. Sci Rep. 2018;8. doi:10.1038/s41598-018-32887-0

15. Chang NC, Chevalier FP, Rudnicki MA. Satellite Cells in Muscular Dystrophy - Lost in Polarity. Trends Mol Med. 2016;22: 479–496. doi:10.1016/j.molmed.2016.04.002

16. Cantó C, Houtkooper RH, Pirinen E, Youn DY, Oosterveer MH, Cen Y, et al. The NAD+ precursor nicotinamide riboside enhances oxidative metabolism and protects against high-fat diet induced obesity. Cell Metab. 2012;15: 838–847. doi:10.1016/j.cmet.2012.04.022

17. Zhang H, Ryu D, Wu Y, Gariani K, Wang X, Luan P, et al. NAD+ repletion improves mitochondrial and stem cell function and enhances life span in mice. Science. 2016;352: 1436–1443. doi:10.1126/science.aaf2693

18. Kastenhuber ER, Lowe SW. Putting p53 in Context. Cell. 2017;170: 1062–1078. doi:10.1016/j.cell.2017.08.028

19. Williams AB, Schumacher B. p53 in the DNA-Damage-Repair Process. Cold Spring Harb Perspect Med. 2016;6. doi:10.1101/cshperspect.a026070

20. Salminen A, Kaarniranta K, Kauppinen A. Crosstalk between Oxidative Stress and SIRT1: Impact on the Aging Process. Int J Mol Sci. 2013;14: 3834–3859. doi:10.3390/ijms14023834

21. Webster GA, Perkins ND. Transcriptional Cross Talk between NF-κB and p53. Mol Cell Biol. 1999;19: 3485–3495.

22. Yi J, Luo J. SIRT1 and p53, effect on cancer, senescence and beyond. Biochimica et biophysica acta. 2010;1804: 1684. doi:10.1016/j.bbapap.2010.05.002

23. Saleem A, Hood DA. Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53–Tfam–mitochondrial DNA complex in skeletal muscle. J Physiol. 2013;591: 3625–3636. doi:10.1113/jphysiol.2013.252791

24. Park J-Y, Wang P, Matsumoto T, Sung HJ, Ma W, Choi JW, et al. p53 Improves Aerobic Exercise Capacity and Augments Skeletal Muscle Mitochondrial DNA Content. Circ Res. 2009;105: 705–712. doi:10.1161/CIRCRESAHA.109.205310

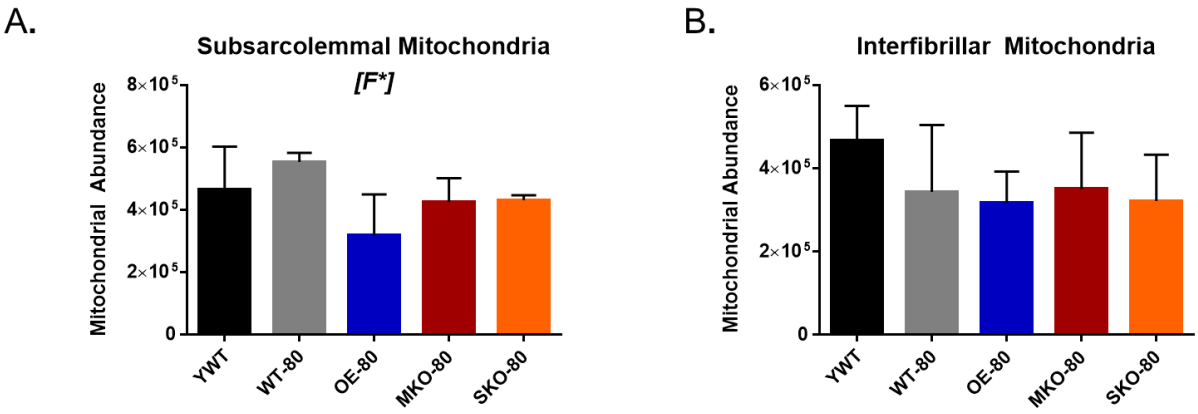
25. Zhao Y, Chaiswing L, Velez JM, Batinic-Haberle I, Colburn NH, Oberley TD, et al. p53 Translocation to Mitochondria Precedes Its Nuclear Translocation and Targets Mitochondrial Oxidative Defense Protein-Manganese Superoxide Dismutase. Cancer Res. 2005;65: 3745–3750. doi:10.1158/0008-5472.CAN-04-3835

26. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, et al. Stress-Dependent Regulation of FOXO Transcription Factors by the SIRT1 Deacetylase. Science. 2004;303: 2011–2015. doi:10.1126/science.1094637

27. Ryall JG, Dell'Orso S, Derfoul A, Juan A, Zare H, Feng X, et al. The NAD+-Dependent SIRT1 Deacetylase Translates a Metabolic Switch into Regulatory Epigenetics in Skeletal Muscle Stem Cells. Cell Stem Cell. 2015;16: 171–183. doi:10.1016/j.stem.2014.12.004

3.5 Figures & Figure Legends

3.5.1. Figure 1.



3.5.2. Figure 1. Mitochondrial abundance among SIRT1 transgenic mouse models. Mitochondria were isolated and then quantified with a FACSCalibur flow cytometer equipped with a 15-MW 488nm argon laser and 633nm red diode laser using 488 nm excitation with 530 nm and 590 nm bandpass emission filters to selectively probe and gate for respiring mitochondria, identified by JC-1 dye. The abundance was quantified as the mean ± SD number of events for the YWT, WT-80, OE-80, MKO-80, and SKO-80 transgenic mouse groups (*n*=5) for (**A**.) the subsarcolemmal and (**B.**) interfibrillar mitochondrial populations. [*F**] denotes that a significant difference was observed for the global two-way ANOVA tests, but not for specific comparisons.

Curriculum Vitae

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Education & Employment

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Oliveira JRS, Mohamed JS, **Myers MJ**, Brooks MJ, Alway SE. Effects of hindlimb suspension and reloading on gastrocnemius and soleus muscle mass and function in geriatric mice. Exp Gerontol. 2019 Jan;115:19-31.

Alway SE, Mohamed JS, **Myers MJ**. Mitochondria initiate and regulate sarcopenia. Exerc Sport Sci Rev. 2017 Apr; 45(2):58-69.

Alway SE, **Myers MJ**, Mohamed JS. Regulation of satellite cell function in sarcopenia. Front Aging Neurosci. 2014 Sep 22;6:246.

Mohamed JS, Wilson JC, **Myers MJ**, Sisson KJ, Alway SE. Dysregulation of SIRT-1 in aging mice increases skeletal muscle fatigue by a PARP-1 dependent mechanism. Aging (Albany NY). 2014 Oct;6(10):820-34.