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DETERMINING THE ROLE OF SATELLITE CELLS DURING SKELETAL MUSCLE ADAPTATION

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DETERMINING THE ROLE OF SATELLITE CELLS DURING SKELETAL
MUSCLE ADAPTATION

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DISSERTATION
_____

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Health Sciences
at the University of Kentucky

By

Davis Alan Englund

Lexington, Kentucky

Co-Directors: Dr. Charlotte A. Peterson, Professor of Physical Therapy

and   Dr. Esther Dupont-Versteegden, Professor of Physical Therapy

Lexington, Kentucky

2020

ABSTRACT OF DISSERTATION

DETERMINING THE ROLE OF SATELLITE CELLS DURING SKELETAL
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Physical inactivity, advancing age, limb immobilization, degenerative diseases and
various systemic diseases (many cancers, sepsis, HIV, COPD, kidney disease) all lead to
skeletal muscle wasting. The loss of muscle mass is of major clinical importance because
it leads to an increased risk for morbidity, disability, and the loss of independence;
collectively contributing to a substantive increase in healthcare utilization and cost. The
prevalence of cachexia (disease-induced muscle wasting) can reach as high as 80% in
certain patient populations and the average cost per hospital stay is $4,641 more than in
non-cachectic patients. Direct healthcare costs attributable to sarcopenia were estimated
to be $18.5 billion in 2002. Therefore, developing effective skeletal muscle restoration
therapies is critical to improve quality of life and prolong functional independence in the
broad patient population afflicted by the loss of muscle mass.

There are few promising strategies emerging to treat loss of muscle mass. Currently,
exercise (e.g., resistance training) is the most effective lifestyle intervention used to
promote muscle growth, and testosterone is a well-known pharmacological intervention
used to increase muscle mass. A greater understanding of the underlying cellular
mechanisms that promote muscle growth and adaptation in response to exercise and
pharmacological agents will enable the design of more targeted and effective
interventions. Moreover, identifying the mechanisms that regulate muscle growth
provides an opportunity to discover novel therapeutic targets for those who cannot or are
unwilling to participate in exercise. In particular, a fundamental understanding of the role
of muscle stem cells (satellite cells) during regeneration and recovery from volumetric
muscle loss made targeting and improving satellite cell function and restorative capacity
during these conditions possible. Gaining an in depth understanding for the function of
satellite cells during muscle growth may likewise allow them to be targeted and increase
their ability to promote muscle growth.

Our lab previously showed that while a lack of satellite cells does not limit short-term
muscle growth, satellite cells are required to support sustained growth, at least in the
plantaris (100% type 2 fibers). As myonuclear accretion and satellite cell abundance are
tightly associated with growth in satellite cell replete muscle, the compensatory pathways
activated in the absence of satellite cell fusion to enable short-term muscle growth are of

interest. In line with this, the mechanism precipitating a shift in the requirement for satellite cells during sustained muscle growth remains to be elucidated. Due to the method of mechanical overload (synergist ablation) used to induce muscle growth in previous studies, our understanding for satellite cell-mediated muscle growth in adult mice is currently restricted to the plantaris. Emerging evidence suggests that these findings may not extend to muscles with a more oxidative phenotype, like the soleus (50% type 1 and 50% type 2) or to muscles which are undergoing robust metabolic adaptations in response to exercise. Whether these finding extend to testosterone induced muscle growth is currently unknown. The mode and length of perturbation utilized to induce muscle growth, the duration of satellite cell depletion, the timing of depletion (pre, post or at some point during adaptation) all may influence requirements for satellite cells.

In order to address these gaps in our understanding of the regulation of muscle growth, I utilized the Pax7-DTA mouse strain, which allows for the inducible depletion of satellite cells and 3 distinct interventions to drive muscle growth and adaptation. I utilized testosterone to determine if testosterone-induced myonuclear accretion by satellite cells is required for skeletal muscle hypertrophy (Chapter 2), a lifelong wheel running stimulus to determine if reduced satellite cell content throughout adulthood influences the transcriptome-wide response to physical activity and diminishes the adaptive response of skeletal muscle Chapter (3) and a short (4-wk) and long (8-wk) term weighted wheel running model to induce hypertrophy in the plantaris and soleus to determine the muscle-specific requirements for satellite cells during growth and elucidate the intracellular mechanisms regulating satellite cell independent and dependent muscle growth over a time course of muscle hypertrophy (Chapter 4). My overarching hypothesis is that satellite cells are required for growth in response to exercise induced muscle growth and that these requirements are more stringent in muscles with an oxidative phenotype. The findings from these studies will enhance the ability to target satellite cells as a method to increase muscle mass and provide information necessary to evaluate the therapeutic potential of this strategy, and potentially identify compensatory mechanisms enabling growth in the absence of satellite cells that may also be potential therapeutic targets.

The findings from these studies show that while satellite cells are not *required* for skeletal muscle hypertrophy, they are critical for optimal adaptation to exercise, including maximal muscle growth. Our results show distinct differences for the reliance on satellite cells based on the stimulus used to induce muscle growth and length of the intervention. We show no differences in SC+ and SC- muscle growth in response to testosterone, but blunted growth and adaptation in response to short and long term exercise. This may be due to an inability of resident myonuclei to meet the increased transcriptional demands of metabolic adaptation to exercise in the absence of satellite cell communication and myonuclear addition. In response to lifelong wheel running we show myonuclear fusion in the soleus and increased GPCR signaling in the plantaris which are absent in SC-skeletal muscle and likely contributed to blunted adaptation. In response to 4-weeks and 8-weeks of weighted wheel running, our transcriptional data reveal an aberrant response in SC- skeletal muscle leading to a maladaptive response in the soleus, including a failure to promote ECM remodeling, attenuated capillarization and muscle growth. To what

extent these processes are related directly to fusion or more related to satellite cell related signaling to the muscle fiber and other cell types remains to be established.

Davis Alan Englund
(Name of Student)

06/03/2020
Date

DETERMINING THE ROLE OF SATELLITE CELLS DURING SKELETAL
MUSCLE ADAPTATION

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Co-Director of Dissertation

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06/03/2020
Date

DEDICATION

To My father Warren David Englund PhD., a lifelong educator and knowledge-seeker.

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I would like to acknowledge my Dissertation Committee: Dr. Charlotte Peterson, Dr. Esther Dupont-Versteegden, Dr. John McCarthy and Dr. Hiroshi Saito for their exceptional mentorship and continued support.

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CHAPTER 1. INTRODUCTION

1.1 Satellite cells and the Myonuclear Domain

Skeletal muscle is a unique tissue for many reasons, the most striking of which is that its cells are multi-nucleated (i.e. syncytial). Further, as skeletal muscle nuclei are post-mitotic, there is a reliance on the dedicated myogenic stem cell population, called satellite cells, to fuse into the syncytium for the purposes of myonuclear addition or possibly replacement. These properties make satellite cells essential for post-natal muscle growth and muscle fiber regeneration after injury [1-5]. Following the discovery of satellite cells [6-8], scientists postulated that a given myonucleus can only transcriptionally govern a fixed volume of cytoplasm, or "myonuclear domain" [9-11]. This hypothesis was reinforced by early experiments in rodents that indicated load-induced hypertrophy was accompanied by myonuclear accretion [12].

The myonuclear domain theory in skeletal muscle can be traced back to the work of Dr. Charles Epstein, who reported that cell size was directly proportional to gene dosage in polyploidy liver cells [13]. Incidentally, hepatocytes have a marked capacity for hypertrophy without DNA synthesis [14,15], and resident hepatocyte nuclei can transcriptionally support at least a doubling in cell size [16]. The idea of a rigid nuclear domain is nevertheless ascribed to highly plastic, multi-nucleated skeletal muscle fibers and is pervasive and engrained within the context of skeletal muscle fiber hypertrophy. The controversy surrounding this idea is evidenced by a recent debate regarding the necessity of satellite cell-mediated myonuclear accretion for loading-induced hypertrophy [17,18], as well as recent studies supporting the existence of a rigid myonuclear domain during hypertrophy [19-24].

1.2 Satellite cells and skeletal muscle maintenance

In the absence of injury, satellite cells are thought to maintain quiescence. However, a few studies indicate myonuclear addition via satellite cell fusion in uninjured muscle fibers [25-29]. While this is not a consistent findings, it suggests myonuclear turnover may occur and that satellite cell fusion may be required for skeletal muscle maintenance over time. In fact, it has been suggested that the decline in satellite cell content seen with advancing age may contribute to the loss of skeletal muscle mass (sarcopenia) [28,30]. However, when our group depleted satellite cells in mature mice throughout adulthood, we found this did not influence the onset or progression of sarcopenia in sedentary mice [31]. A limitation to this study is that its findings can be extended only to sedentary cohorts. As exercise is known to influence myonuclear and satellite cell content, a long term exercise stimulus may alter the requirements for satellite cells throughout adulthood in physically active mice [32,33]. We explore this idea in Chapter 3 [34].

1.3 Evidence for Myonuclear Domain Flexibility During Hypertrophy

Adult humans, mice, and rats have nearly identical myonuclear domain sizes [35]. Numerous studies in humans and rodents report that satellite cell proliferation and myonuclear accretion occur with skeletal muscle fiber hypertrophic growth [reviewed in [30,36]]. The often-observed incidence of large muscle fibers with a high proportion of myonuclei after training reinforces the notion that the myonuclear domain may expand modestly [37], but generally remains stable during hypertrophy. Muscle fiber hypertrophy and myonuclear accretion via testosterone supplementation further implies that myonuclear accretion directly contributes to adult muscle growth [38,39], a hypothesis that we

directly tested in Chapter 2. Conversely, time-course studies in rats [40] and humans [41] show a substantial degree of loading-induced muscle fiber hypertrophy can occur prior to or in the absence of myonuclear accretion, resulting in significant expansion of the myonuclear domain.

Type 1 and 2 muscle fibers demonstrate differential hypertrophic plasticity under a wide variety conditions in humans [42-44], and myonuclear domain flexibility during hypertrophy may differ by fiber type. Our laboratory [45] and others [46,47] showed that the myonuclear domain of human Type 2 muscle fibers, which comprise ~50% of muscle fibers in most muscles, is highly flexible. Herman-Montemayor et al. reported Type 2 fiber hypertrophy >30% with non-statistically significant myonuclear accretion (9.5%, P<0.10) and a 29% expansion of the myonuclear domain after resistance training in untrained women [47]. These findings do not necessarily rule out the existence of a "myonuclear domain ceiling", but do challenge the idea of a muscle fiber growth "threshold" beyond which myonuclear accretion is theoretically required to sustain hypertrophy [37,41,48]. This threshold was derived most recently from correlations in human work and, while translational outcomes in humans are the ultimate goal, loss-of-function studies in mice are necessary to compliment the indirect, correlative evidence gained in humans and to determine causality. It has also been proposed that differences between "low" and "high" hypertrophic responders to resistance training was explained by the extent of myonuclear accretion [48,49], but this has recently been challenged in the literature by evidence showing myonuclear accretion is uncoupled from muscle fiber hypertrophy [50].

Following conditional genetic deletion of satellite cells, our laboratory reports substantial hypertrophy in the plantaris muscle, primarily composed of Type 2 fibers,

following synergist ablation surgery in adult mice (>4 month old) [2,18,30,51-54]. Importantly, the lack of myonuclear accretion during hypertrophy does not negatively affect single muscle fiber contractile function [2,52]. Furthermore, resident myonuclei possess a transcriptional reserve that compensates for a lack of satellite cells during Type 2 fiber hypertrophy [53].

A maximal transcriptional rate for a given myonucleus that corresponds to a specific myonuclear domain size has yet to be identified. Manipulating signaling pathways that are central to muscle fiber size regulation (e.g. Myostatin, AKT, JunB), in the absence of muscle overload or damage, also induces significant hypertrophy without myonuclear accretion and provides further evidence for myonuclear domain flexibility [55-61]. Interestingly, when Type 2 fibers are genetically modified to be more oxidative, hypertrophy is associated with myonuclear accretion [57]. This suggests there may a greater reliance on myonuclear addition during a response to exercise (where numerous metabolic adaptations occur) than to pharmacological interventions. Different roles for satellite cells during a pharmacological (Chapter 2) vs an exercise intervention (Chapters 3 and 4) are explored in this dissertation [34,62]. In order to determine whether increased biosynthetic activity associated with the high metabolic demands of oxidative muscles and coordinating a response to exercise influences requirements for myonuclear accretion during hypertrophy we utilized an alternative non-surgical method of inducing hypertrophy in muscles other than the plantaris (Chapter 4) [63].

1.4 Evidence for Prerequisite Myonuclear Accretion to Support Adult Muscle Fiber Hypertrophy

It is clear that the myonuclear domain is more flexible than previously appreciated, but some evidence suggests that myonuclear accretion is absolutely required for, and in fact precedes hypertrophy. Type 2 fibers in young mice (<4 months old) appear to have an absolute requirement for myonuclear accretion to mount a hypertrophic response [19-22,64]. Further, findings from the Myomaker[scKO] mouse strain (a model of impaired satellite cell fusion) suggest the absence of myonuclear addition completely inhibits muscle growth in response to synergist ablation and uphill treadmill running [20,24]., Counter to the idea that myonuclear accretion drives hypertrophy, significant myonuclear accretion can ensue during heavy endurance training, in the absence of hypertrophy [65-67].

The effects of anabolic steroid usage provide circumstantial evidence that myonuclear accretion facilitates hypertrophy. A hypothesis that we directly test in Chapter 2. Supraphysiological testosterone levels elicit hypertrophy in conjunction with myonuclear addition [38,39], which is compounded during heavy resistance training and pronounced hypertrophy [68,69]. However, androgen signaling can increase myogenic cell proliferation and differentiation *in vitro* and *in vivo* [70-73], making it difficult to tease out the contribution of testosterone-directed fusion versus inflammation/damage versus hypertrophy on myonuclear accretion. Recent evidence also suggests that an inflammatory environment alone causes myonuclear accretion in the absence of exercise training and altered muscle fiber size [74]. We interpret these findings to mean that satellite cell fusion to muscle fibers *in vivo* can be mediated solely by the signaling milieu and uncoupled from muscle fiber size. During short-term muscle atrophy induced by hind limb suspension in

mice, the myonuclear domain is dramatically reduced in size, further demonstrating the flexibility of the myonuclear domain [30,75-77]. That is to say, if the myonuclear domain were truly rigid, one may expect myonuclear loss to consistently scale with atrophy in order to maintain the myonucleus-to-protein ratio, which would be consistent with the original "DNA unit" concept that is the foundation of the myonuclear domain theory (Cheek et al. 1971).

1.5 Perspectives on the Early Satellite Cell Response to Resistance Exercise

Following severe muscle fiber injury, satellite cells mediate skeletal muscle regeneration via myogenesis and interactions with fibroblasts to coordinate proper extracellular matrix remodeling [3]. Resistance exercise in humans is not typically associated with muscle degeneration, but muscle injury, inflammation, and sarcolemmal damage may ensue [78]. One hypothesis is that the magnitude of early satellite cell proliferation after unaccustomed resistance exercise reflects myonuclear accretion potential and predicts hypertrophic adaptation; however, the relationship to muscle fiber growth is unclear [79]. Once muscle damage subsides, satellite cell proliferation after resistance exercise is attenuated [46,80], and is not predictive of the ~16% Type 2 muscle fiber growth reported by Damas et al. [46]. Consistent with initial conjectures on early satellite cell proliferation with overload [81], it seems that muscle fiber damage and satellite cell niche disruption primarily dictates satellite cell responses to resistance exercise, and not hypertrophy *per se*. The relationship between exercise-mediated muscle fiber damage and satellite cell proliferation is underscored by greater satellite cell density after highly- versus minimally-damaging contractions in exercise-naïve men [82,83].

Under certain conditions, the propensity to fuse is an inherent property of activated satellite cells [84]. As such, it is conceivable that satellite cell-mediated myonuclear accretion observed with resistance training is simply a response to muscle fiber damage and the accompanying milieu, and not a requirement to maintain myonuclear domain size. In support of this hypothesis, Type 2 fiber myonuclear accretion occurs during 12 weeks of eccentric (i.e. damaging) but not concentric (i.e. minimally damaging) resistance training, despite similarly modest hypertrophic responses to both modes [85]. The most strenuous exercise may induce the most damage, and subsequently the most myonuclear accretion. Worth mentioning is that the synergist ablation model of skeletal muscle overload used to induce hypertrophy in rodents can be rather severe and damage-inducing depending on how the surgery is conducted [81], which is an obvious drawback of the model that may affect translatability to humans. Interestingly, our laboratory showed that the early satellite cell proliferative response to synergist ablation is crucial for proper extracellular matrix remodeling during hypertrophy [51]. Instead of myonuclear addition to muscle fibers, we contend that the critical role for activated satellite cells during hypertrophy is to participate in extracellular matrix remodeling which facilitates growth independent of fusion [30,51,52]. To determine if these findings extend to a more translatable hypertrophy model, we will utilize progressive weighed wheel running, to avoid excessive muscle damage (Chapter 4).

CHAPTER 2. RESIDENT MUSCLE STEM CELLS ARE NOT REQUIRED FOR

TESTOSTERONE-INDUCED SKELETAL MUSCLE HYPERTROPHY

Abstract of Chapter 2

It is postulated that testosterone-induced skeletal muscle hypertrophy is driven by myonuclear accretion as the result of satellite cell fusion. To directly test this hypothesis we utilized the Pax7-DTA mouse model to deplete satellite cells in skeletal muscle followed by testosterone administration. Pax7-DTA mice (6 months of age) were treated for 5 days with either vehicle (satellite cell replete, SC+) or tamoxifen (satellite cell depleted, SC-). Following a washout period, a testosterone propionate or sham pellet was implanted for 21 days. Testosterone administration caused a significant increase in muscle fiber cross sectional area (CSA) in SC+ and SC- mice in both oxidative (soleus) and glycolytic (plantaris and extensor digitorum longus (EDL)) muscles. In SC+ mice treated with testosterone, there was a significant increase in both satellite cell abundance and myonuclei that was completely absent in testosterone-treated SC- mice. These findings provide direct evidence that testosterone-induced muscle fiber hypertrophy does not require an increase in satellite cell abundance or myonuclear accretion.

2.1 Introduction

Satellite cells are required for post-natal skeletal muscle growth and regeneration, but their role during periods of adult skeletal muscle hypertrophy remains to be fully elucidated [86-89]. Most studies that have investigated the requirement for satellite cells during hypertrophic growth in both juvenile and mature mice have utilized the surgical synergist ablation model to induce hypertrophy, which is supra-physiological and non-reversible [19,20,87,90-92]. These studies have shown that satellite cells are required for muscle hypertrophy prior to complete skeletal muscle maturation (< 4 months of age) [19,20,91,92]. In mature skeletal muscle, considerable muscle growth can occur over a relatively short time frame in the absence of satellite cell-dependent myonuclear accretion; however, satellite cells are required for sustained periods of muscle growth [87,90,91]. To further our understanding of satellite cell-mediated muscle hypertrophy we utilized the Pax7-DTA mouse model to enable depletion of satellite cells and a pharmacological approach to induce muscle hypertrophy. We chose continuous-release (21 days) testosterone pellet implantation as our treatment strategy since high doses of testosterone have been consistently shown to promote an increase in muscle fiber cross sectional area (CSA), myonuclear number and satellite cell content. [93-95].

There are multiple mechanisms by which testosterone treatment has been reported to stimulate muscle hypertrophy [93,96-98]. The primary mechanism is dependent upon androgen receptor (AR) content to induce androgen stimulated increases in protein synthesis [99,100]. Once bound by testosterone, the AR is released from the membrane to induce genomic and non-genomic actions that include increasing IGF-1 expression, Akt/mTORC1 phosphorylation events and subsequent elevated protein synthesis [100-102]. It

is well-documented that muscle fibers, satellite cells and other mononuclear cell populations (fibroblasts, endothelial cells and mast cells) found within skeletal muscle all express the AR; in particular, testosterone treatment of satellite cells *in vitro* induces a 25% increase in proliferation and global knockout of the AR in mice alters regulators of proliferation and differentiation [103-105]. While testosterone administration induces satellite cell proliferation and muscle growth, more work is needed to determine the direct relationship between increased satellite cell content and muscle hypertrophy in response to testosterone [98,104].

Based on the effects of testosterone on satellite cell proliferation and fusion, and indirect evidence in humans and in mice demonstrating a relationship between myonuclear accretion and increased muscle size, the general consensus is that testosterone-induced muscle hypertrophy is driven directly by satellite cell activation and subsequent myonuclear accretion; however, direct evidence to support this consensus is currently lacking. [93,94]. We therefore set out to test the hypothesis that testosterone-induced myonuclear accretion by satellite cells is required for skeletal muscle hypertrophy. Female Pax7-DTA mice were treated with vehicle (satellite cell replete, SC+), to serve as controls or tamoxifen (satellite cell depleted, SC-) and were supplied with testosterone propionate or sham pellets for 21 days after which hindlimb muscles were isolated and analyzed.

2.2 Methods

The Pax7CreER/+-R26RDTA/+ strain, called Pax7-DTA, was generated by crossing the Pax7CreER/CreER mouse strain with the Rosa26DTA/DTA mouse strain [3]. The Pax7-DTA mouse allows for the specific and inducible depletion of satellite cells

upon tamoxifen treatment, through *Cre*-mediated activation of the diphtheria toxin A gene in Pax7-expressing cells. [87]. Mice were housed at 14:10 hour light:dark cycle and had access to water and food ad libitum. All animal procedures were conducted in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Experimental design

Thirty-two mature (6-months old) female Pax7-DTA mice were treated via intraperitoneal injection with either vehicle (15% ethanol in sunflower seed oil) or tamoxifen at a dose of 2.5 mg/day for five days [87]. Following a 2-week washout period, either 2.5 mg testosterone propionate pellet or sham pellet, with a release time of 21 days (A-211 Innovative Research America), was implanted subcutaneously into the lateral side of the neck with a precision trocar (MP-182 Innovative Research America) under anesthesia (1–2% inhaled isoflurane). After 21 days, mice were euthanized and muscles (plantaris, soleus, and EDL) were excised and weighed, then pinned to a cork at resting length and covered with Tissue Tek Optimal Cutting Temperature (Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen cooled isopentane and stored at -80°C.

Testosterone Enzyme-Linked Immunosorbent Assay (ELISA)

To determine the efficacy of the testosterone pellets, eight mice were implanted with either sham (n = 3) or testosterone (n = 5) pellets. Three days post implantation, mice were sacrificed and blood draws were conducted under light anesthesia (1–2% inhaled isoflurane). Blood samples were spun at 2,000x*g* for 15 min. Serum was collected and analyzed using Mouse/Rat Testosterone Elisa (ALPCO, Salem, NH) per manufacturer's protocol.

Immunohistochemistry

Immunohistochemical (IHC) analyses for determination of cross sectional area (CSA) and myonuclear number were performed as previously described [91]. Frozen muscle samples were sectioned at the mid-belly of the muscle at -23°C (7 µm), air-dried and stored at -20°C. For determining muscle fiber CSA and myonuclear density, cross sections were incubated overnight in an anti-dystrophin antibody (1:100, ab15277, Abcam, St. Louis, MO, USA). Sections were incubated with a secondary antibody (1:150, anti-rabbit IgG, CI-1000, Vector), diluted in phosphate-buffered saline (PBS). Sections were mounted using VectaShield with DAPI (H-1200, Vector).

Detection of Pax7+ cells was performed as previously described [87]. Sections were fixed in 4% paraformaldehyde (PFA) followed by antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS followed by a blocking step with 1% Tyramide Signal Amplification (TSA) blocking reagent (TSA kit, T20935, Invitrogen) and Mouse-on-Mouse blocking reagent (Vector Laboratories, Burlingame, CA, USA). Pax7 primary antibody (1:100, DSHB, Iowa City, Iowa, USA) and laminin primary antibody (1:100, Sigma-Aldrich, St. Louis, MO, USA) were diluted in 1% TSA blocking buffer and applied overnight. Samples were then incubated with an isotype specific anti-mouse biotin-conjugated secondary antibody against the Pax7 primary antibody (1:1000, 115-065-205, Jackson ImmunoResearch, West Grove, PA, USA) and anti-rabbit secondary for laminin (1:250, A11034, Alexa Fluor 488, Invitrogen, Carlsbad, CA, USA). Slides were washed in PBS followed by streptavidin-horseradish peroxidase (1:500, S-911, Invitrogen) for 1 hour. AlexaFluor 594 was used to

visualize antibody-binding for Pax7 (1:100, TSA kit, Invitrogen). Sections were mounted and nuclei were stained with Vectashield with DAPI (H-1200, Vector).

Image quantification

All images were captured at 20x magnification at room temperature using a Zeiss AxioImager M1 upright fluorescent microscope (Zeiss, Oberkochen, Germany). Whole muscle sections were obtained using the mosaic function in Zeiss Zen 2.3 imaging software (Zeiss). To minimize bias and increase reliability, muscle fiber CSA and myonuclear number were quantified on cross sections using MyoVision automated analysis software [106]. MyoVision enables muscle fibers and myonuclei in the whole muscle cross section to be counted and quantified for measures of CSA and myonuclei/fiber [106]. To determine satellite cell density (Pax7+ cells/fiber), satellite cells (Pax7+/DAPI+) were counted manually on entire muscle cross sections using tools in the Zen software. Satellite cell counts were normalized to fiber number via laminin. All manual counting was performed by a blinded, trained technician.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Data were analyzed with GraphPad Prism software (GraphPad Software, San Diego, California, USA) via a two factor ANOVA for Pax7$^+$ cells/fiber, muscle weight, mean fiber CSA and myonuclei/fiber, and a two factor repeated measures ANOVA for body weight over time. When interactions were detected, post hoc comparisons were made with Holm-Sidak post hoc tests. Serum testosterone levels were compared using an unpaired two-tailed Student's t-test. Statistical significance was accepted at $P < 0.05$.

2.3 Results

Testosterone pellet implantation leads to higher satellite content in SC+ mice

The implantation of testosterone pellets resulted in significantly higher levels of serum testosterone, measured in a sub-group of mice 3 days after implantation of the pellet (Figure 2.1A). A representative image of immunohistochemical (IHC) detection of Pax7 positive cells in the soleus muscle is shown in Figure 2.1B. IHC image quantification demonstrated effective depletion in the soleus following tamoxifen treatment (Figure 2.1C). Tamoxifen treatment effectively depleted satellite cells in the oxidative soleus (Figure 2.1D) and the glycolytic plantaris (Figure 2.1E). Three weeks of testosterone administration led to higher satellite cell number in soleus (22%, $p = 0.007$) and plantaris (29%, $p < 0.001$) from satellite cell replete (SC+), but not depleted (SC-) mice (Figure 2.1 D&E).

Testosterone administration induces higher whole-body weight, muscle weights and muscle fiber CSA, independent of satellite cell content.

Three weeks of testosterone administration led to a significant increase in body weight ($p = 0.044$), resulting in a higher body weight when compared to sham treated mice ($p = 0.023$) (Figure 2.2A), and higher soleus ($p = 0.023$), plantaris ($p < 0.001$) and EDL ($p < 0.001$) wet weights (Figure 2.2B-D, respectively), independent of satellite cell content. Representative images of plantaris muscle immunoreacted with an antibody against dystrophin to measure CSA are shown in Figure 2.3A&B. Testosterone led to higher muscle fiber CSA in the soleus (17%, $p = 0.002$), plantaris (16%, $p = 0.001$), and EDL (14%, $p = 0.038$) regardless of the presence of satellite cells (Figure 2.3C-E, respectively).

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Myonuclear addition in response to testosterone administration is dependent on the presence of satellite cells

Representative images of soleus muscle immunoreacted with an antibody against dystrophin and DAPI staining to determine myonuclear number are shown in Figure 2.4A&B. Myonuclear density was higher in the soleus (43%, $p < 0.001$), plantaris (32%, $p < 0.001$) and EDL (31%, $p < 0.001$) muscles of testosterone-treated SC+ mice (Figure 2.4C-E). In mice depleted of satellite cells, no myonuclear accretion occurred with testosterone treatment, which did not affect growth, as SC- mice exhibited the same hypertrophic response to testosterone as SC+ mice (see Figs. 2.2 and 2.3).

2.4 Discussion

This study reports the novel finding that neither the presence of satellite cells nor myonuclear addition via satellite cell fusion are required for testosterone-induced muscle hypertrophy, contrary to our hypothesis. It has previously been hypothesized that satellite cells play a direct role in stimulating muscle hypertrophy in response to testosterone [93,94]. However, the findings of the current study demonstrate that the hypertrophic response to testosterone is unaffected by absence of satellite cells arguing against such a mechanism. It is also worth noting that testosterone administration did not lead to the accrual of myonuclei through fusion of an extraneous stem cell population in tamoxifen treated muscle, as evidenced by the lack of myonuclear addition in satellite cell-depleted mice treated with testosterone.

Our report supports the finding of Egner et al. that robust myonuclear accretion is observed in response to testosterone administration in muscles that have a full complement of satellite cells [95]. Interestingly, the hypertrophic growth response noted by

Egner et al. (~77% hypertrophy in the soleus) greatly exceeds what we report here (~17% hypertrophy in soleus). This discrepancy is most likely due to differences in the skeletal muscle maturity of the mice used at the onset of testosterone administration. We utilized fully mature 6 month old mice, resulting in a correspondingly larger mean muscle fiber CSA (~1,400 μm) in sham-treated mice than those used by Egner et al. (mean muscle fiber CSA ~1,000 μm) [95]. It therefore seems likely that skeletal muscle maturation in combination with testosterone administration acted synergistically to augment muscle fiber growth in the report by Egner et al [95].

It is currently unknown whether the higher number of satellite cells and myonuclei observed after testosterone administration is driven by a direct effect of androgen action on the satellite cells or an indirect effect from increased levels of androgen acting directly on the muscle fiber (e.g. elevated IGF-1 expression) [102]. Based on our findings, it is apparent that testosterone increases satellite cell abundance and myonuclear content, providing further evidence for the proliferative effects of testosterone on satellite cells, either directly or indirectly. Our findings in satellite cell-depleted mice also demonstrate that the genomic and/or non-genomic actions of testosterone within the muscle fibers are sufficient to drive skeletal muscle hypertrophy [100-102]. This finding is supported by a study in rat myotubes *in vitro*, in which no change in myonuclear number occurred following 12 hours of testosterone administration despite the reported 35% higher myotube diameter than vehicle-treated cells [100]. Further, our lab has shown that satellite cells are not required for an increase in the activation of the Akt/mTOR signaling pathway and others have shown muscle fiber hypertrophy resulting from the overexpression of Akt occurs without satellite cell participation [87,107]. The

importance of testosterone activity directly on the muscle fiber itself seems to be somewhat lost in the literature, as many groups have hypothesized that increases in skeletal muscle size are a direct consequence of satellite cell fusion and not the transcriptional activity of resident myonuclei [39]. This is an important concept, since mechanisms internal to the muscle fiber should also be considered, particularly in designing interventions to increase muscle size. In line with this, we previously showed that resident myonuclei appear to have a transcriptional reserve capacity which can compensate for lack of satellite cells, enabling hypertrophy in response to synergist ablation (8). We show here that resident myonuclei also appear to be able to compensate in satellite-depleted muscle to enable hypertrophy in response to testosterone administration. Interestingly, it is known that satellite cells are required to support sustained periods of muscle growth in response to mechanical overload (5). Whether satellite cells are also required for continued muscle growth in response to testosterone is currently unknown. Emerging technology and novel transgenic mouse models will allow for future in-depth analysis of resident myonuclei during periods of skeletal muscle growth [108]. In particular, the transcriptional profile of resident myonuclei in response to a hypertrophic stimulus in the absence of satellite cell fusion is of interest.

In summary, this paper provides novel insight into the role of satellite cells in the muscle growth response to testosterone. Although testosterone normally increases satellite cell number and promotes myonuclear accretion during hypertrophy, satellite cell-dependent myonuclear accretion is not required in adult muscle for hypertrophic muscle growth. Thus, understanding the compensatory mechanism that enables healthy adult resident myonuclei to support growth in the absence of satellite cells may point to

new targets for intervention in conditions where satellite cell abundance and/or activity declines and growth is compromised, such as aging.
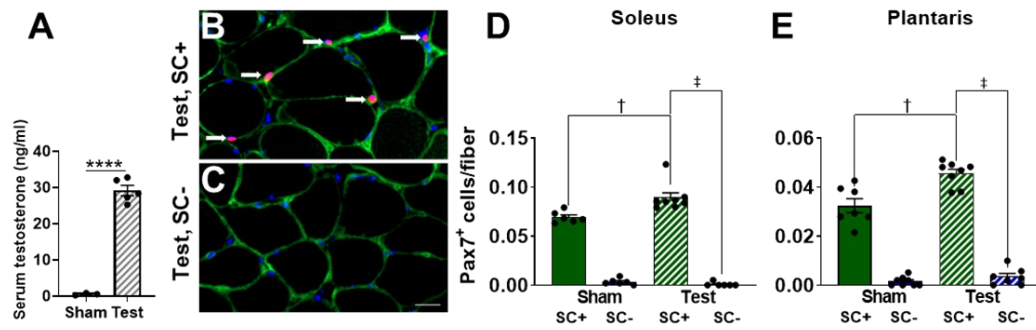
Figure 2.1

Higher serum testosterone (Test) and higher satellite cell density in vehicle (SC+) compared to tamoxifen-treated (SC-) mice after Test administration. (A) Serum Test concentration after 3 days of Test or Sham administration. (B&C) Representative images of satellite cell immunohistochemistry in the soleus showing laminin (green) nuclei (blue) and Pax7 (red; white arrows). Scale bar = 20 μm. (D&E) Satellite cell density in the soleus and plantaris. Data represent mean ± SEM. n = 6-8 mice per group. † $P < 0.05$, Test SC+ vs Sham SC+; ‡ $P < 0.05$, Test SC+ vs Test SC-. **** $P < 0.0001$ Test (n = 5) vs. Sham (n = 3) mice at 3-days.
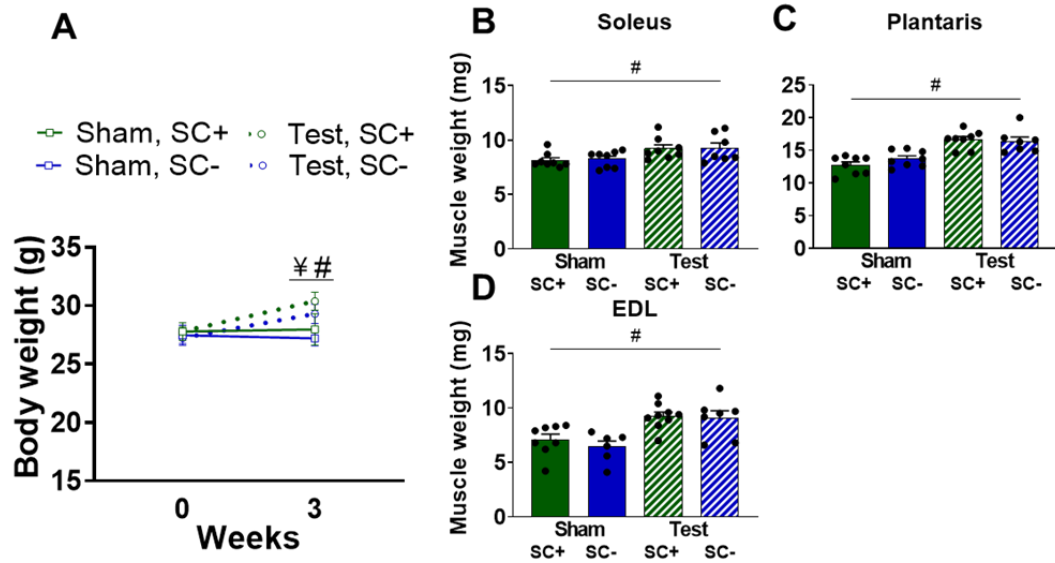
Figure 2.2

Testosterone (Test) increases whole-body and muscle weight in mice with (SC+) or without (SC-) satellite cells. (A) Body weight at baseline and after 3-weeks of Test or Sham administration. (B-D) Muscle wet weights in the soleus, plantaris and EDL. Data represent mean ± SEM. n = 6-8 mice per group. ¥ $P < 0.05$, within group change over time in test treated mice. # $P < 0.05$ Test vs Sham mice at 3-weeks.
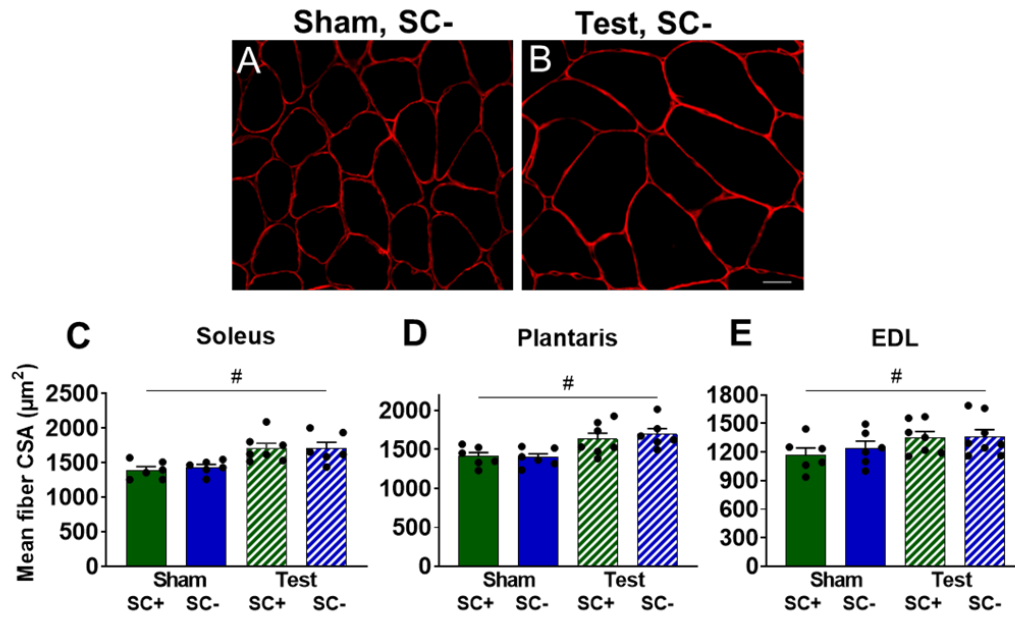
Figure 2.3

Testosterone (Test) leads to greater muscle fiber size independent of satellite cell content.

(A&B) Representative images of muscle fiber cross sectional area (CSA) in the plantaris

visualized with an antibody against dystrophin. Scale bar = 20 μm. (C-E) Mean fiber

CSA in the soleus, plantaris and EDL. Data represent mean ± SEM. n = 6-8 mice per

group. # $P < 0.05$ Test vs Sham mice.
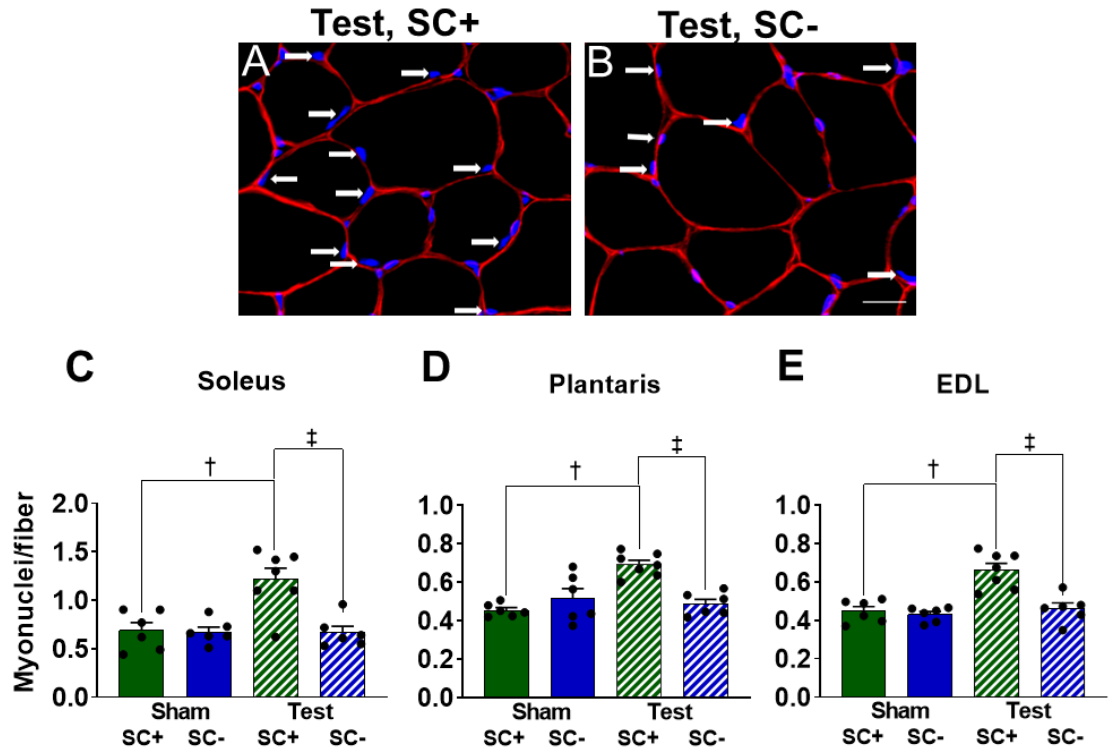
Figure 2.4

Elevated myonuclear number in response to testosterone (Test) is dependent on satellite

cell content. (A&B) Representative images of dystrophin immunohistochemistry (red)

and DAPI (blue) staining from soleus cross sections to identify myonuclei (white

arrows). Scale bar = 20 μm. (C-E) Myonuclear density of the soleus, plantaris and EDL.

Data represent mean ± SEM. n = 6-7 mice per group. † P < 0.05, Test SC+ vs Sham SC+;

‡ P < 0.05, Test SC+ vs Test SC-.

CHAPTER 3. DEPLETION OF RESIDENT MUSCLE STEM CELLS NEGATIVELY IMPACTS RUNNING VOLUME, PHYSICAL FUNCTION AND MUSCLE FIBER HYPERTOPHY IN RESPONSE TO LIFELONG PHYISCAL ACTIVITY

Abstract of Chapter 3

To date, studies that have aimed to investigate the role of satellite cells during adult skeletal muscle adaptation and hypertrophy have utilized a non-translational stimulus and/or have been carried out over a relatively short time frame. While it has been shown that satellite cell depletion throughout adulthood does not drive skeletal muscle loss in sedentary mice, it remains unknown how satellite cells participate in skeletal muscle adaptation to long term physical activity. The current study was designed to determine if reduced satellite cell content throughout adulthood would influence the transcriptome-wide response to physical activity and diminish the adaptive response of skeletal muscle. We administered vehicle or tamoxifen to adult Pax7-DTA mice to deplete satellite cells and assigned them to sedentary or wheel-running conditions for 13 months. Satellite cell depletion throughout adulthood reduced balance and coordination, overall running volume and the size of muscle proprioceptors (spindle fibers). Further, satellite cell participation was necessary for optimal muscle fiber hypertrophy but not adaptations in fiber type distribution in response to lifelong physical activity. Transcriptome-wide analysis of the plantaris and soleus revealed that satellite cell function is muscle type-specific; satellite cell-dependent myonuclear accretion was apparent in oxidative muscles, whereas initiation of GPCR signaling in the glycolytic plantaris may require satellite cells to induce optimal adaptations to long term physical activity. These findings suggest satellite cells play a role in preserving physical function during aging and influence muscle adaptation during sustained periods of physical activity.

3.1 Introduction

Genetic mouse models enabling an inducible depletion of satellite cells, the resident skeletal muscle stem cell, have demonstrated that satellite cells are required for regeneration after injury and hypertrophic growth prior to skeletal muscle maturity (4 months of age) [86,87,91]. Once mature, robust muscle fiber hypertrophy and alterations in fiber type distribution can occur over a relatively short time frame in the absence of satellite cells [62,87,109]. However, satellites cells become necessary during sustained periods of muscle growth and adaptation [51,90]. While intriguing, the translational relevance of these findings is limited due to the applied stimulus and/or an inadequate length of intervention needed to determine the long term implications of reduced satellite cell content [87,90,109]. The majority of these studies have utilized synergist ablation as a model of mechanical overload, which induces a supraphysiological and damaging stimulus and restricts findings to the overloaded plantaris muscle, which is comprised of 100% type 2 fibers [87,90,91]. As type 1 fibers comprise ~50% of human skeletal muscle and are known to positively influence physical function and health, determining the role of satellite cells during type 1 fiber adaptation is clinically relevant [110,111]. Further, it is becoming clear the length of satellite cell depletion, stimulus applied and muscle(s) analyzed influence findings on the necessity of satellite cells to support muscle adaptation [90,109,112]. Many signaling cascades have been identified that promote muscle adaptation, among the most studied are the IGF1/PI3K/AKT hypertrophy signaling pathways and AMPK/PGC-1α metabolic signaling pathways. More recently, G protein coupled receptors (GPCRs) and specific G proteins have emerged as regulators of muscle adaptation [113-116]. How the presence or absence of satellite cells influences these signaling cascades and phenotypic adaptation over a prolonged period of time is unknown.

While there is a well-documented association between reduced satellite cell content and the loss of muscle mass with advancing age, we reported that significantly reducing satellite cell content at 4 months of age did not influence the onset or progression of muscle loss in sedentary mice as they aged [31,33]. However, to date, no study has taken a translational approach to directly assess the impact of satellite cell loss on skeletal muscle adaptability in physically active mice with aging. The current study

was designed to determine if satellite cell depletion throughout adulthood would diminish improvements in physical function and skeletal muscle adaptation, assessed across several hindlimb muscles, in response to lifelong physical activity. Further, we wanted to determine to what degree satellite cell participation influenced the transcriptome-wide response to physical activity and signaling pathways specific to muscle adaptation, enabling the identification of satellite cell-dependent processes. In order to address these questions, we utilized the Pax7-DTA mouse to deplete satellite cells for over one year and gave the mice free access to a running wheel during this time to simulate lifelong physical activity. Our results show that satellite cell depletion reduced measures of balance and coordination, as well as overall running volume. These outcomes were likely influenced by the smaller muscle proprioceptors found in the absence of satellite cells. Further, we show the presence of satellite cells is critical for optimal muscle fiber hypertrophy, but not for alterations in fiber type distribution, in response to lifelong physical activity. Our findings suggest a muscle-specific role for satellite cell participation during growth, with fusion playing a more direct role in the oxidative soleus, whereas initiation of GPCR signaling is influenced by satellite cell depletion in the glycolytic plantaris. Taken together, these findings suggest satellite cells play a role in preserving physical function during aging and influence muscle adaptation during sustained periods of physical activity.

3.2 Methods

Animals

The Pax7$^{CreER/+}$-R26$^{DTA/+}$ strain, called Pax7-DTA, was generated by crossing the Pax7$^{CreER/CreER}$ mouse strain with the Rosa26$^{DTA/DTA}$ mouse strain [3]. The Pax7-DTA mouse allows for the specific and inducible depletion of satellite cells upon tamoxifen treatment, through *Cre*-mediated recombination to induce expression of the diphtheria toxin A (DTA) gene in Pax7-expressing cells, effectively killing satellite cells. All animal procedures were conducted in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Experimental design

Thirty-nine adult (5-months old) female Pax7-DTA mice were treated via intraperitoneal injection with vehicle (15 % ethanol in sunflower seed oil) or tamoxifen at a dose of 2.5 mg/day for five days, as previously described [87]. Following a 2-month washout period, mice were singly housed and randomly assigned to the sedentary group (cage with locked running wheel) or the running group (free access to running wheel) at 7-months of age (n=9-10 per group). A mechanical counter was used to record wheel rotations and was connected to a desktop computer via ClockLab software (Actimetrics, Wilmette, IL). The animals had access to food and water ad libitum and were checked daily for health and wellness. Mice were sacrificed 13 months later, at a mean age of 20 months. Running wheels were locked 48 hours before sacrifice and mice were fasted overnight. Hindlimb muscles (plantaris, soleus, tibialis anterior (TA) and extensor digitorum longus (EDL) were harvested and prepared for immunohistochemical (IHC) analysis and the contralateral limb for RNA extraction, and then stored at −80 °C. Five mice (3 vehicle-treated and 2 tamoxifen-treated) died over the duration of the study and upon sacrifice tumors were found in one additional mouse (vehicle-treated); these six mice were excluded from analyses.

Immunohistochemistry

IHC analyses were performed as previously described [91]. Briefly, hindlimb muscles were extracted and weighed, then were pinned to a cork block at resting length and covered with Tissue Tek Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA, USA), and quickly frozen in liquid nitrogen cooled isopentane and stored at -80°C. Frozen muscles were sectioned at -23°C (7 μm), air-dried for at least one hour, and then stored at -20°C. For determining fiber type distribution, muscle fiber cross-sectional area (CSA), fiber type-specific CSA, and myonuclear density, cross sections were incubated overnight in a cocktail of isotype specific anti-mouse antibodies for MyHC 1 (1:75, IgG2B, BA.D5), MyHC 2a (neat, IgG1, SC.71), and MyHC 2b (neat, IgM, BF.F3) from Developmental Studies Hybridoma Bank (DSHB, Iowa City, Iowa, USA), along with an antibody against dystrophin (1:100, ab15277, Abcam, St. Louis, MO, USA), to delineate fiber borders for CSA quantification. Sections were subsequently

incubated with secondary antibodies (1:250, goat anti-mouse IgG2b Alexa Fluor 647, #A21242; 1:500, IgG1 Alexa Fluor 488, #A21121; 1:250, IgM Alexa Fluor 555, #A21426) from Invitrogen (Carlsbad, CA, USA), along with the secondary antibody for dystrophin (1:150, anti-rabbit IgG AMCA, CI-1000, Vector), diluted in PBS. Sections were mounted using VectaShield with DAPI (H-1200, Vector). For an additional measure of myonuclear density, soleus cross sections were stained for pericentriolar material 1 (PCM1, Sigma-Aldrich), as previously described [117].

Detection of Pax7+ cells was performed as previously described [87]. Briefly, sections were fixed in 4% paraformaldehyde (PFA) followed by antigen retrieval using sodium citrate (10 mM, pH 6.5) at 92°C. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS) followed by an additional blocking step with 1% Tyramide Signal Amplification (TSA) blocking reagent (TSA kit, T20935, Invitrogen) and Mouse-on-Mouse blocking reagent (Vector Laboratories, Burlingame, CA, USA). Pax7 primary antibody (1:100, DSHB) and laminin primary antibody (1:100, Sigma-Aldrich) were diluted in 1% TSA blocking buffer and applied overnight. Samples were then incubated with anti-mouse biotin-conjugated secondary antibody against the Pax7 primary antibody (1:1000, 115-065-205, Jackson ImmunoResearch, West Grove, PA, USA) and anti-rabbit secondary for laminin (1:250, A11034, Alexa Fluor 488, Invitrogen, Carlsbad, CA, USA). Slides were washed in PBS followed by streptavidin-horseradish peroxidase (1:500, S-911, Invitrogen) for 1 hour. AlexaFluor 594 was used to visualize antibody-binding for Pax7 (1:100, TSA kit, Invitrogen). Sections were mounted and nuclei were stained with Vectashield with DAPI (H-1200, Vector).

Detection of N-acetyl-d-glucosamine was evaluated on muscle sections using Texas Red-conjugated wheat germ agglutinin (WGA (eBiosciences, San Diego, CA)). Sections were fixed in 4 % PFA and then incubated with WGA conjugate for 2 hours at room temperature. Sections were also stained with Picrosirius red (PSR) for 1 hour per the manufacturer's instructions (24901-500; Polysciences, Warrington, PA, USA). For quantification, the area of red-stained collagen was quantified relative to the total muscle area.

Image quantification

All WGA and PSR-stained images were captured at 10x magnification, and the total stained area quantified using the thresholding feature of the AxioVision Rel software, as described previously [109]. Muscle spindles were identified by the presence of a thick basement membrane (the muscle spindle capsule) which surrounds the intrafusal fibers [118,119]. Spindles and lobes (located within muscle spindles) were manually traced for CSA analysis. All remaining images were captured at 20x magnification at room temperature using a Zeiss upright fluorescent microscope (Zeiss AxioImager M1 Oberkochen, Germany). Whole muscle sections were obtained using the mosaic function in Zeiss Zen 2.3 imaging software. To minimize bias and increase reliability, fiber type distribution, muscle fiber CSA, fiber type-specific CSA and myonuclear density were quantified on cross sections using MyoVision automated analysis software [106]. To determine satellite cell density (Pax7+ cells/fiber), satellite cells (Pax7+/DAPI+) were counted manually on entire muscle cross sections using tools in the Zen software. Satellite cell counts were normalized to fiber number, delineated by laminin boundaries. All manual counting was performed by a blinded, trained technician.

RNA isolation

Soleus and plantaris muscles were homogenized in QIAzol (Qiagen, Hilden, Germany) and RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was eluted in nuclease-free water for subsequent microarray and RT-qPCR analyses. RNA quantity and purity was checked by measuring the optical density (260 and 280 nm) using Nanodrop. All 260/280 ratios were above 1.8.

RT-qPCR

Complementary DNA was synthesized from 500 ng of total RNA using the SuperScript IV VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, California). RT-qPCR was performed on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, California), with Fast SYBR Green master mix (Thermo Fisher Scientific), a 10- fold dilution of cDNA into a 10μl final reaction, using primers against *Gpr4, Gnai2, Dgkh, Prkcsh* and *Vcp* (Table 3.1). RT-qPCRs were performed using the following

thermal cycler conditions: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles of a two-step reaction, denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds. RT-qPCR efficiency was calculated by linear regression from fluorescence increase in the exponential phase in the LinRegPCR software v11.1[120]. Expression was normalized to *Vcp* using the delta-delta Ct method [121].

Microarray analysis

The microarray hybridization and processing were performed at the University of Kentucky Microarray Core Facility, according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Affymetrix chips (mouse Clariom S array) were used with 50 ng/µl of RNA (500 ng total RNA) derived from a pooled sample of right plantaris and soleus muscles (n=7/8 group). In an additional analysis, we pooled RNA (50 ng/µl) samples from a subset of mice that performed an equal volume of running (n=3/group). We pooled RNA samples based on the experimental results reported by Kendziorski et al. showing that gene expression from RNA pools are similar to averages of individuals that comprise the pool [122].

Gene expression analysis

Enriched (≥ 1.5 fold) gene expression data sets were transferred to the Venn diagram tool created by the Van de Peer lab (http://bioinformatics.psb.ugent.be/webtools/Venn/). Data sets were then transferred to ConsensusPathDB for over-representation and enrichment analyses, carried out with default parameters [123].

Functional testing

Rotarod and balance beam measurements were performed by a trained technician (blinded to the group assignments) at the University of Kentucky Rodent Behavior Core a week prior to sacrifice.

Rotarod test

Sensorimotor coordination was assessed using a rotarod apparatus consisting of a rotating rod suspended 18 inches above a padded floor (San Diego Instruments, San

Diego, CA). This system uses a mouse's natural fear of falling as a motivational tool to test gross motor function. Mice were placed on the rotating rod at a speed of 4 revolutions per minute (rpm) for 60 seconds for their training sessions (two training sessions separated by at least 10 minutes). After successful completion of the training sessions and adequate rest, the speed of the rod was gradually increased to a maximum of 40 rpms for each of the three testing sessions. The trial was complete when the animal fell, or the time period ended (300 second max). Latency to fall (seconds) was recorded, and the average of the three testing trials was reported for each animal.

Balance beam

A beam walking protocol was used to evaluate mice on their ability to traverse beams of decreasing widths. The beam walking protocol evaluates motor balance and coordination by assessing both the time it takes the mice to traverse the beam. Following standard acclimatization and training, three beam widths were used to assess balance (28, 17, and 11 mm) and the mice were given 1 minute per trial to complete the crossing of the beam to a bedding filled safe-room on the far end of the beam. The mean of the five trials for the 11mm beam was used for analysis. Difficulty traversing only occurred with the 11 mm beam.

Data Analysis

Results are presented as mean ± SEM. Data were analyzed with GraphPad Prism software via a two factor ANOVA, a two factor repeated measures ANOVA (running over time), or an unpaired Student's t-test (cross sectional running data). When interactions were detected, post hoc comparisons were made with Sidak post-tests. Statistical significance was accepted at $P < 0.05$.

3.3 Results

Satellite cell replete mice (SC+) had higher satellite cell and myonuclear density in response to lifelong physical activity than satellite cell depleted mice (SC-)

Pax7-DTA mice treated with tamoxifen at 5 months of age demonstrated ≥90% depletion of satellite cells (SC-) in hindlimb skeletal muscles (plantaris, soleus, TA and

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EDL) at the time of sacrifice at 20 months of age (representative image of Pax7 staining shown in Figure 3.1A and B; quantified in Figures 3.1C and D, Table 3.2). Free access to a running wheel for 13 months, beginning at 7 months of age, did not result in higher satellite cell content in SC- mice (Figures 3.1C and D, Table 3.2). By contrast, in satellite cell replete mice (SC+), satellite cell density (satellite cells/fiber) in both the plantaris and soleus muscles was higher in response to lifelong physical activity, (Figures 3.1C and D). Satellite cell content was not affected by running in the SC+ TA or EDL muscles (Table 3.2).

Lifelong physical activity resulted in higher myonuclear density in the soleus, but not the plantaris, of SC+ mice; as expected there was no change in myonuclear density in these muscles in SC- mice (representative images of myonuclear staining in Figures 3.2A and B; quantified in Figures 3.2C and D, Table 3.2). The observation that myonuclear density was not higher in the soleus of SC- runners suggests that an alternative stem cell population did not substitute for satellite cells in contributing myonuclei in response to lifelong physical activity. To confirm the higher myonuclear density in the soleus of SC+ mice following running, we performed pericentriolar material 1 (PCM1) staining to definitively identify myonuclei [117]. As reported previously, PCM1 staining identified more myonuclei compared to DAPI/dystrophin staining; however, the change in myonuclear density was the same between the two methods (Figure 3.2D) [117]. Similar to satellite cell content, there was no myonuclear addition in the EDL and TA hindlimb muscles in response to running (Table 3.2).

Lifelong physical activity influenced whole-body and heart weight independent of satellite cell content

Lifelong physical activity led to lower body weight (Figure 3.3A) and higher normalized heart weight (Figure 3.3B). These findings highlight that lifelong voluntary wheel running was a powerful stimulus capable of inducing positive phenotypic adaptations independent of satellite cell content.

Satellite cells were not required for an oxidative shift in fiber type composition in response to lifelong physical activity

In response to lifelong physical activity, IHC analysis showed a robust shift from glycolytic 2b fibers to oxidative 2a fibers in the plantaris (representative images shown in Figures 3.4A and B; quantified in Figure 3.4C). This is the first report to demonstrate that a lack of satellite cells in combination with aging does not impair this metabolic adaptation to wheel running. There was no significant shift in the fiber-type composition of the soleus with physical activity, likely due to its inherently oxidative phenotype (Figure 3.4D).

Satellite cell depletion limited hypertrophic growth in response to lifelong physical activity

Muscle fiber type-specific CSA was larger in plantaris and soleus muscles following lifelong physical activity in SC+ compared to SC- mice (representative images shown in Figures 3.5A-D; quantified in Figures 3.5E-H). Total mean muscle fiber CSA was larger in the SC+ compared to SC- soleus (Figure 3.5H); however, due to the prominent shift towards smaller 2a fibers in the plantaris of SC+ runners (as shown above in Figure 3.4C), plantaris mean fiber CSA was not different between sedentary and running mice (Figure 3.5E). We also analyzed whole-muscle wet weights, corroborating our findings at the CSA level (Figure 3.6). Neither lifelong physical activity nor satellite cell depletion influenced muscle fiber size in the TA or EDL (Table 3.2).

Satellite cell content influenced the transcriptome-wide response to lifelong physical activity

We next performed a transcriptome-wide analysis to identify signaling pathways involved in satellite cell-mediated muscle fiber hypertrophy in response to lifelong physical activity. We pooled RNA samples from the plantaris (n=7/group) and soleus (n=7/group) and performed microarray analysis to uncover pathways preferentially enriched in SC+ compared to SC- runners. We compared the highly expressed genes (≥1.5-fold) in the plantaris to those of the soleus and found considerable differences

between the muscles in their satellite cell-dependent response to lifelong physical activity (Figure 3.7A). Next, we utilized an over-representation analysis to identify signaling pathways significantly enriched in SC+ compared to SC- muscles (Figures 3.7B and C) and a gene set enrichment analysis which identified a distinct Gα_{i2} signaling pathway with a well-established role in hypertrophic growth to be preferentially enriched in SC+ plantaris (Figure 3.7D) [114,115]. We validated this finding by performing qPCR on key genes involved in the Gα_{i2} signaling cascade (Figure 3.7E). Collectively, these data suggest that Gα_{i2} signaling is a potential satellite cell-dependent process promoting muscle fiber growth in the plantaris in response to lifelong physical activity.

Satellite cell depletion throughout adulthood negatively influenced physical function and running volume

We utilized a rotarod test to quantify motor coordination and a balance beam test to assess balance and coordination. SC- mice performed worse on both tests of function, spending less time on the rotarod and taking a longer period of time to transverse the balance beam (Figure 3.8A and 3.8B). Lifelong physical activity did not improve performance on the tests of physical function in SC+ or SC- mice. These findings could, in part, explain declines in physical function with aging that are independent of muscle mass and/or a physically active lifestyle. Depletion of satellite cells reduced voluntary wheel running volume (km/day) over the duration of the study (Figures 3.8C and 3.8D). This is likely explained by the demonstrated impairments in balance and coordination. In an effort to control for differences in running volume we performed microarray analysis on a subset of SC+ and SC- mice (n = 3/group) that ran equal volumes (~9 km/day) over the course of the study. We analyzed the genes enriched ($\geq$1.5 fold) in SC+ mice when compared to SC- mice of equal running volume and found that the most highly enriched pathways and key genes in the Gαi2 signaling network appeared to be mediated by the presence of satellite cells, as they were still enriched in SC+ compared to SC- mice when accounting for running volume (Figure 3.9)

Satellite cell depletion led to aberrant muscle spindle characteristics

To investigate the underlying cause for reduced physical function in the absence of satellite cells, we tested whether satellite cell depletion influenced spindle fiber (muscle proprioceptor) morphology. We quantified N-acetyl-D-glucosamine (a key component of glycosaminoglycan polymers) utilizing a fluorescent wheat germ agglutinin (WGA) to stain the extracellular matrix (ECM). We found no differences in ECM accumulation within the spindle fibers themselves (ECM index (Figure 3.10C)). However, satellite cell depleted mice had lower muscle spindle size and trended towards lower spindle-lobe size (Figure 3.10D and 3.10E). Interestingly, lifelong physical activity did not rescue this phenotype, having no impact on muscle spindle size in SC- or SC+ mice.

Satellite cell depletion did not lead to fibrosis in response to lifelong physical activity

As ECM accumulation, or fibrosis, has previously been shown to be associated with blunted muscle fiber hypertrophy in response to mechanical overload in the absence of satellite cells, we utilized Picrosirius red (PSR) and WGA staining to quantify levels of ECM [90]. We found no differences in ECM accumulation in the entire cross section (total PSR and WGA staining) regardless of satellite cells or running status, ruling this out as a potential mechanism underlying blunted hypertrophic growth in the absence of satellite cells (Figure 3.11A-J).

3.4 Discussion

We report for the first time that a lack of satellite cells throughout adulthood limits muscle fiber hypertrophy in response to lifelong physical activity. Further, satellite cell depletion adversely affects muscle proprioceptor size, physical function (balance and coordination), as well as running volume. On the other hand, we show that satellite cells are not required for an oxidative shift in fiber type in response to wheel running during aging, demonstrating that processes regulating hypertrophic growth and the metabolic properties of the muscle can be uncoupled. It is not surprising that lifelong physical activity leads to higher heart weight and attenuates age-related gains in body weight that accompany murine aging. Satellite cell ablation does not influence these long term

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outcomes in response to physical activity. While wheel running induced robust training adaptations in the soleus and plantaris, the TA and EDL were not influenced by the training stimulus, likely due to the different functional demands being placed on the muscles via the wheel running stimulus [124,125].

Greater muscle fiber CSA in response to voluntary wheel running is a common but not universal finding in mice [109,126-128]. We previously reported that 2 months of running did not result in larger fiber CSA, suggesting that a protracted period of running is necessary to elicit a significant increase in muscle fiber size [109]. Importantly, the mice analyzed in this report were 20 months old at the time of sacrifice, enabling the study of skeletal muscle adaptation prior to the confounding effects of age-related reduction in muscle fiber size that occur $\geq$24 months of age [31,129,130]. Given the decline in satellite cell content with advancing age, our findings suggest increasing satellite cell content specifically in physically active older adults could be a potential therapeutic strategy to increase muscle mass in response to exercise [33,131,132]. Beyond this, falls and fractures are common in older adults, which can result in extended bouts of physical rehabilitation in otherwise sedentary individuals [133]. Our results suggest targeting satellite cells may augment the hypertrophic response to sustained periods of rehabilitation. Further, satellite cell depletion throughout adulthood reduces muscle proprioceptor size, voluntary physical activity and physical function. Declines in balance and coordination with advancing age increase the risk of falls and are associated with slower walking speeds, a major risk factor for mobility disability and the loss of independence [134-136]. Whether restoring satellite cell number may improve physical function remains to be determined but has important clinical implications.

Satellite cell depletion for 13 months does not influence ECM accumulation in sedentary or physically active mice. While the ablation of satellite cells has been shown to induce excessive ECM development in response to mechanical overload as a result of synergist ablation surgery, the adaptive response to wheel running is distinct from that of synergist ablation [90]. This distinction is likely due to the damaging and supraphysiological nature of synergist ablation-induced muscle overload [137]. In line with our previous findings, which also utilized female Pax7-DTA mice, there was no effect of

satellite cell depletion on ECM accumulation in running or sedentary mice at 8 months of age in the plantaris or at 8 and 24 months in the diaphragm muscle [109,138]. However, ECM accumulation may be exacerbated by lifelong satellite cell depletion in mice ≥ 2 years of age, as reported previously [31,139].

Satellite cell participation in muscle fiber hypertrophy differed between the soleus and plantaris in response to lifelong physical activity, consistent with the idea that distinct mechanisms regulate muscle growth in different muscles and fiber types [29,140,141]. While satellite cell content is higher in both the SC+ soleus and plantaris muscles in response to running, significant myonuclear accretion only occurs in the soleus. It may be that in response to lifelong physical activity, satellite cells potentiate growth in the soleus directly through fusion, whereas they may stimulate growth in the plantaris through other mechanisms, such as influencing the muscle microenvironment to facilitate remodeling [51]. This finding supports previous work that has indicated the reliance on myonuclear addition during muscle growth is greater in oxidative than in glycolytic muscles [89,112,139].

Based on the results of our transcriptome analysis, it appears that satellite cells are critical for the initiation of certain anabolic signaling events in response to lifelong physical activity and may function via GPCR and downstream $G\alpha_{i2}$ signaling, specifically in the plantaris. This effect may be indirect, through negative impact on spindle fiber function, thereby reducing running volume, or through direct effects on myofiber CSA in response to running, or a combination thereof. Subgroup analysis of SC- and SC+ mice that ran equal volumes suggests a direct effect on muscle GPCR signaling. GPCR-mediated signaling events are integral to anabolic signaling and muscle growth [113,116]. Further, the work of Minetti et al. suggests activation of the $G\alpha_{i2}$ signaling cascade in the presence of satellite cells likely initiates downstream phosphorylation events necessary for the stimulation of muscle protein synthesis [114,115]. While available deep sequencing data provide insight into the molecular profile of activated satellite cells and information can be gleaned as to the signaling molecules/ligands that are likely candidates influencing GPCR-related signaling, the GPCRs upregulated in the plantaris are orphan receptors, making the identification of bonafide ligands challenging [142,143]. Further research is required to identify and integrate the mechanisms whereby satellite

cells maximize physical activity and promote increases in muscle fiber size over the lifespan, that extend beyond fusion to muscle fibers.

Our findings are limited by assigning the two muscles that adapted to life-long wheel running (the plantaris and soleus) to either IHC or transcriptomic analysis, preventing further validation of findings at the protein level. Also, as all mice were female, sacrificed at 1 time point (20-months old) and given free access to running wheels, we are unable to investigate sex-specific or long term skeletal muscle adaptation in a temporal fashion while controlling for running volume. This study has several strengths including a thorough characterization of skeletal muscle adaptation to a translation stimulus with and without satellite cell participation. This work has produced clinically relevant findings with important implications for the skeletal muscle field.

In conclusion, while our previous work shows that satellite cells are not required for short term adaptation to a growth stimulus in adult mice, or maintenance of muscle mass during aging in sedentary mice, satellite cells are necessary for the maintenance of physical function and the increase in muscle fiber size in response to lifelong physical activity [31,87]. Our study provides novel insight into the muscle-specific role of satellite cells in skeletal muscle hypertrophy in response to lifelong physical activity. In more oxidative muscles, satellite cell fusion may be essential, whereas in glycolytic muscles, satellite cells may promote signaling in muscle fibers to facilitate growth. These findings suggest that preserving satellite cell content with aging could preserve physical function and improve muscle growth in older adults engaged in regular physical activity.

Table 3.1 qPCR primer list

		Sequence 5' → 3'
Gpr4	Forward primer	CTGTGCAGAGTCGGGACCAA
	Reverse primer	AGATTGCTAGGCCTGCTCCG
Gnai2	Forward primer	CTGGCAGGATGGGCCAC
	Reverse primer	CATGGTCTTCTTGCCCCCAT
Dgkh	Forward primer	CAGCCTGGACCTGGGGATT
	Reverse primer	AAGTTGGGAGGGTTCCGTTC
Prkcsh	Forward primer	CGGAGCGTTCCGTTCTCTTA
	Reverse primer	ATGGCCTCTCAGCGAGGTA
Vcp	Forward primer	TGCCAGGCCAACTTCATCTC
	Reverse primer	CCGGACATTGGCCTCAGATT

Table 3.2 TA and EDL characteristics

	TA				EDL			
	Sed, SC+	Sed, SC-	Run, SC+	Run, SC-	Sed, SC+	Sed, SC-	Run, SC+	Run, SC-
Pax7$^+$ cells/100 fibers	3.36 ± 0.19	0.09 ± 0.02	3.81 ± 0.33	0.27 ± 0.18	3.28 ± 0.51	0.14 ± 0.05	3.30 ± 0.12	0.13 ± 0.08
Myonuclei/fiber	0.91 ± 0.09	0.88 ± 0.05	0.88 ± 0.06	0.92 ± 0.07	0.85 ± 0.04	0.71 ± 0.09	0.81 ± 0.05	0.80 ± 0.06
Mean fiber CSA, μm^2	1,666 ± 60.3	1,678 ± 55.9	1,647 ± 102.9	1,636 ± 42	1,240 ± 54.7	1,192 ± 41.2	1,244 ± 42.6	1,248 ± 40.17

Values are mean ± SEM. N = 6-9 mice/group. Sed, Sedentary conditions; Run, Running conditions.
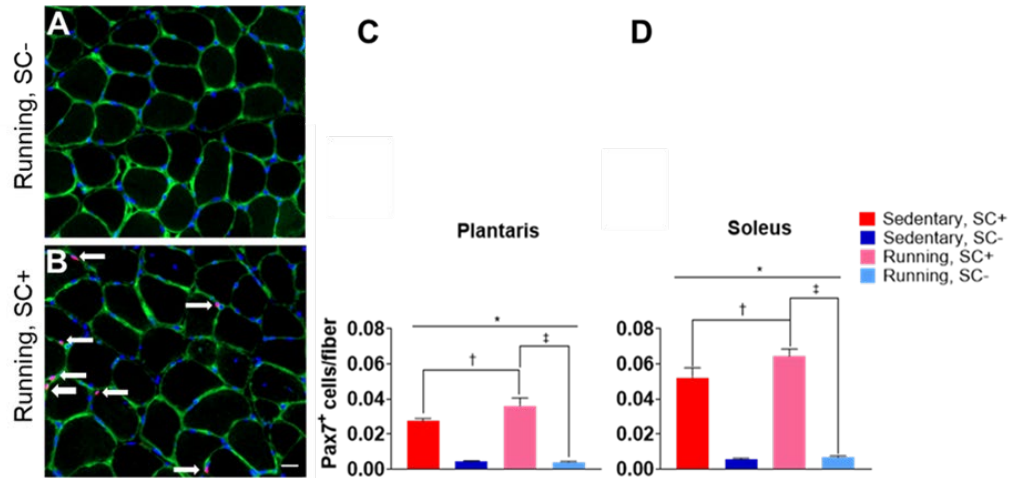
Figure 3.1

Higher satellite cell density in the plantaris and soleus muscles of running satellite cell replete (SC+) compared to depleted (SC-) mice. (A-B) Representative image of satellite cell immunohistochemistry showing laminin (green), nuclei (blue), and Pax7 (red; white arrows). Scale bar = 20 μm. (C-D) Satellite cell density in the plantaris and soleus. Data represent mean ± SEM. n = 6-9 mice per group. Data were analyzed via a two factor ANOVA. Post hoc comparisons were made with Sidak post-tests. *$P < 0.05$, interaction effect between condition and treatment; † $P < 0.05$, running SC+ vs sedentary SC+; ‡ $P < 0.05$, running SC+ vs running SC-.
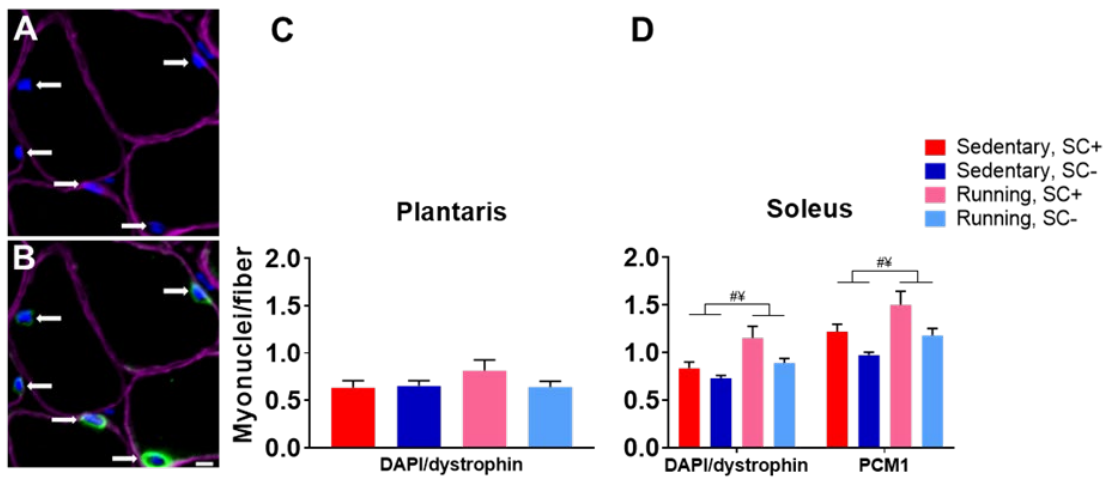
Figure 3.2

Myonuclear accretion with lifelong physical activity was dependent on the presence of satellite cells. (A) Representative image of dystrophin (purple) and DAPI (blue) staining from muscle cross sections to identify myonuclei (white arrows). (B) Representative image of dystrophin (purple) and DAPI (blue) and PCM1 (green) staining from muscle cross sections to identify myonuclei (white arrows). Scale bar = 5 μm. (C-D) Myonuclear density of the plantaris and soleus. Data represent mean ± SEM. n = 6-9 mice per group. Data were analyzed via a two factor ANOVA. #$P < 0.05$, running vs sedentary mice; ¥ $P < 0.05$, SC+ vs SC- mice.
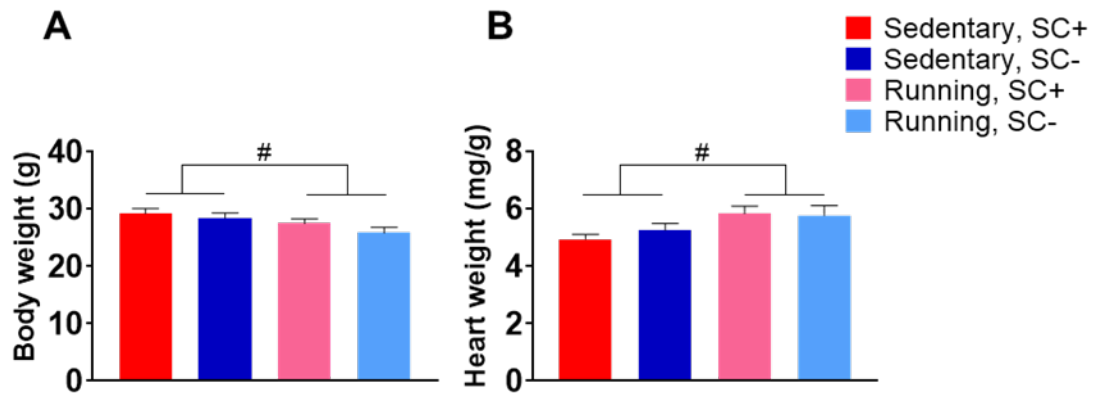
Figure 3.3

Lifelong physical activity led to a lower body weight and higher heart weight

independent of satellite cells. (A) Body weight. (B) Heart weight normalized to body

weight. Data represent mean ± SEM. n = 7-9 mice per group. Data were analyzed via a

two factor ANOVA. #$P < 0.05$, running vs sedentary mice.
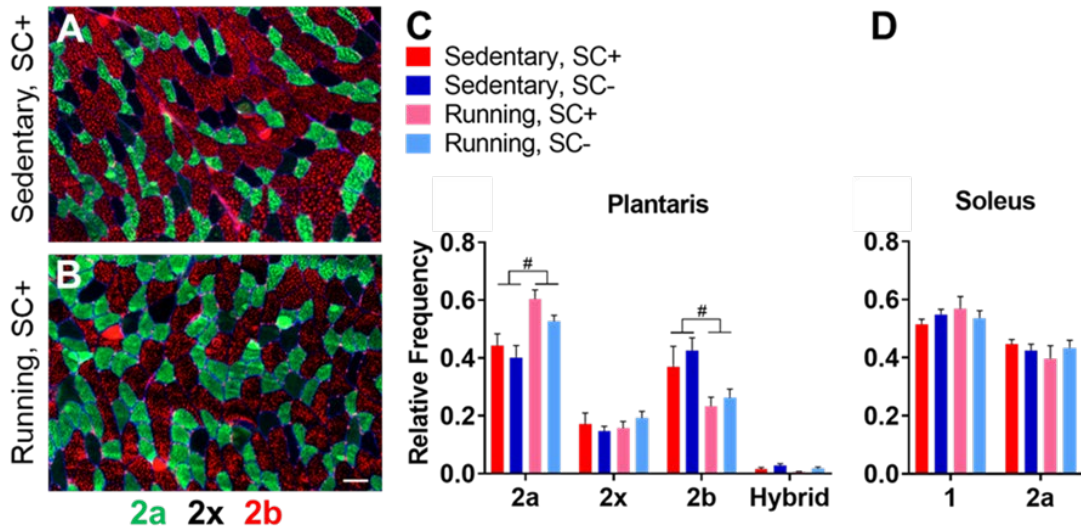
Figure 3.4

Lifelong physical activity led to an oxidative shift in muscle fiber composition in the plantaris. (A-B) Representative images of the plantaris muscle stained for dystrophin (blue), myosin heavy chain 2a (green) and myosin heavy chain 2b (red). Scale bar = 50 μm. (C-D) Relative frequency of fiber type in the plantaris and soleus. Data represent mean ± SEM. n = 6-9 mice per group. Data were analyzed via a two factor ANOVA. #$P < 0.05$, running vs sedentary mice.
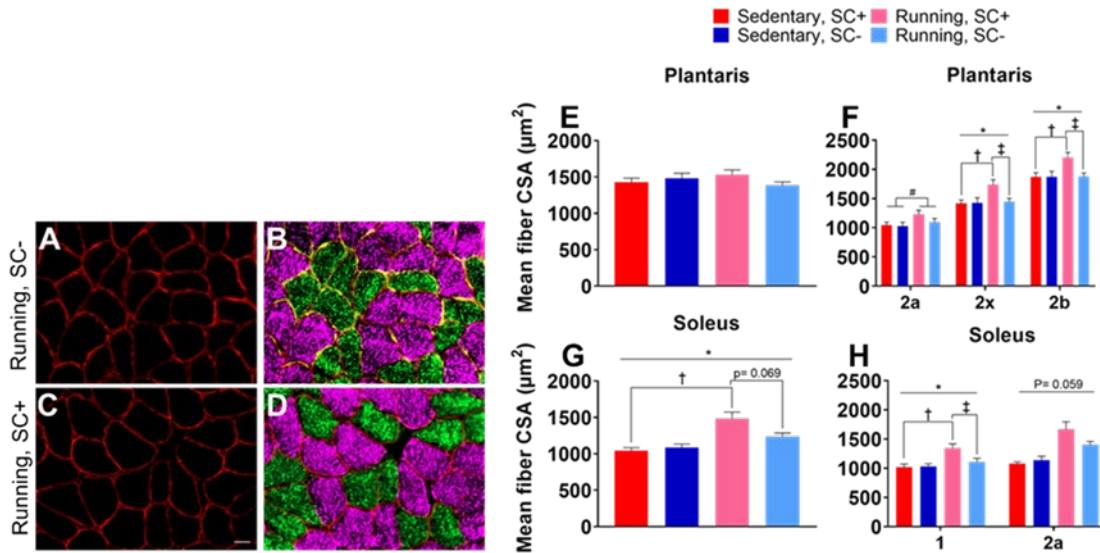
Figure 3.5

Satellite cell depletion limited the hypertrophic response induced by lifelong physical activity. (A-D) Representative images of muscle fiber size in the soleus stained for dystrophin (red), type 1 myosin heavy chain (pink) and type 2a (green) myosin heavy chain. Scale bar = 20 μm. (E) Whole muscle mean fiber CSA in the plantaris. (F) Mean fiber CSA by fiber type in the plantaris. (G) Whole muscle mean fiber CSA in the soleus. (H) Mean fiber CSA by fiber type in the soleus. Data represent mean ± SEM. n = 6-9 mice per group. Data were analyzed via a two factor ANOVA. Post hoc comparisons were made with Sidak post-tests. *$P < 0.05$, interaction effect between condition and treatment; † $P < 0.05$, running SC+ vs sedentary SC+; ‡ $P < 0.05$, running SC+ vs running SC-.
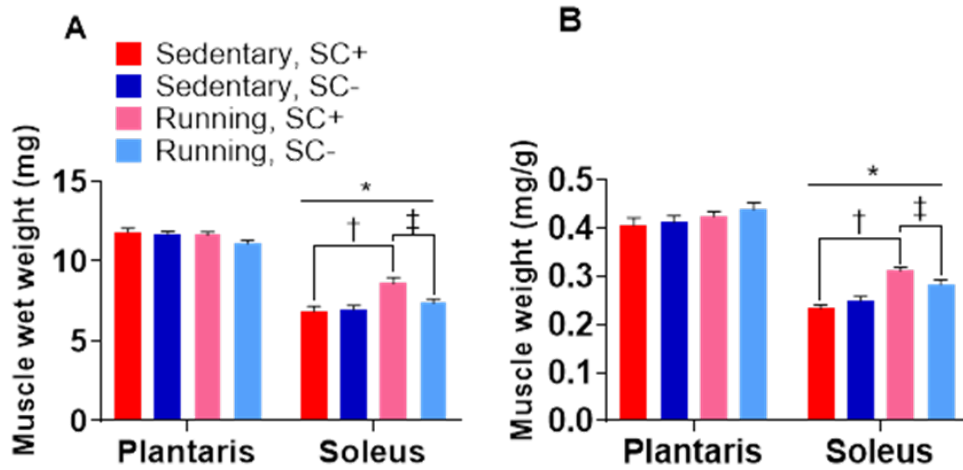
Figure 3.6

Satellite cell depletion limited muscle growth in the soleus induced by lifelong physical activity. (A) Muscle wet weights in the plantaris and soleus. (B) Muscle weights normalized to body weight. Data represent mean ± SEM. n = 7-9 mice per group. Data were analyzed via a two factor ANOVA. Post hoc comparisons were made with Sidak post-tests. *$P < 0.05$, interaction effect between condition and treatment; † $P < 0.05$, running SC+ vs sedentary SC+; ‡ $P < 0.05$, running SC+ vs running SC-.

A Plantaris / Soleus Venn diagram: 1325 | 105 | 1318

B

P-value	Pathway
1.47E-17	Olfactory transduction - Mus musculus (mouse)
2.57E-09	GPCR downstream signaling
3.88E-09	Signaling by GPCR
6.62E-09	Olfactory Signaling Pathway
1.58E-07	Signal Transduction
0.000	Formyl peptide receptors bind formyl peptides and many other ligands
0.001	Arachidonic acid metabolism - Mus musculus (mouse)
0.002	G alpha (i) signalling events
0.002	GPCR ligand binding
0.002	Cytokine-cytokine receptor interaction - Mus musculus (mouse)

C

P-value	Pathway
7.46E-06	mRNA 3,-end processing
7.46E-06	Post-Elongation Processing of Intron-Containing pre-mRNA
6.65E-05	Cleavage of Growing Transcript in the Termination Region
6.65E-05	RNA Polymerase II Transcription Termination
6.65E-05	Post-Elongation Processing of the Transcript
8.67E-05	mRNA Splicing - Major Pathway
8.67E-05	mRNA Splicing
0.001	Nucleotide-like (purinergic) receptors
0.002	Processing of Capped Intron-Containing Pre-mRNA
0.003	P2Y receptors

D

E
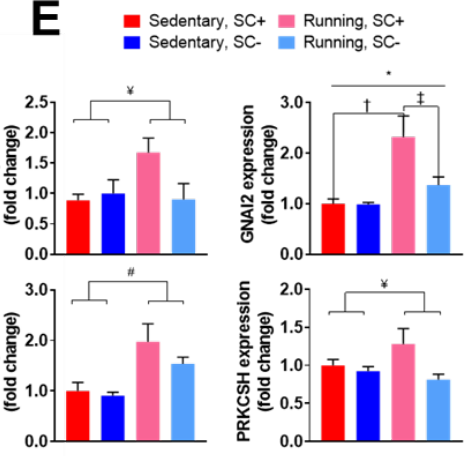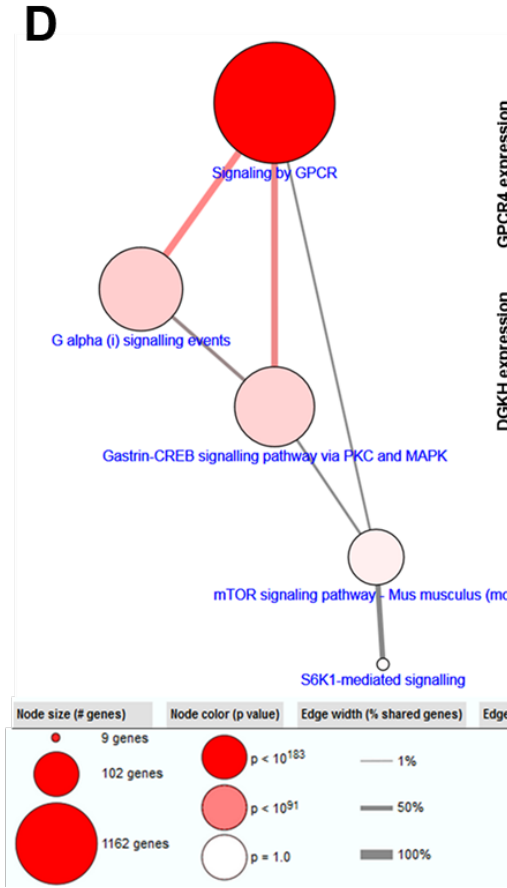
Figure 3.7

Lifelong exercise in the presence of satellite cells influenced global transcription and up-regulated expression of genes involved in GPCR signaling pathways in the plantaris. (A) Venn diagram of genes enriched in the soleus and plantaris of satellite cell replete runners. Top 10 gene sets overrepresented in the (B) plantaris and (C) soleus. (D) Gene set enrichment analysis highlighting the $G\alpha_{i2}$ signaling network in the plantaris. (E) RT-qPCR results of $G\alpha_{i2}$ genes verified the results seen in our gene set enrichment analysis. Data represent mean $\pm$ SEM. n = 6-9 mice per group. Data were analyzed via a two factor ANOVA. Post hoc comparisons were made with Sidak post-tests. *$P < 0.05$, interaction effect between condition and treatment; † $P < 0.05$, running SC+ vs sedentary SC+; ‡ $P <$ 0.05, running SC+ vs running SC-. #$P < 0.05$, running vs sedentary mice; ¥ $P < 0.05$, SC+ vs SC- mice.

A

P-value	Pathway
1.12E-22	Olfactory transduction - Mus musculus (mouse)
8.83E-14	Olfactory Signaling Pathway
1.04E-09	GPCR downstream signaling
2.13E-09	Signaling by GPCR
1.31E-07	Signal Transduction

B

Figure 3.8

Differences in enriched gene sets persisted after controlling for running volume. (A) Top 5 gene sets overrepresented in the plantaris of satellite cell replete mice when compared to depleted mice that ran equal volumes. (B) Gene set enrichment analysis highlighting the Gα_{i2} signaling network.

Figure 3.9

Satellite cell depletion affected muscle spindle characteristics but not ECM accumulation.

(A-B) Representative images of muscle spindle fibers from the plantaris muscle. Scale

bar = 5 μm. Quantification of, (C) ECM index, (D) spindle area and (E) spindle lob size.

Data represent mean ± SEM. n = 4 mice per group. Data were analyzed via a two factor

ANOVA. ¥ P < 0.05, SC+ vs SC- mice.
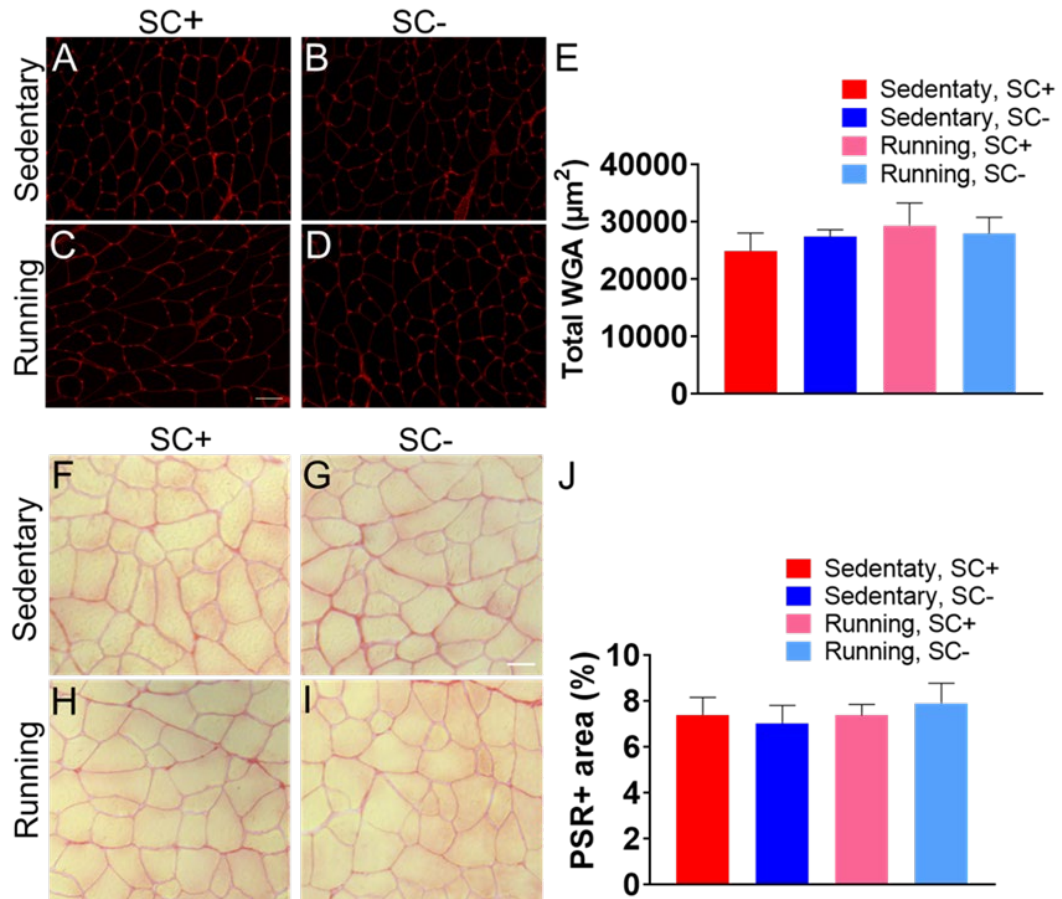
Figure 3.10

Satellite cell depletion did not cause fibrosis in response to lifelong physical activity. (A-E) Representative WGA-stained muscle cross sections and quantification. Scale bar = 50 μm. (F-J) Representative PSR-stained muscle cross sections and quantification. Scale bar = 20 μm. Data represent mean ± SEM. n = 7-8 mice/group.

CHAPTER 4: RESIDENT MUSCLE STEM CELL DEPLETION BLUNTS

MUSCLE FIBER HYPERTOPHY AFTER SHORT AND LONG TERM

WEIGHTED WHEEL RUNNING

Abstract of Chapter 4

Satellite cells, the resident muscle stem cell, are required for post-natal development, skeletal muscle regeneration across the lifespan and skeletal muscle growth before maturity ($\geq$ 4 months old). Certain groups, including our own, have aimed to specifically address whether satellite cells are required for hypertrophic growth in mature skeletal muscle. We have reported that satellite cells are not required for hypertrophy in response to short-term testosterone administration or synergist ablation induced mechanical overload; however, we have discovered that sustained periods of muscle growth during long term-term synergist ablation or wheel running requires satellite cells for maximal adaptation. Certain studies even suggest the absence of myonuclear addition completely inhibits muscle growth. These divergent results highlight distinct cellular processes regulating muscle adaptation in response to satellite cell depletion based on the chosen intervention. Here, we aimed to generate a comprehensive characterization and transcriptome-wide profiling of skeletal muscle during growth and adaptation in the presence and absence of satellite cells in order to identify distinct phenotypes and signaling networks influenced by satellite cell content. We administered vehicle or tamoxifen to adult Pax7-Diptheria Toxin A (Pax7-DTA) mice and subjected them to 4 and 8 weeks of progressive weighted wheel running (PoWeR) and performed immunohistochemical (IHC) analysis on the soleus (oxidative phenotype) and plantaris (mixed oxidative and glycolytic phenotype) of vehicle- (SC+) and tamoxifen-treated (SC-) mice and whole-muscle RNA-seq on the soleus. We further performed single myonuclear (smn) RNA-seq on the soleus muscle of SC+ and SC- mice after PoWeR to provide distinct information on how satellite cell fusion affects myonuclear transcription.

We show that while skeletal muscle can mount a robust hypertrophic response to PoWeR in the absence of satellite cells, growth is ultimately blunted at 4 and 8 weeks in the soleus and at 8 weeks in the plantaris. Transcriptome-wide profiling revealed several signaling networks key to muscle adaptation are dysregulated in the absence of satellite cells in the soleus at the 4 and 8 week time points.

4.1 Introduction

Satellite cells, the resident muscle stem cell, are required for post-natal development, skeletal muscle regeneration across the lifespan and skeletal muscle growth before maturity (≥ 4 months old) [19,86,87,91]. Certain groups, including our own, have aimed to specifically address whether satellite cells are required for hypertrophic growth in response to mechanical loading, testosterone administration (see Chapter 2), and exercise (see Chapter 3), in mature skeletal muscle [20,24,34,62,87,90]. Findings vary and seem to be influenced by a host of confounding variables across studies, such as the mode and length of perturbation utilized to induce muscle growth, the duration of satellite cell depletion, the timing of depletion (pre, post or at some point during adaptation) and the mouse model utilized [20,24,34,62,90,109]. Our group has leveraged the Pax7-Diptheria Toxin A (Pax7-DTA) strain to deplete satellite cells at several time points and over various durations to gain a fundamental understanding for the requirements of satellite cells during adaptation and growth [31,34,90,91,139]. To date, we have reported that satellite cells are not required for hypertrophy in response to short-term testosterone administration or synergist ablation induced mechanical overload; however, we have discovered that sustained periods of muscle growth during long term-term synergist ablation or wheel running requires satellite cells for maximal adaptation [34,62,90].

Our findings in response to synergist ablation show activated satellite cells play a critical role in regulating fibrosis during growth via exosome delivery of miR-206 to fibroblasts, targeting Rrbp1 and suppressing collagen biosynthesis [51]. Interestingly, the presence of satellite cells was only required for the first week of muscle overload in order to mount effective extracellular matrix remodeling and growth [51]. This suggests that

blunted long-term growth in the absence of satellite cells is related more to secretory signaling via activated satellite cells than to a lack of satellite cell fusion and myonuclear accretion, at least in response to synergist ablation [51,90]. Interestingly, in response to a more translational model of muscle adaptation, long term wheel running, hypertrophy in the absence of satellite cells was attenuated, independent of fibrosis, as shown in Chapter 3 [34]. Adding to this complexity, in Chapter 3, our data show there may be muscle-specific roles for satellite cells, with glycolytic muscles being more reliant on the secretory function of satellite cells and oxidative muscles more reliant on fusion. Conversely, findings from the Myomaker[scKO] mouse strain (a model of impaired satellite cell fusion) suggest the absence of myonuclear addition completely inhibits muscle growth in response to synergist ablation and uphill treadmill running [20,24]. These divergent results highlight distinct cellular processes regulating muscle adaptation in response to satellite cell depletion based on the applied stimuli and potentially the mouse strain being used.

While synergist ablation has classically been utilized to study muscle hypertrophy in mice, several groups have recently developed models of muscle growth and adaptation that can be more easily translated to humans [24,63,144-146]. Our group published a report characterizing a progressive weighted wheel running (PoWeR) protocol that induces robust skeletal muscle adaptation and growth with a resulting phenotype very similar to that of progressive cycle training in humans [112]. This new model, along with others, provide an opportunity to perform rigorous and translational studies to generate novel information that further elucidates how satellite cells influence skeletal muscle adaptation.

Here, we aimed to generate a comprehensive characterization and transcriptome-wide profiling of skeletal muscle during growth and adaptation in the presence and absence of satellite cells in order to identify distinct phenotypes and signaling networks influenced by satellite cell content. We subjected Pax7-DTA mice to 4 and 8-weeks of PoWeR and performed immunohistochemical (IHC) analysis on the soleus (oxidative phenotype) and plantaris (mixed oxidative and glycolytic phenotype) of vehicle- (SC+) and tamoxifen-treated (SC-) mice and whole-muscle RNA-seq on the soleus. We further performed single myonuclear (smn) RNA-seq on the soleus muscle of SC+ and SC- mice after PoWeR to provide distinct information on how satellite cell fusion affects myonuclear transcription. We show that while skeletal muscle can mount a robust hypertrophic response to PoWeR in the absence of satellite cells, growth is ultimately blunted at 4 and 8 weeks in the soleus and at 8 weeks in the plantaris. Transcriptome-wide profiling revealed several signaling networks key to muscle adaptation are dysregulated in the absence of satellite cells in the soleus at the 4 and 8 week time points.

4.2 Methods

Animals

The Pax7$^{CreER/+}$-R26$^{DTA/+}$ strain, called Pax7-DTA, was generated by crossing the Pax7$^{CreER/CreER}$ mouse strain with the Rosa26$^{DTA/DTA}$ mouse strain [3]. The Pax7-DTA mouse allows for the specific and inducible depletion of satellite cells upon tamoxifen treatment, through *Cre*-mediated recombination to induce expression of the diphtheria toxin A (DTA) gene in Pax7-expressing cells, effectively killing satellite cells. All animal procedures were conducted in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Experimental design

For initial whole-muscle RNA-seq and IHC experiments, forty-eight adult (6-months old) female Pax7-DTA mice were treated via intraperitoneal injection with vehicle (SC+) (15 % ethanol in sunflower seed oil) or tamoxifen (SC-) at a dose of 2.5 mg/day for five days, as previously described [87]. Following a 1-wk washout period, mice were singly housed and randomly assigned to 4-wk Sedentary SC+/SC- or 8-wk Sedentary groups (cage with locked running wheel), 4-wk PoWeR SC+/SC- groups or 8-wk PoWeR SC+/SC- groups (n=8 per group). After 1-week of acclimation to an unweighted wheel, 4-wk PoWeR consisted of 2 g of weight in week 1, 3 g in week 2, 4 g in week 3, 5 g in week 4; 8-wk PoWeR consisted of 2 g of weight in week 1, 3 g in week 2, 4 g in week 3, 5 g in weeks 4 and 5, and 6 g in weeks 6–8 **(Figure 1A)**. The wheels were loaded with 1 g magnets (product no. B661, K&J Magnetics, Pipersville, PA). ClockLab software (Actimetrics, Wilmette, IL) was used to record running behavior. The animals had access to food and water ad libitum and were checked daily for health and wellness. Running wheels were locked 24 hours before sacrifice and mice were fasted overnight. The soleus and plantaris muscles were excised, weighed and prepared for IHC analysis and the contralateral limb for RNA extraction, and then stored at −80 °C. As there were no differences in body or muscle weights between the 4 and 8-week time points for both SC+ and SC- sedentary mice, time points were collapsed and treated as one group. An additional 6 mice were randomly assigned to the 4-wk PoWeR SC+/SC- groups. Wheels were locked 4 or 24 hours before sacrifice and mice were fasted overnight. The soleus muscle was excised at both time points and prepared immediately

for smnRNA-seq. Another group of 6 mice were randomly assigned to the 8-wk PoWeR SC+/SC- groups for in vivo force testing. Wheels were locked 48 hours before testing.

Immunohistochemistry

IHC analyses were performed as previously described [91]. Briefly, hindlimb muscles were extracted and weighed, then were pinned to a cork block at resting length and covered with Tissue Tek Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA, USA), and quickly frozen in liquid nitrogen cooled isopentane and stored at -80°C. Frozen muscles were sectioned at -23°C (7 µm), air-dried for at least one hour, and then stored at -20°C. For determining fiber type distribution, muscle fiber cross-sectional area (CSA), fiber type-specific CSA, and myonuclear density, cross sections were incubated overnight in a cocktail of isotype specific anti-mouse antibodies for MyHC 1 (1:75, IgG2B, BA.D5), MyHC 2a (neat, IgG1, SC.71), and MyHC 2b (neat, IgM, BF.F3) from Developmental Studies Hybridoma Bank (DSHB, Iowa City, Iowa, USA), along with an antibody against dystrophin (1:100, ab15277, Abcam, St. Louis, MO, USA), to delineate fiber borders for CSA quantification. Sections were subsequently incubated with secondary antibodies (1:250, goat anti-mouse IgG2b Alexa Fluor 647, #A21242; 1:500, IgG1 Alexa Fluor 488, #A21121; 1:250, IgM Alexa Fluor 555, #A21426) from Invitrogen (Carlsbad, CA, USA), along with the secondary antibody for dystrophin (1:150, anti-rabbit IgG AMCA, CI-1000, Vector), diluted in PBS. Sections were mounted using VectaShield with DAPI (H-1200, Vector).

Detection of Pax7+ cells was performed as previously described [87]. Briefly, sections were fixed in 4% paraformaldehyde (PFA) followed by antigen retrieval using sodium citrate (10 mM, pH 6.5) at 92°C. Endogenous peroxidase activity was blocked

with 3% hydrogen peroxide in phosphate-buffered saline (PBS) followed by an additional blocking step with 1% Tyramide Signal Amplification (TSA) blocking reagent (TSA kit, T20935, Invitrogen) and Mouse-on-Mouse blocking reagent (Vector Laboratories, Burlingame, CA, USA). Pax7 primary antibody (1:100, DSHB) and laminin primary antibody (1:100, Sigma-Aldrich) were diluted in 1% TSA blocking buffer and applied overnight. Samples were then incubated with anti-mouse biotin-conjugated secondary antibody against the Pax7 primary antibody (1:1000, 115-065-205, Jackson ImmunoResearch, West Grove, PA, USA) and anti-rabbit secondary for laminin (1:250, A11034, Alexa Fluor 488, Invitrogen, Carlsbad, CA, USA). Slides were washed in PBS followed by streptavidin-horseradish peroxidase (1:500, S-911, Invitrogen) for 1 hour. AlexaFluor 594 was used to visualize antibody-binding for Pax7 (1:100, TSA kit, Invitrogen). Sections were mounted and nuclei were stained with Vectashield with DAPI (H-1200, Vector).

Detection of N-acetyl-d-glucosamine was evaluated on muscle sections using Texas Red-conjugated wheat germ agglutinin (WGA (eBiosciences, San Diego, CA)). Sections were fixed in 4 % PFA and then incubated with WGA conjugate for 2 hours at room temperature. Sections were stained with Picrosirius red (PSR) for 1 hour per the manufacturer's instructions (24901-500; Polysciences, Warrington, PA, USA). PSR staining was used according to the manufacturer's recommendations to visualize the collagen network under polarized light. Detection of capillaries was evaluated on muscle sections using lectin [147]. Fresh muscle sections were cut and allowed to air dry for 4 hours, rehydrated with PBS, blocked in 2.5% normal horse serum (Vector Laboratories,

S-2012) and incubated in Texas Red conjugated Lectin from Griffonia Simplicifolia Lectin I (1:50) diluted in 2.5% NHS (Sigma, L4889).

Image quantification

All WGA images were captured at 10x magnification, and the total stained area quantified using the thresholding feature of the AxioVision Rel software, as described previously [109]. PSR images were captured at 10x magnification and quantifications were obtained with ImageJ software. For analysis, polarized images were analyzed and the amounts of densely (red) and loosely (green) packed collagen were quantified in the muscle cross sections relative to muscle area with ImageJ. Lectin images were captured at 10x magnification and the thresholding feature of the AxioVision Rel software was used to identify Lectin+ capillaries. All remaining images were captured at 20x magnification at room temperature using a Zeiss upright fluorescent microscope (Zeiss AxioImager M1 Oberkochen, Germany). Whole muscle sections were obtained using the mosaic function in Zeiss Zen 2.3 imaging software. To minimize bias and increase reliability, fiber type distribution, muscle fiber CSA, fiber type-specific CSA and myonuclear density were quantified on cross sections using MyoVision automated analysis software [106]. To determine satellite cell density (Pax7+ cells/fiber), satellite cells (Pax7+/DAPI+) were counted manually on entire muscle cross sections using tools in the Zen software. Satellite cell counts were normalized to fiber number, delineated by laminin boundaries. All manual counting was performed by a blinded, trained technician.

RNA isolation

Soleus muscles were homogenized in QIAzol (Qiagen, Hilden, Germany) and RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's

instructions. Two µg of RNA was eluted in 50 µL of nuclease-free water (concentration

50ng/µL) and purity checked: RIN ≥6.8 OD260/280 >2.0 and sent to Novogene for

subsequent library construction, sequencing and preliminary bioinformatic analysis. RNA

samples where pooled (n=4/group) based on the experimental results reported by

Kendziorski et al. showing that gene expression from RNA pools are similar to averages

of individuals that comprise the pool [122].

RNA-seq analysis

Downstream analysis was performed using a combination of programs including

STAR, HTseq, Cufflink and our wrapped scripts. Alignments were parsed using Tophat

program and differential expressions were determined through DESeq2/edgeR. Reference

genome and gene model annotation files were downloaded from genome website browser

(NCBI/UCSC/Ensembl) directly. Indexes of the reference genome was built using STAR

and paired-end clean reads were aligned to the reference genome using STAR (v2.5).

STAR used the method of Maximal Mappable Prefix(MMP) which can generate a precise

mapping result for junction reads. HTSeq v0.6.1 was used to count the read numbers

mapped of each gene. And then FPKM of each gene was calculated based on the length

of the gene and reads count mapped to this gene. FPKM, Reads Per Kilobase of exon

model per Million mapped reads, considers the effect of sequencing depth and gene

length for the reads count at the same time, and is currently the most commonly used

method for estimating gene expression levels [148]. Differential expression analysis

between two conditions/groups (two biological replicates per condition) was performed

using the DESeq2 R package (2_1.6.3). DESeq2 provide statistical routines for

determining differential expression in digital gene expression data using a model based

on the negative binomial distribution. The resulting P-values were adjusted using the

Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR).

Genes with an adjusted P-value <0.1 found by DESeq2 were assigned as differentially

expressed.

Myonuclear isolation

Soleus muscles were excised immediately following euthanasia and the protocol

developed by Cutler el al. was followed for isolation of myonuclei [149]. Briefly, muscles

were minced with scissors in homogenization buffer (500 µl HEPES (1 M) 3 ml KCl (1

M) 250 µl spermidine (100 mM) 750 µl spermine tetrahydrochloride (10 mM) 10 ml

EDTA (10 mM) 250 µl EGTA (100 mM) 2.5 ml MgCl (100 mM) 5.13 g sucrose Ultra-

pure water to 50 ml Filter sterilize and store at 4 °C for up to 1 month. Immediately prior

to use add: 100 µl DTT (1 M) 5 Roche complete mini protease inhibitor tablets), dounced

on ice in homogenization buffer and passed through a 40 µm filter into sorting buffer.

DAPI was added to label nuclei and fluorescently-labeled nuclei were purified via

Fluorescence-activated cell sorting (FACS) and collected in reverse transcription (RT)

buffer.

Construction of libraries and generation of cDNA on the 10x genomics platform

Nuclei were loaded into the 10X Chromium system using the Single Cell Reagent

Kit v3 according to the manufacturer's protocol. Following library construction, libraries

were sequenced on the Illumina NovaSeq 6000 system at the University of Florida.

Single myonuclear (smn)RNA-seq analyses

The Cell Ranger Single-Cell Software Suite was used to perform sample

demultiplexing, barcode processing and single-cell 3′ gene counting

(http://software.10xgenomics.com/single-cell/overview/welcome). The cDNA insert was aligned to an appropriate reference genome using STAR. For mouse cells, mm10 was used. Partek Flow was used for all downstream analysis from Filtered_Barcode_Matrix.h5 files. Filter criteria included total reads: (Min: 499, Max: 20,567), expressed genes: (Min: 400, Max: 4,018) and mitochondrial reads percent: (Min: 0%, Max: 15.00%). Additional filtering was performed which excluded features where values were < 1.0 in at least 99.9% of the samples yielding a total of 9,358 genes for downstream analysis. Samples were normalized using counts per million and log transformation. Principal component analysis was used prior to t-SNE for visual based clustering. Myonuclei for each sample were classified by Acta1 expression. Gene set analysis was performed between selected time points and treatments. Low-value filtering was set to 1.0 for lowest average coverage and FDR step-up (Adj p < 0.01) was used for multiple test correction.

Pathway analysis

Differentially expressed gene lists (DEGs) were uploaded to g:Profiler for pathway analysis and uploaded to Cytoscape v3.8 for subsequent enrichment analysis and data visualization. The GeneMANIA application within Cytoscape was also used for network category analysis and data visualization. Nodes were generated with an adjusted p value < 0.1 for enrichment. The size of each node is scaled to the gene set size, the shape of each node is specific to GO (circle) or pathway (diamond) enrichment. The color of the node is scaled to the adjusted p value. Edges connecting nodes are scaled to the similarity coefficient (genes shared between nodes). Network categories included: co-expression: genes are linked if their expression levels are similar across conditions in a

gene expression study. Co-localization: genes are linked if they are both expressed in the same tissue or if their gene products are both identified in the same cellular location. Genetic interaction: genes are functionally associated if the effects of perturbing one gene were found to be modified by perturbations to a second gene. Pathway: two gene products are linked if they participate in the same reaction within a pathway. Physical interaction: genes are linked if they were found to interact in a protein-protein interaction study. Predicted: predicted functional relationships between genes.

Strength testing

Following PoWeR training, strength of plantar flexor muscles was determined by in vivo isometric tetanic torque. Mice were anesthetized by 2.5% isoflurane with oxygen set at 1.5 l/min (VetEquip vaporizer) in an induction chamber and transferred to a nose cone. The right hind limb was assessed for all mice. Fur on the lower two-thirds of the hind limb was trimmed (Wahl Bravmini), and the mouse was placed on a 37°C temperature regulated platform (809c in-situ mouse apparatus, Aurora Scientific, Aurora, ON, Canada). The hind limb was securely positioned using a clamp at the knee, and the foot was placed in a footplate attached to a dual-mode lever and motor (300D-300C-LRFP, Aurora Scientific). Tape was wrapped around the foot and footplate to prevent compensatory movement or placement shifting, and the apparatus was adjusted to ensure the tibia was parallel with the platform with a 90-degree angle at the ankle. Platinum needle electrodes were placed percutaneously lateral to the knee to stimulate the tibial nerve via an electric stimulator (High Power Bi-Phase Stimulator, Aurora Scientific). Needles were adjusted to identify the optimal placement to generate maximum torque production and eliminate activation of dorsiflexors, using the Instant Stim function with

Live View in Dynamic Muscle Control LabBook (DMC v6.000). The level of electrical

current to stimulate maximal torque output was determined by a series of twitches (0.05 s

pulse duration) beginning at 10 mA and increasing to approximately 50 mA until the

maximum isometric torque stimulated by the minimum current was determined. This

current setting remained constant throughout the subsequent torque-frequency curve to

determine maximum isometric tetanic torque (10 Hz, 40 Hz, 80 Hz, 120 Hz, 150 Hz, 180

Hz, and 200 Hz, 0.25s pulse duration with a 2 min rest period between each stimulus).

All data were collected with DMC v6.000 and analyzed using Dynamic Muscle Analysis

(DMA v5.501).

Statistical Analysis

Results are presented as mean ± SEM. Data were analyzed with GraphPad Prism

software, via a two-way ANOVA with Tukey's correction for multiple comparisons, a

one-way repeated measures ANOVA with Sidak's correction for multiple comparisons or

an unpaired two-tailed Student's t-test. Significance was set at a p value <0.05.

4.3 Results

PoWeR-induced muscle growth is blunted in the absence of satellite cells

Neither satellite cell depletion nor PoWeR influenced body weight over the

duration of the study (Figure 4.1B). There was no difference in average daily running

volume over the 4-wk and 8-wk PoWeR protocols and this was not influenced by the

presence of satellite cells (Figure 4.1C). PoWeR induced higher normalized soleus

weight after 4-wks and 8-wks of PoWeR; this effect was blunted at both time points in

the absence of satellite cells (Figure 4.1D). PoWeR induced higher normalized plantaris

weight after 4-wks and 8-wks of PoWeR; this effect was blunted at 8-wks in the absence of satellite cells (Figure 4.1E).

Higher satellite cell density in the soleus and plantaris of SC+ PoWeR trained mice

Tamoxifen administration depleted satellite cells ≥90% in the soleus and plantaris in sedentary and PoWeR trained mice. This is shown qualitatively in representative satellite cell IHC from the soleus (Figure 4.2A) and quantitatively in the soleus and plantaris (Figures 4.2B and C). PoWeR training led to higher satellite cell density at 4-wks and 8-wks in the soleus and plantaris of SC+ mice only. This is shown qualitatively in representative satellite cell IHC from the soleus (Figure 4.2A) and quantitatively in the soleus and plantaris (Figures 4.2B and C).

PoWeR led to a greater proportion of oxidative fibers in the plantaris, independent of satellite cell content

Four-wks of PoWeR training led to an oxidative shift in fiber type distribution in the plantaris. This is shown qualitatively in representative fiber type IHC (Figure 4.3A) and quantitatively in Figure 4.3B. Fiber type distribution did not differ between 4-wks and 8-wks of PoWeR in the plantaris (Figure 3B). The soleus did not experience any alterations in fiber type distribution in response to PoWeR (Figure 4.3C).

Higher myonuclear density and fiber size in the soleus and plantaris of SC+ PoWeR trained mice

PoWeR training led to higher myonuclear density at 4-wks in the soleus and plantaris in PoWeR trained mice, which did not increase further at 8-wks. This is shown

qualitatively in representative dystrophin/DAPI staining from the soleus (Figure 4.4A) and quantitatively in the soleus and plantaris (Figures 4.4B and C). PoWeR training led to higher muscle fiber size at 4-wks and 8-wks in both the soleus and plantaris. Satellite cell depletion blunted growth at 4-wks and 8-wks in the soleus and at 8-wks in the plantaris. Fiber size assessment is shown qualitatively in the soleus (Figure 4.4A) and quantified for both the soleus and plantaris (Figures 4.4D and E).

Extracellular matrix accumulation did not limit muscle fiber hypertrophy in response to PoWeR

As we have previously shown excess extracellular matrix (ECM) accumulation is associated with blunted skeletal muscle hypertrophy in response to 8-wks of overload induced by synergist ablation in the absence of satellite cells, we wanted to assess ECM accumulation in response to PoWeR [90]. Neither PoWeR nor satellite cell depletion led to increased levels of glycosaminoglycans surrounding the muscle fibers, measured by wheat germ agglutinin (WGA) staining. This is shown qualitatively in WGA-stained plantaris cross sections (Figure 4.5A), quantified in Figure 4.5B.

Whole-muscle and smnRNA-seq reveal dysregulated signaling during adaptation to 4-wks of PoWeR in SC- skeletal muscle

In an effort to gain an understanding of the underlying mechanisms blunting muscle fiber hypertrophy in SC- skeletal muscle we performed whole-muscle RNA-seq and single myonuclear (smn)RNA-seq on satellite cell replete and deplete soleus. After 4-wks of PoWeR training 1902 genes were overexpressed in SC+ skeletal muscle and 2600 were overexpressed in SC- skeletal muscle relative to their sham controls (Figure 4.6A). Gene ontology (GO) and pathway analyses revealed substantial differences in gene

expression in response to 4-wks of PoWeR between SC+ and SC- skeletal muscle (Figures 4.6B and C). Genes involved in ECM remodeling (both in GO and pathway analyses) and capillarization (GO analysis) were significantly overexpressed only in the presence of satellite cells in response to PoWeR. The SC- group had no statistically enriched pathways. Enrichment mapping, combining GO and pathway analyses, allowed more detailed analyses of the pathways that comprise the nodes of gene networks differentially enriched in SC+ (blue) or SC- (pink) soleus (Figure 4.7A). Figure 4.7B expands the ECM remodeling network and Figure 4.7C expands the capillarization network, with the color of each node scaled to the adjusted p value and the node size scaled to the gene set size. Edge sizes connecting nodes were scaled to the similarity coefficient (genes shared between nodes). Highly enriched nodes in the ECM remodeling network, collagen activated tyrosine kinase receptor signaling pathway (Figure 4.7D), and the capillarization network, artery morphogenesis (Figure 4.7E), specific to SC+ soleus muscle were further analyzed in GeneMANIA to visualize and provide information around the genes within each node. The color of the node for each gene is scaled to an interaction score, defined as the effect of a given gene on the selected pathway. Edges represent network categories (see Methods for detailed description).

After 4-wks of PoWeR training 1844 genes were underexpressed in SC+ skeletal muscle and 2502 were underexpressed in SC- skeletal muscle compared to their sham controls (Figure 4.8A). Enrichment mapping and pathway analysis showed a great deal of similarity in the pathways downregulated in response to 4-wks of PoWeR between SC+ and SC- skeletal muscle (Figures 4.8B-C and 4.9A). Pathways involved in ribosome

biogenesis (Figure 4.9B) and oxidative metabolism (Figure 4.9C) were suppressed in SC+ and SC- skeletal muscle after 4-wks of PoWeR.

In order to examine the myonuclear transcriptome in relation to the whole-muscle transcriptome, we performed RNAseq on individual isolated myonuclei (smnRNA-seq) from SC+ and SC- soleus muscles after 4-wks of PoWeR. Represented are a schematic of the work flow (Figure 4.10A). An unbiased cluster of smnRNA-seq data, including all cell types and myonuclei from SC+ and SC- mice represented on a UMAP plot (Figure 4.10B, left) and a distinct myonuclear cluster showing myonuclei from SC+ muscle in blue and SC- muscle in pink (Figure 4.10B, right). Myonuclei from SC- soleus showed 964 underexpressed genes and 83 overexpressed genes when compared to SC+ myonuclei (Figure 4.10C). Pathway analysis revealed a lower expression of genes in oxidative and ribosomal pathways, revealing a greater suppression of these pathways after exercise in myonuclei in the absence of satellite cells (Figures 4.10D-F).

After 8-wks of PoWeR SC- skeletal muscle displayed delayed transcriptional activity

After 8-wks of PoWeR training only 166 genes were overexpressed in SC+ skeletal muscle while 1415 were overexpressed in SC- skeletal muscle relative to 4-wk PoWeR (Figure 4.11A). Consistent with a robust transcriptional response, GO and pathway analyses displayed a greater degree of pathway enrichment in SC- muscle in response to 8-wks of PoWeR (Figures 4.11B and C), which was confirmed by enrichment mapping (Figure 4.12A). In particular, the ribosome biogenesis network was only enriched in the absence of satellite cells (Figures 4.11B-C and 4.12C and E) and there

was higher degree of enrichment in oxidative pathways in SC- skeletal muscle (Figures 4.11B and C).

Pathways most highly enriched in the presence of satellite cells (e.g., RNA/mRNA stability) were also upregulated in SC- skeletal muscle (Figures 4.12B and D).

After 8-wks of PoWeR training 157 genes were underexpressed in SC+ skeletal muscle and 1368 were underexpressed in SC- skeletal muscle (Figure 4.13A), consistent with the large difference in overexpressed genes seen between groups (see Figure 4.11A). GO and pathway analyses revealed few pathways to be heavily enriched in either group but heterogeneity existed in enrichment analysis between SC+ and SC- skeletal muscle (Figures 4.13B and C). This was driven largely by the downregulation of gene sets involved in collagen remodeling at the 8-wk time point in the absence of satellite cells. The enriched pathways and biological processes in SC+ skeletal muscle were driven by a small group of genes related to immune function and another small gene set involved in suppressing glycolysis. Given the relatively large number of underexpressed genes in SC-skeletal muscle, the low level of pathway enrichment is surprising and indicates that the vast majority of these underexpressed genes do not map to known biological processes or pathways. This may represent an uncoordinated transcriptional response or be related to meaningful unmapped pathways being altered in response to PoWeR in the absence of satellite cells.

Aberrant skeletal muscle adaptation in response to PoWeR in the absence of satellite cells

Based on the dysregulation of pathways key to skeletal muscle adaptation in the absence of satellite cells, we aimed to determine if there was a resultant phenotype after PoWeR. We found that total collagen area was slightly lower in SC+ skeletal muscle after PoWeR. This is shown qualitatively in PSR-stained cross sections of the soleus imaged under bright field excitation (Figure 4.14A), quantified in Figure 4.14B. This finding may have been influenced by the larger fibers present in SC+ muscle, as total PSR was quantified in regions of interest and not the whole muscle cross section.

We found that only in the presence of satellite cells was there an increase in densely and loosely organized collagen networks, suggesting altered collagen organization/remodeling in the absence of satellite cells. This is shown qualitatively in PSR-stained cross sections of the soleus imaged under polarized-light excitation (Figure 4.14C), quantified in Figure 4.14D. We also assessed capillarization and found a trend for reduced capillary density in response to PoWeR in SC- skeletal muscle. This is shown qualitatively in lectin-stained cross sections (Figure 4.14E) and quantitatively (Figure 4.14F). To determine if this maladaptive response to exercise influence muscle strength, we assessed peak torque over a force frequency curve and found that plantar flexor muscles from SC- mice produced lower levels of peak torque in response to supramaximal stimulation when compared to SC+ muscles (Figure 4.14G).

4.4 Discussion

Whether satellite cells are required for skeletal muscle hypertrophy is a long-standing debate in the field. It is now apparent that robust muscle fiber hypertrophy can

occur in the absence of satellite cell participation in response to anabolic pharmacological interventions [56,62,107,150-153]. What remains less clear is the need for satellite cells in order to mount a growth response to an exercise (or overload) stimulus [20,24,90]. The findings of this study show that while the soleus and plantaris hypertrophy and adapt to a considerable degree in the absence of satellite cells, these effects are blunted when compared to satellite cell replete muscle. Further, we identify the dysregulation of several exercise-induced gene networks in the soleus in the absence of satellite cells which may contribute to a maladaptive response to exercise.

At the end of 4-wks of PoWeR, soleus muscles from SC- mice have blunted muscle fiber hypertrophy and fail to mount a transcriptional response conducive to optimal skeletal muscle adaptation to exercise. When compared to SC+ mice, there is a failure to increase gene networks regulating collagen turnover/remodeling. In order to determine if this aberrant transcriptional response to PoWeR in SC- mice led to phenotypic alterations, we assessed ECM composition, quantifying both glycosaminoglycans and total collagen content. Although the amount of ECM was not different between groups, immunohistochemical analysis revealed lower proportions of densely and loosely organized collagen networks in soleus muscles from SC+ compared to SC- mice after 8-weeks of PoWeR. ECM remodeling during skeletal muscle hypertrophy is a well characterized phenomenon that is critical for transmitting force generated by muscle fibers [154,155]. Forces are transmitted laterally through basement membrane collagens and longitudinally via fibrillar collagens and the capacity to transmit force is determined by the molecular composition and arrangement of the ECM [154,156].

Based on the different composition of collagens comprising of satellite cell deplete and replete skeletal muscle after PoWeR, we wanted to determine if there was a resultant strength phenotype by examining measures of in vivo plantar flexion torque. Our data show that while both SC+ and SC- mice reach comparable levels of peak force, force levels drop off rapidly in SC- mice over the duration of strength testing, while peak force levels are maintained in SC+ mice. In line with this, we have previously shown that force generation of the plantaris muscle is attenuated after synergist ablation in SC- skeletal muscle. Here, we show increased remodeling and stiffness of collagen in skeletal muscle only occurs in the presence of satellite cells. It seems likely that in response to PoWeR or synergist ablation altered ECM organization in the absence of satellite cells is contributing to reduced strength outcomes [157] [156].

Gene expression profiling also showed that artery morphogenesis/capillarization pathways were activated in SC+ compared to SC- soleus, which was associated with a trend for lower capillary density in the absence of satellite cells. Capillaries are a critical component of adaptation as they allow for adequate muscle fiber perfusion which supports the delivery of oxygen, growth factors and nutrients to the muscle [158]. In line with this, low capillary density is associated with blunted skeletal muscle growth in humans [159] [160]. It is now well established that satellite cells are anatomically close to capillaries and participate in bidirectional signaling with endothelial cells (e.g., VEGF signaling) [161-163]. Satellite cell differentiation supports angiogenesis and a recent report shows high levels of VEGFA in satellite cells, which serves to recruit blood vessel endothelial cells [161-163]. Further, the report by Verma et al., shows ECM organization to

be a highly enriched satellite cell to endothelial cell interaction pathway, which may in part explain our ECM findings [162].

Our soleus whole-muscle RNA-seq data at the 4-wk time point show both SC+ and SC- mice transcriptionally downregulate several pathways involved in oxidative metabolism and ribosome biogenesis in response to PoWeR. To determine if gene expression responses observed at the whole muscle level were generated from the muscle fiber, as opposed to other cell populations in the muscle, we analyzed the myonuclear transcriptome of the soleus after 4-wks of PoWeR in both SC+ and SC- mice, isolating myonuclei 24 hours after the last running bout, consistent with our whole-muscle data. smnRNA-seq data reveal a greater suppression of metabolic pathways in SC- myonuclei when compared to SC+ myonuclei. Ribosomal biogenesis and oxidative phosphorylation pathways are substantially lower in SC- compared to SC+ myonuclei. A downregulation of several rRNAs after chronic resistance training has been shown previously in humans, corroborating what we report here after 4-wks of PoWeR [164]. Unique to this study, we show a greater degree of downregulation of ribosomal pathways at the myonuclear level in the absence of satellite cells. While the extent to which these differences can be attributed to newly fused satellite cell-derived myonuclei remains to be determined, recent publications using single nuclear RNAseq have demonstrated considerable heterogeneity in myonuclei [165].

The transcriptional signature of SC+ skeletal muscle at the end of 8-wks of PoWeR varies by only a few hundred genes compared to the 4-wk time point. This demonstrates SC+ muscle is capable of mounting a robust and effective transcriptional response to a novel stimulus in order to coordinate adaptation, which leads to a new

transcriptional homeostasis that is maintained for the final 4-wks of training. As the overall exercise stimulus is similar to that of the first 4-wks of training, this makes a great deal of sense. On the other hand, in SC- skeletal muscle, there is pronounced transcriptional heterogeneity (thousands of differentially expressed genes) between the 4-wk and 8-wk time points, in stark contrast to SC+ skeletal muscle, showing failure to initiate an appropriate transcriptional response to a novel stimulus. Interestingly, though, pathways downregulated at the 4-wk time point (oxidative phosphorylation and ribo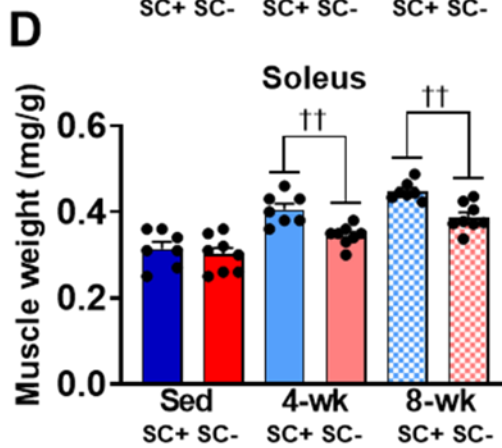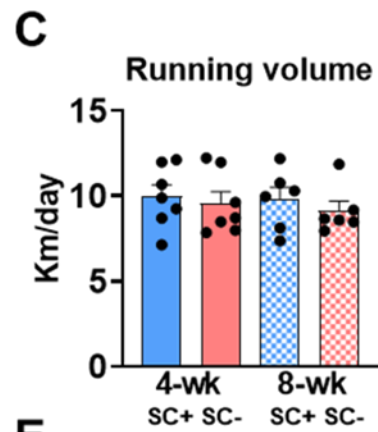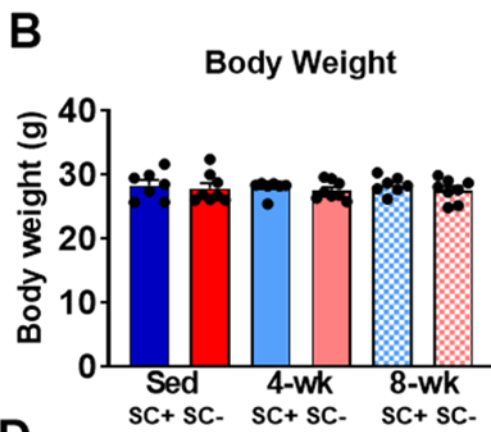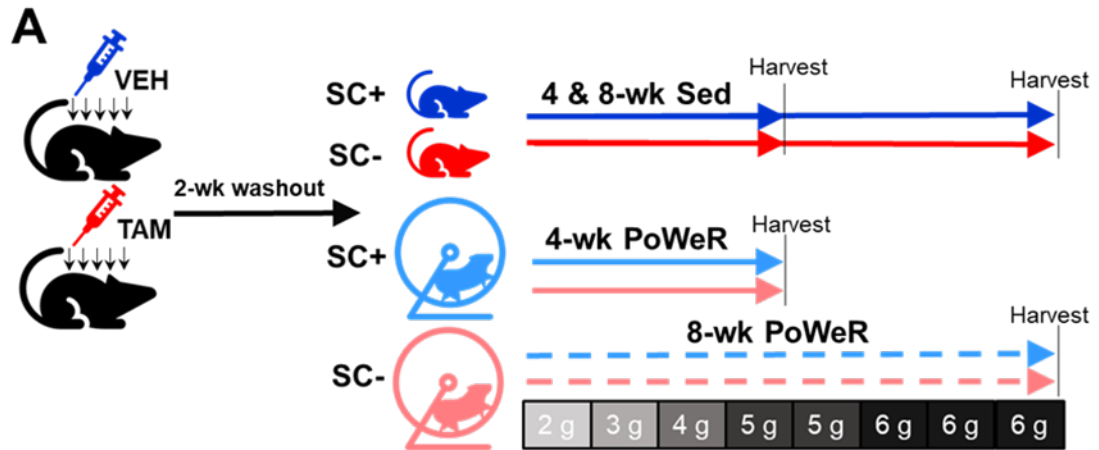some biogenesis) are now upregulated at the 8-wk time point only in SC- skeletal muscle. This may be indicative of delayed, or compensatory, transcriptional activation in resident myonuclei in the absence of satellite cell-dependent myonuclear addition to aid in adaptation.

Our findings reported here are in line with what we have shown previously during synergist ablation induced muscle overload. In response to both mechanical overload and PoWeR, resident myonuclei can transcriptionally support a hypertrophic and adaptive response in the absence of satellite cells and myonuclear addition. However, muscle growth and adaptation are ultimately blunted in satellite cell deplete muscle. The attenuated growth in response to synergist ablation is associated with fibrosis, at least partially due to a lack of communication from satellite cells to fibroblasts to suppress ECM accumulation in response to the severe model of mechanical overload. In response to PoWeR, a more translational model of exercise, muscle does not become fibrotic in the absence of satellite cells, but growth is still attenuated and associated with aberrant ECM/collagen remodeling. Our data reveal satellite cells are critical for a coordinated and effective transcriptional response to a novel stimulus, optimizing adaptations and

muscle hypertrophy. Based on findings from both models, it seems very possible that satellite cells and/or fusion in and of itself is not anabolic, per say, but that satellite cells are a perquisite for a transcriptional response to exercise that will lead to maximal muscle adaption.

A

VEH

2-wk washout

TAM

SC+ SC- 4 & 8-wk Sed Harvest Harvest

SC+ 4-wk PoWeR Harvest

SC- 8-wk PoWeR Harvest

| 2 g | 3 g | 4 g | 5 g | 5 g | 6 g | 6 g | 6 g |

B Body Weight

C Running volume

D Soleus

E Plantaris
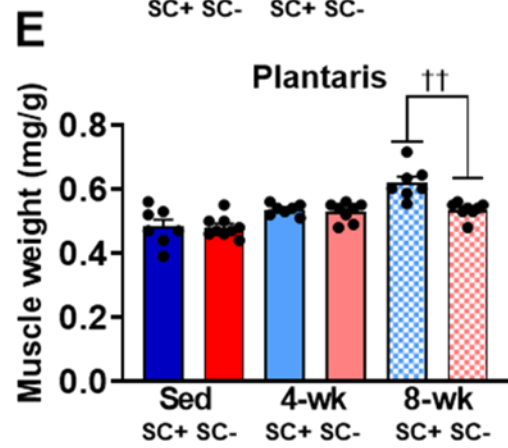
Figure 4.1

Satellite cell depletion blunted higher muscle weights induced by PoWeR. (A) Schematic showing PoWeR protocol. (B) Endpoint body weights across all groups. (C) Average running volume after 4-wks and 8-wks of PoWeR. (D) Normalized muscle weight in the soleus. (E) Normalized muscle weight in the plantaris. Data are represented as mean ± SEM. Statistical analysis: Two-way ANOVA with Tukey's multiple comparisons test. PoWeR led to higher muscle weights at 4-wks ($p<0.0001$) and 8-wks ($p<0.0001$) in the soleus and at 4-wks ($p<0.01$) and 8-wks ($p<0.0001$) in the plantaris. There was a significant effect for satellite cell depletion blunting this adaptation. †† $p<0.01$.
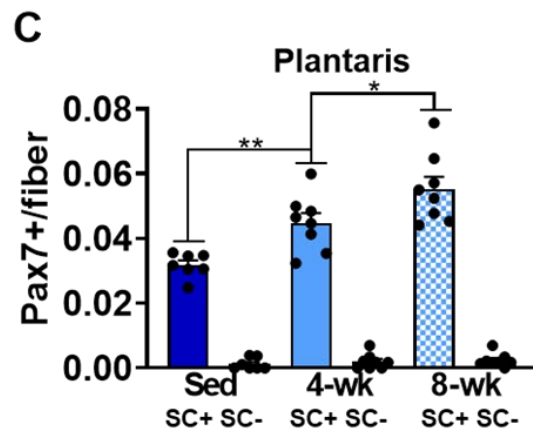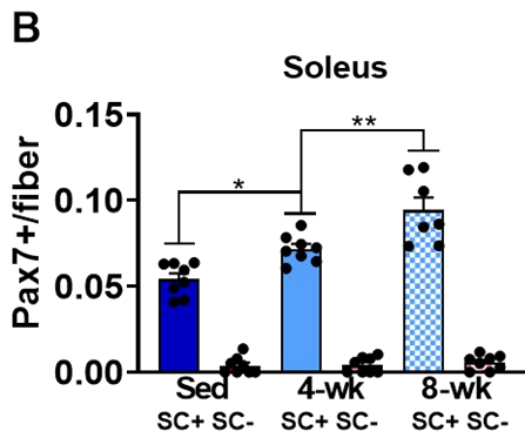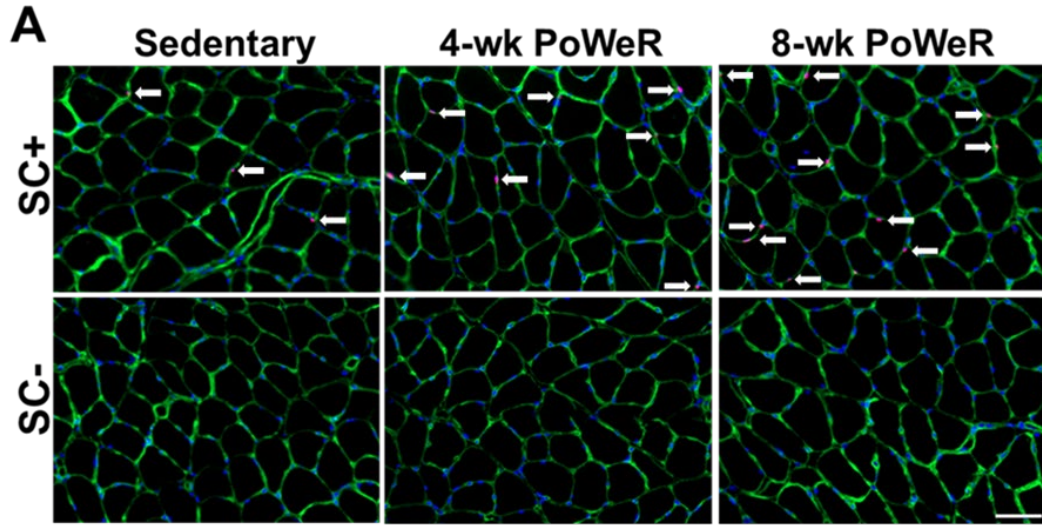
Figure 4.2

PoWeR led to higher satellite cell content in the soleus and plantaris in SC+ mice. (A) Representative images of satellite cell immunohistochemistry from the soleus across all groups showing laminin (green), nuclei (blue), and Pax7 (red; white arrows). (B) Satellite cell density in the soleus. (C) Satellite cell density in the plantaris. Scale bar = 50 μm. Data are represented as mean ± SEM. Statistical analysis: Two-way ANOVA with Tukey's multiple comparisons test. There was a main effect for PoWeR ($p<0.0001$) and satellite cell depletion ($p<0.0001$) in the soleus and a main effect for PoWeR ($p<0.0001$) and satellite cell depletion ($p<0.0001$) in the plantaris. There was a significant interaction between PoWeR and satellite cell content in the soleus ($p<0.001$) and plantaris ($p<0.0001$). *$p<0.05$, **$p<0.01$.
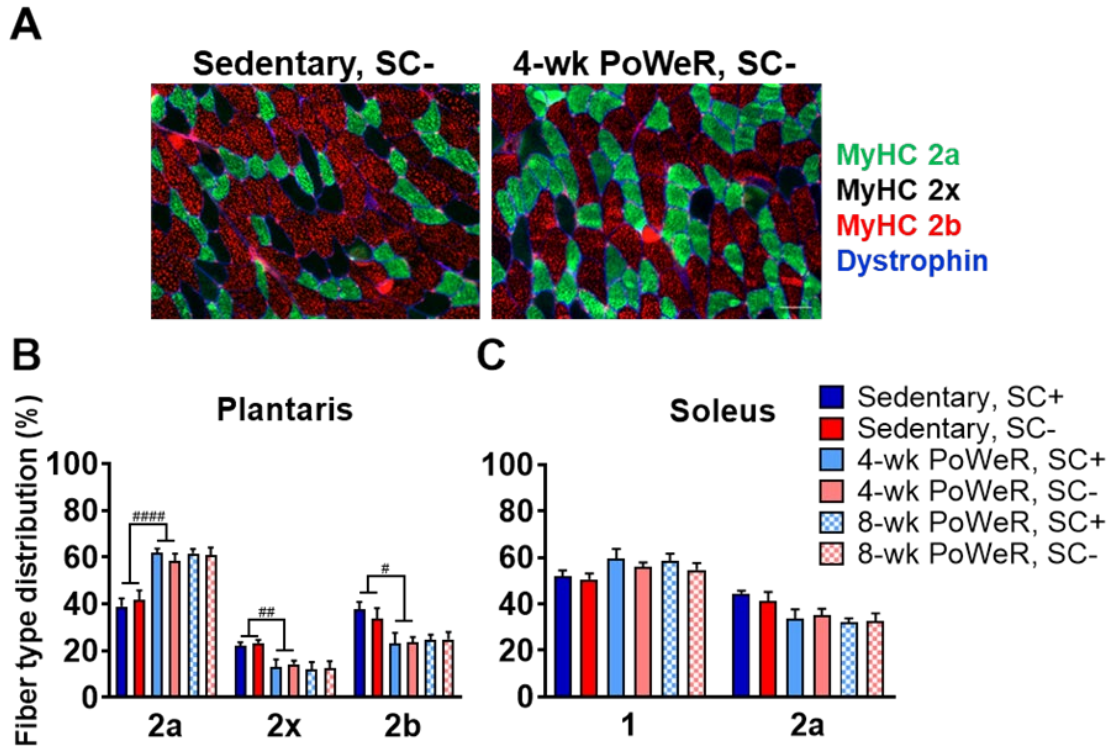
Figure 4.3

PoWeR led to an oxidative shift in fiber type distribution in the plantaris. (A) Representative images of fiber type immunohistochemistry in the plantaris showing dystrophin (blue) myosin heavy chain 2a (green) and myosin heavy chain 2b (red). Negative (black) fibers were scored as myosin heavy chain 2x. (B) Relative frequency of fiber type in the plantaris. (C) Relative frequency of fiber type in the soleus. Scale bar = 50 μm. Data are represented as mean ± SEM. Statistical analysis: Two-way ANOVA with Tukey's multiple comparisons test. PoWeR led to a higher proportion of oxidative fibers. $\#p<0.05$, $\#\#p<0.01$, $\#\#\#\#p<0.0001$.
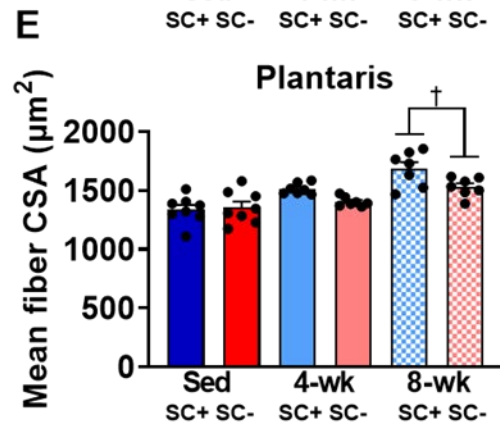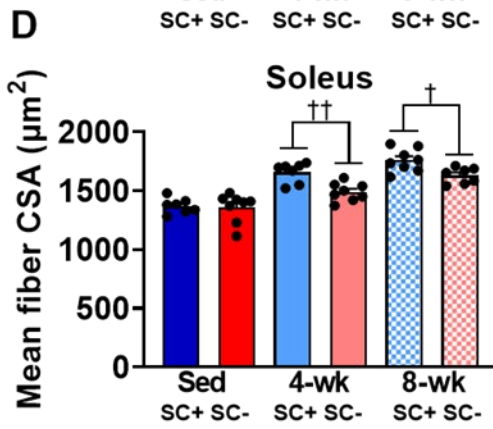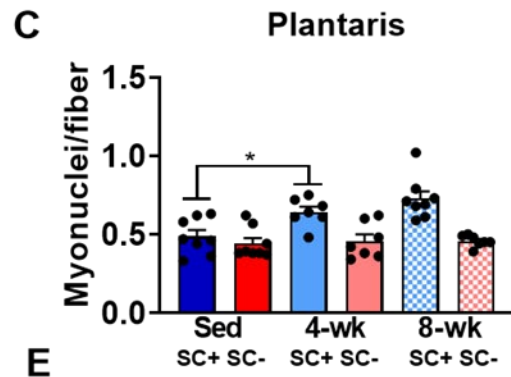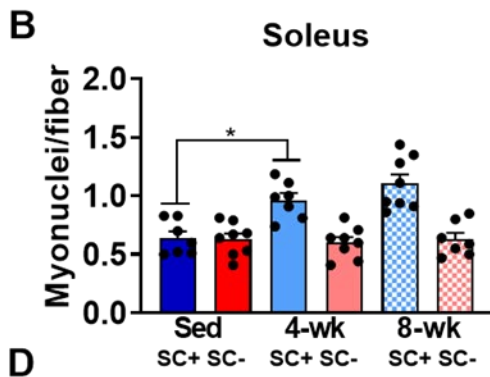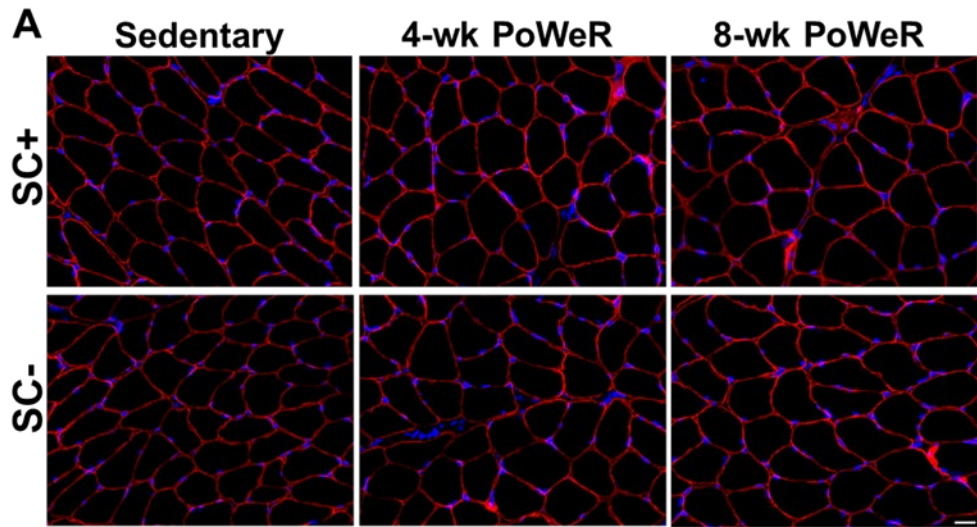
Figure 4.4

Satellite cell depletion inhibited myonuclear accretion and blunted muscle fiber
hypertrophy in response to PoWeR. (A) Representative images of myonuclear and fiber
size immunohistochemistry from the soleus across all groups showing dystrophin (red)
and nuclei (blue). (B) Myonuclear cell density in the soleus. (C) Myonuclear density in
the plantaris. (D) Mean fiber cross sectional area (CSA) in the soleus. (E) Mean fiber
CSA in the plantaris. Scale bar = 20 μm . Data are represented as mean ± SEM.
Statistical analysis: Two-way ANOVA with Tukey's multiple comparisons test. (B) and
(C):there was a main effect for PoWeR ($p < 0.05$) and satellite cell depletion ($p < 0.001$) in
the soleus and a main effect for PoWeR ($p < 0.01$) and satellite cell depletion ($p < 0.0001$)
in the plantaris. There was a significant interaction between PoWeR and satellite cell
depletion in the soleus ($p < 0.05$) and plantaris ($p < 0.05$). *$p < 0.05$. (D) and (E): PoWeR led
to higher muscle fiber CSA at 4-wks ($p < 0.0001$) and 8-wks ($p < 0.0001$) in the soleus and
at 4-wks ($p < 0.05$) and 8-wks ($p < 0.0001$) in the plantaris. There was a significant effect
for satellite cell depletion blunting this adaptation. † $p < 0.05$, †† $p < 0.01$.
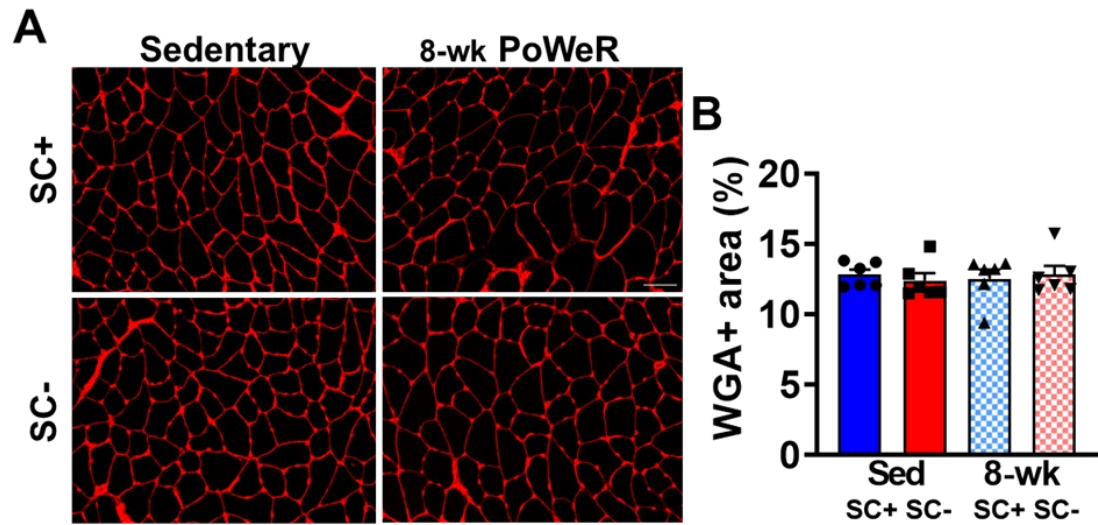
Figure 4.5

Neither PoWeR nor satellite cell content influenced glycosaminoglycan accumulation.

(A) Representative images WGA-stained plantaris muscle. (B) Relative area of WGA

positive staining. Scale bar = 50 µm. Data are represented as mean ± SEM. Statistical

analysis: Two-way ANOVA with Tukey's multiple comparisons test.
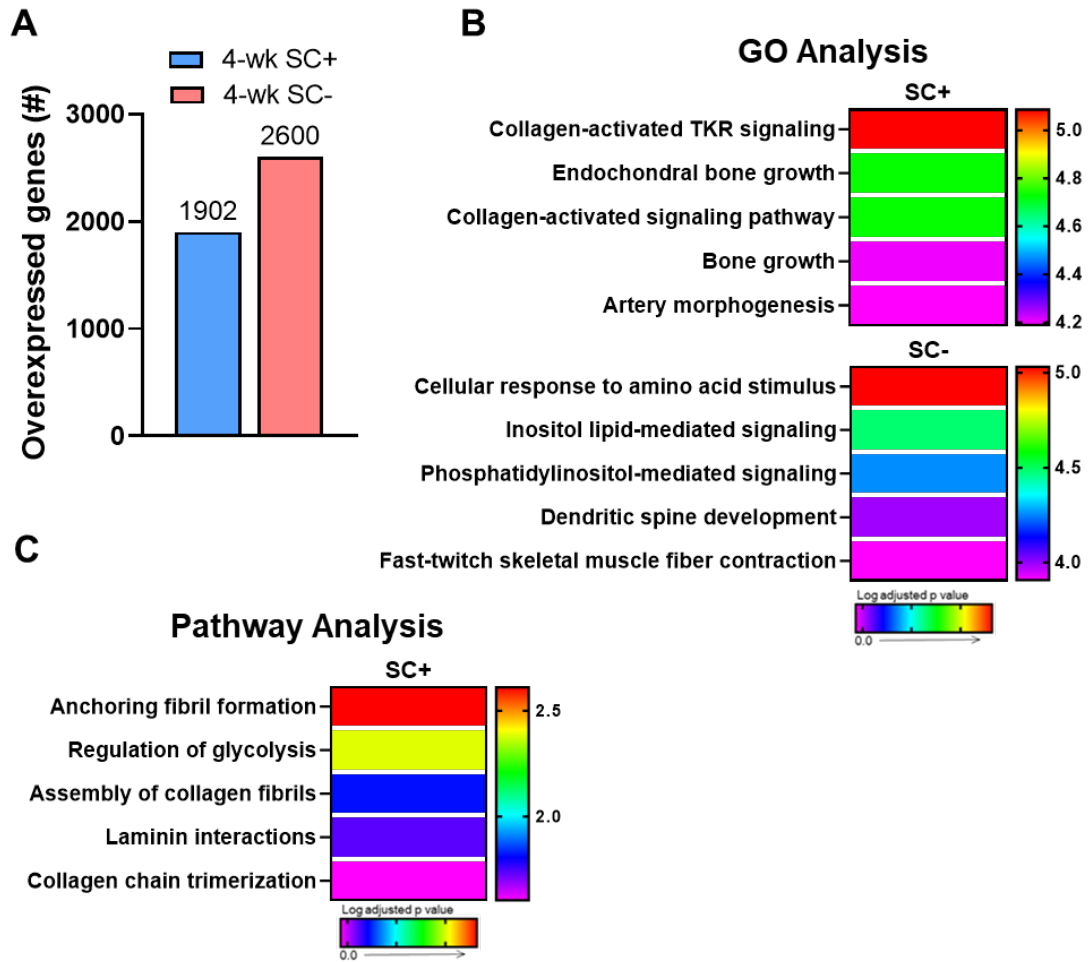
Figure 4.6

Collagen remodeling and capillarization pathways were enriched only in the presence of satellite cells after 4-wks of PoWeR. (A) Bar graph showing the number of overexpressed genes in the soleus (whole-muscle RNA-seq) after 4-wks of PoWeR in SC+ and SC- skeletal muscle 24 hours after the last bout of exercise relative to sham controls. (B) The most highly enriched biological processes (GO Analysis) for both groups. (C) The most highly enriched Reactome pathways (Pathway Analysis) for the SC+ group. The SC- group had no statistically enriched pathways.
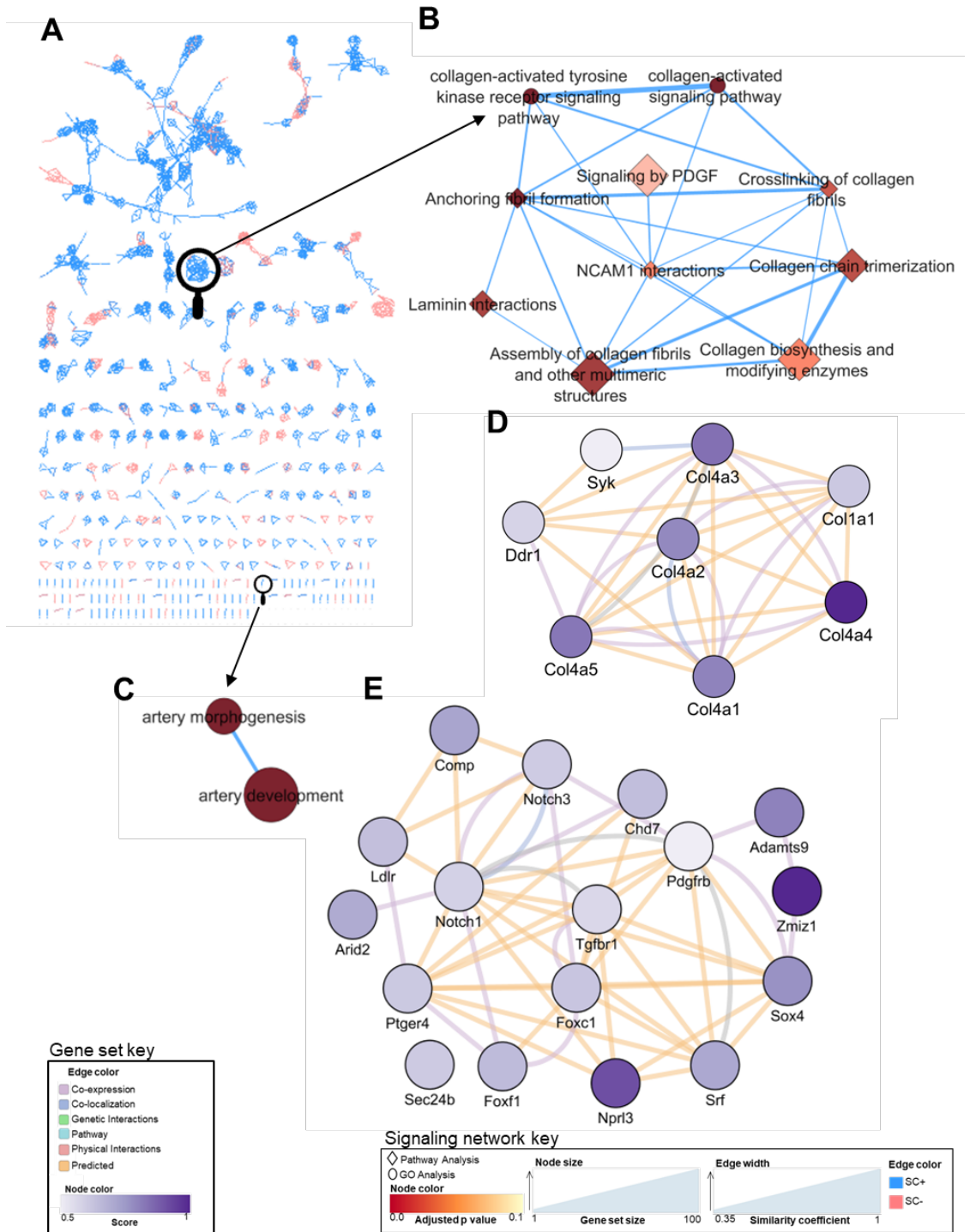
Figure 4.7

Enriched signaling networks and gene sets specific to SC+ skeletal muscle. (A) Unbiased

enrichment map representing all statistically enriched GO biological process terms and

Reactome pathways (nodes) and the number of genes overlapping between biological

processes and pathways (edges); shown in blue for SC+ and pink for SC-. (B) Collagen

signaling network. (C) Capillarization signaling network. (D) Gene set enriching the

collagen activated tyrosine kinase receptor signaling pathway node. (E) Gene set

enriching the artery morphogenesis node.

A

B
GO Analysis

C
Pathway Analysis

91
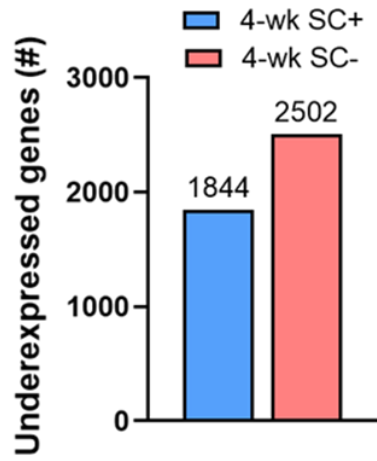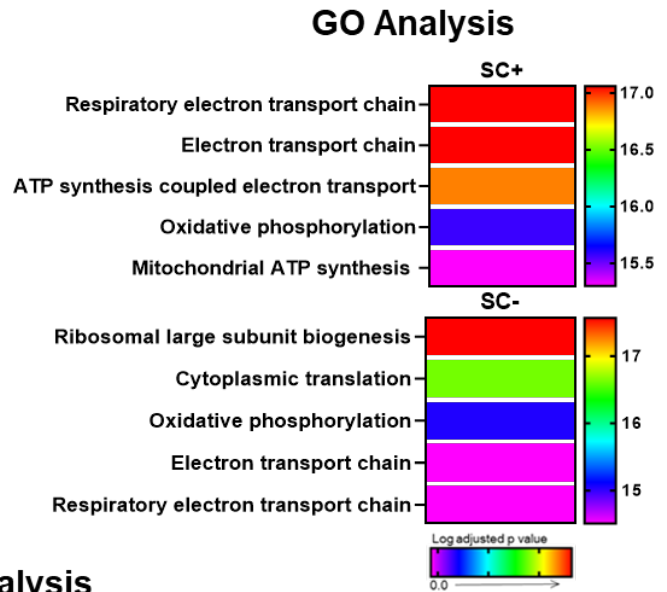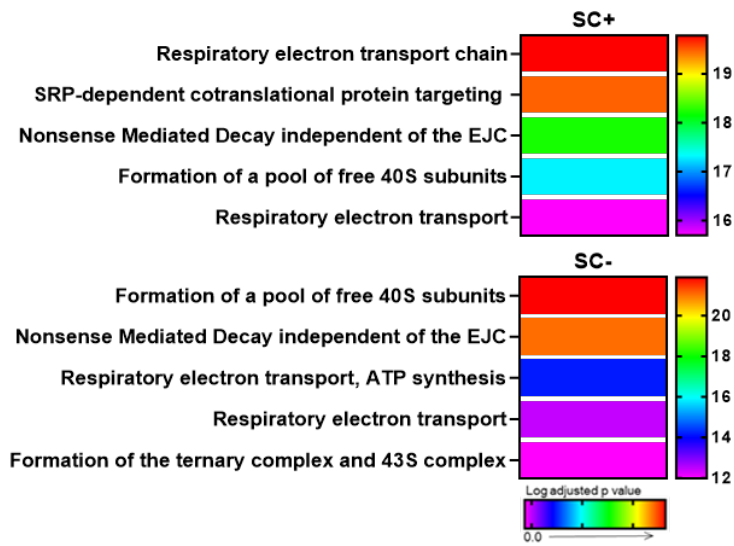
Figure 4.8

Oxidative phosphorylation and ribosome biogenesis are downregulated after 4-wks of

PoWeR. (A) Bar graph showing the number of underexpressed genes in the soleus

(whole-muscle RNA-seq) after 4-wks of PoWeR in SC+ and SC- skeletal muscle 24

hours after the last bout of exercise relative to sham controls. (B) The most highly

enriched biological processes (GO Analysis) for both groups. (C) The most highly

enriched Reactome pathways (Pathway Analysis) for both groups
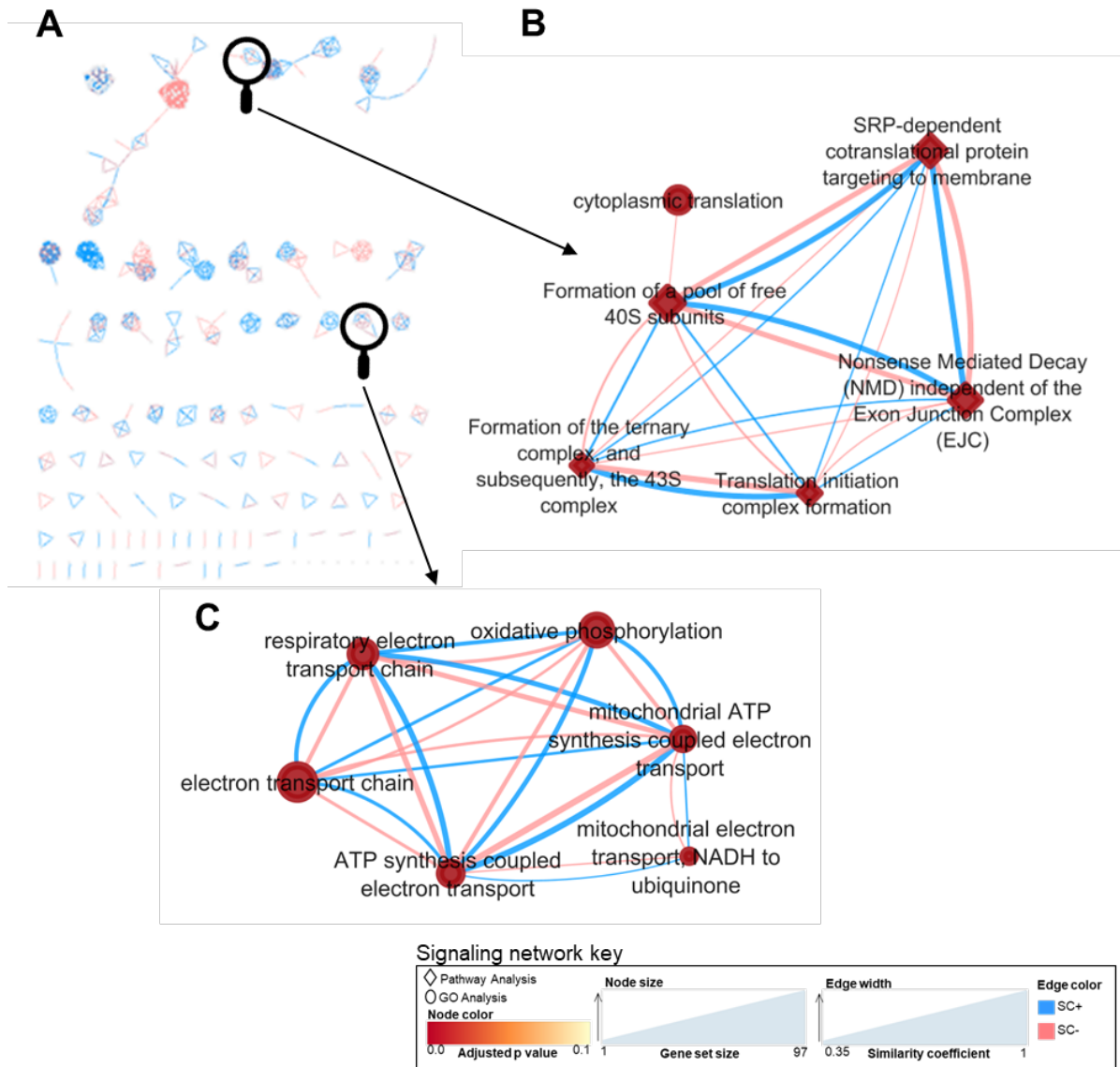
Figure 4.9

Enriched signaling networks are shared between SC+ and SC- skeletal muscle. (A) Unbiased enrichment map representing all statistically enriched GO biological process terms and Reactome pathways (nodes) and the number of genes overlapping between biological processes and pathways (edges); shown in blue for SC+ and pink for SC-. (B) Translation signaling network. (C) Oxidative phosphorylation signaling network.

A

4-wk PoWeR

SC+

SC−

24 h. post last exercise bout

Soleus dissection & myonuclear isolation

GEMs

myonuclei

10x chromium single myonuclear RNA-seq

B

myonuclei

SC+ myonuclei
SC− myonuclei

C

4-wk PoWeR SC−/SC+

Differentially expressed genes (#)

overexpressed
underexpressed

83

964

D

Pathway Analysis

Down (SC−)

SRP-dependent cotranslational protein targeting

Nonsense Mediated Decay independent of the EJC

Formation of a pool of free 40S subunits

Respiratory electron transport, ATP synthesis

Complex I biogenesis

E

GO Analysis

Down (SC−)

cytoplasmic translation

myofibril assembly

oxidative phosphorylation

NADH dehydrogenase complex assembly

mitochondrial respiratory chain complex I assembly

Log adjusted p value

F

Enriched signaling networks

SRP-dependent cotranslational protein targeting to membrane

Formation of a pool of free 40S subunits

cytoplasmic translation

Nonsense Mediated Decay (NMD) independent of the Exon Junction Complex (EJC)

Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S

Translation initiation complex formation

Formation of the ternary complex, and subsequently, the 43S complex

Ribosomal scanning and start codon recognition

Gene set key

Edge color
Co-expression
Co-localization
Genetic Interactions
Pathway
Physical Interactions
Predicted

Node color
0.5   1
Score

Signaling network key

Pathway Analysis
GO Analysis

Node color
0.0   Adjusted p value   0.1

Node size
1   Gene set size   100

Edge width
0.35   Similarity coefficient   1

Edge color
SC+
SC−

94

Figure 4.10

Myonuclear transcriptome reveals a greater downregulation of oxidative and ribosomal pathways in the absence of satellite cells. (A) Schematic of myonuclear isolation and sequencing from skeletal muscle following PoWeR. (B) Unbiased cluster of smnRNA-seq data from SC+ and SC- mice represented on a UMAP and the myonuclear cluster showing myonuclei from SC+ muscle in blue and SC- muscle in pink. (C) Bar graph showing the number of over and underexpressed genes in the soleus after 4-wks of PoWeR in SC+ skeletal muscle when compared to SC- skeletal muscle. The most highly enriched (D) biological processes (GO Analysis) and (E) Reactome pathways (Pathway Analysis). (F) Translation signaling network and the gene set enriching the selected node.

A

8-wk SC+
8-wk SC−

Overexpressed genes (#)

166
1415

B GO Analysis

SC+

regulation of mRNA stability
regulation of RNA stability
ncRNA export from nucleus
ATP synthesis coupled electron transport
RNA export from nucleus

2.7
2.6
2.5
2.4

SC−

mitochondrial respiratory chain complex assembly
ribosomal large subunit biogenesis
ribosomal small subunit biogenesis
mitochondrial respiratory chain complex I assembly
NADH dehydrogenase complex assembly

10
9
8
7

Log adjusted p value
0.0

C Pathway Analysis

SC+

Scavenging by Class B Receptors
Formation of ATP by chemiosmotic coupling
Cristae formation
Mitochondrial biogenesis
Scavenging by Class A Receptors

1.9
1.8
1.7

SC−

SRP-dependent cotranslational protein targeting
Nonsense Mediated Decay independent of the EJC
Formation of a pool of free 40S subunits
Respiratory electron transport, ATP synthesis
Respiratory electron transport
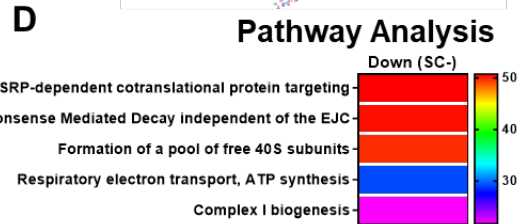
11
10
9
8

Log adjusted p value
0.0

Figure 4.11

Oxidative phosphorylation and ribosome biogenesis are upregulated after 8-wks of PoWeR only in the absence of satellite cells. (A) Bar graph showing the number of overexpressed genes in the soleus (whole-muscle RNA-seq) after 8-wks of PoWeR in SC+ and SC- skeletal muscle 24 hours after the last bout of exercise relative to 4-wk PoWeR. (B) The most highly enriched biological processes (GO Analysis) for both groups. (C) The most highly enriched Reactome pathways (Pathway Analysis) for both groups.
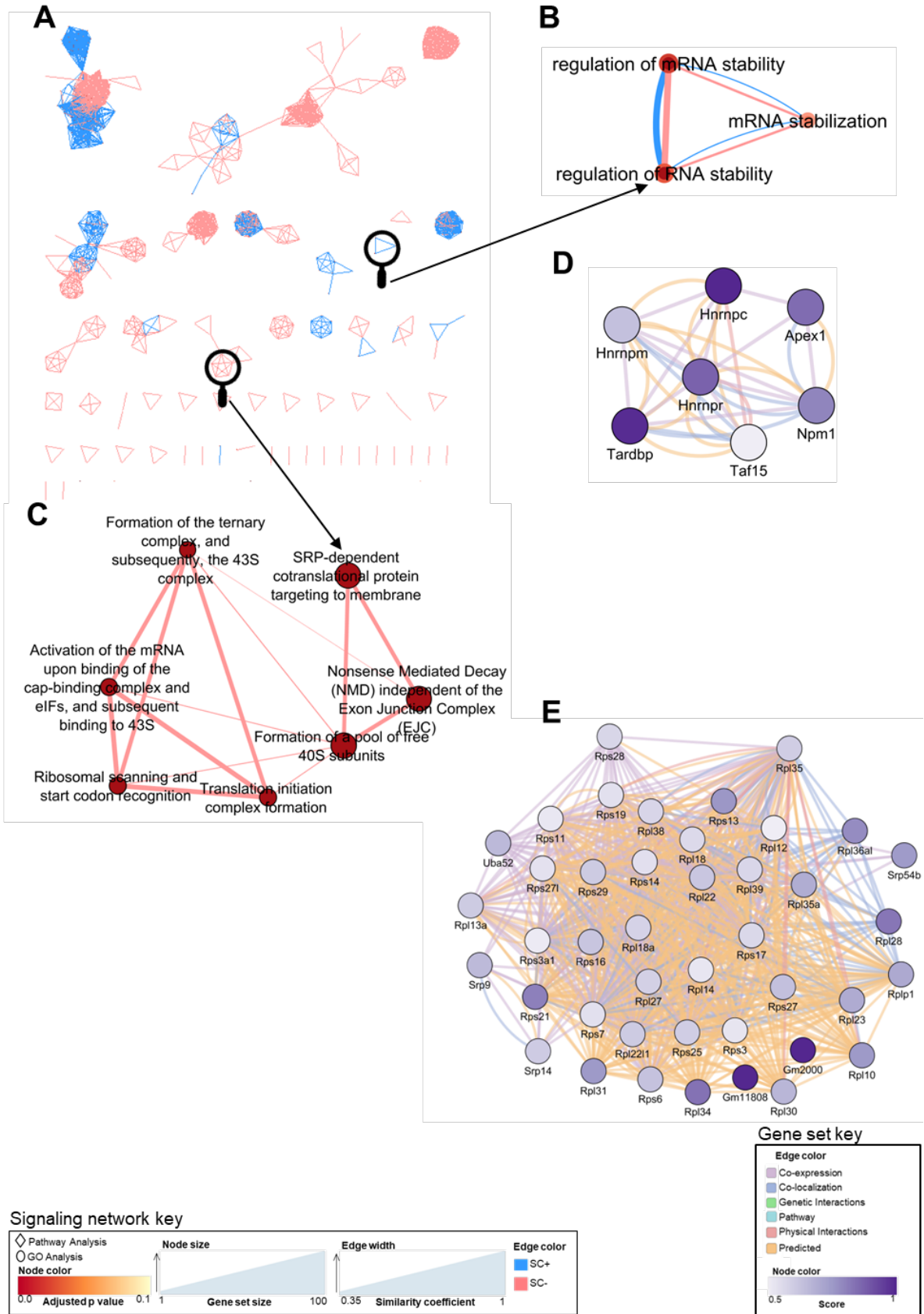
Figure 4.12

Enriched signaling networks and gene sets in SC+ and SC- skeletal muscle. (A) Unbiased enrichment map representing all statistically enriched GO biological process terms and Reactome pathways (nodes) and the number of genes overlapping between biological processes and pathways (edges); shown in blue for SC+ and pink for SC-. (B) RNA stability signaling network. (C) translation signaling network. (D) The gene set enriching the regulation of RNA stability node. (E) The gene set enriching the SRP-dependent cotranslational protein targeting to the membrane node.
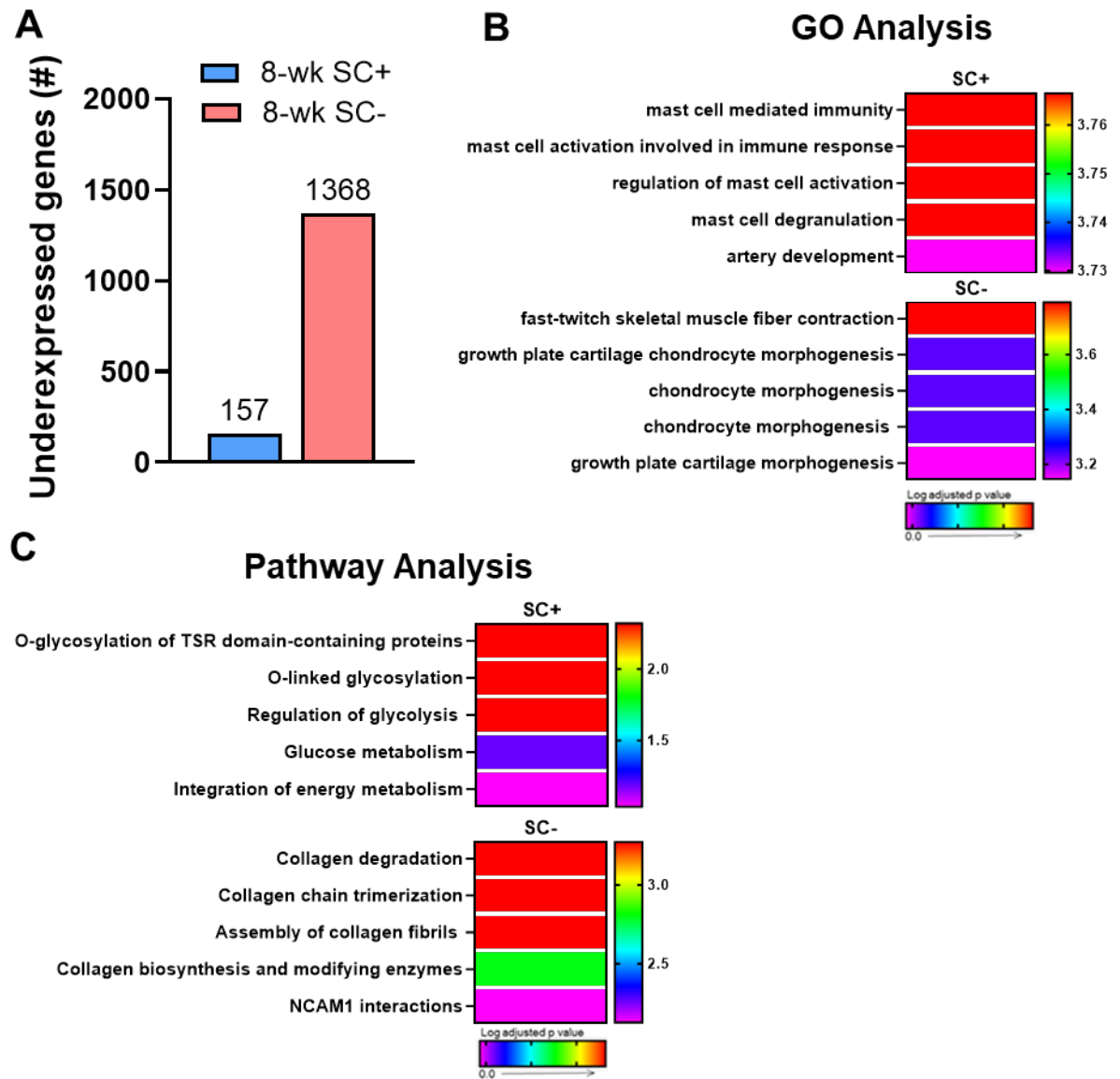
Figure 4.13

Collagen remodeling is suppressed after 8-wks of PoWeR only in the absence of satellite cells. (A) Bar graph showing the number of underexpressed genes in the soleus (whole-muscle RNA-seq) after 8-wks of PoWeR in SC+ and SC- skeletal muscle 24 hours after the last bout of exercise relative to 4-wk PoWeR. (B) The most highly enriched biological processes (GO Analysis) for both groups. (C) The most highly enriched Reactome pathways (Pathway Analysis) for both groups.
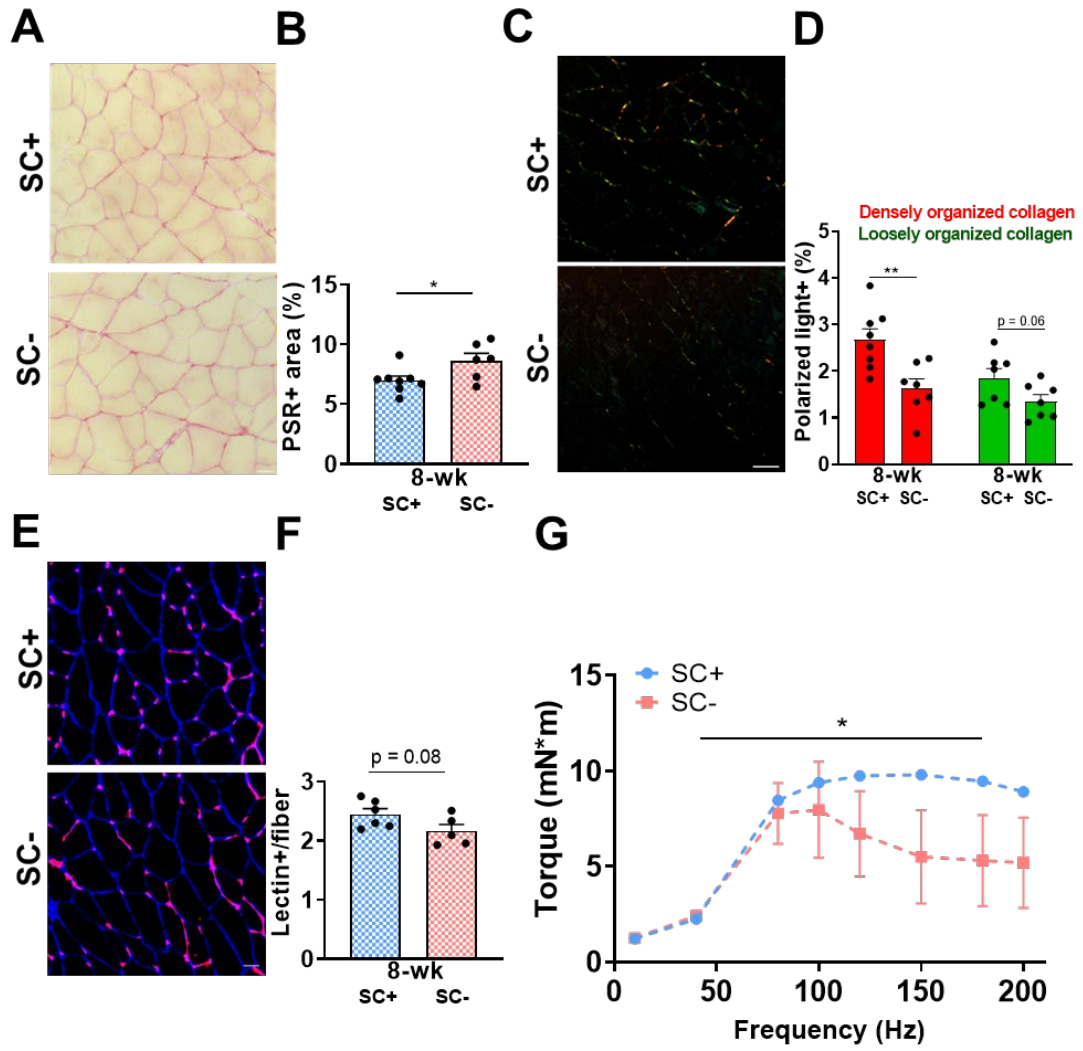
Figure 4.14
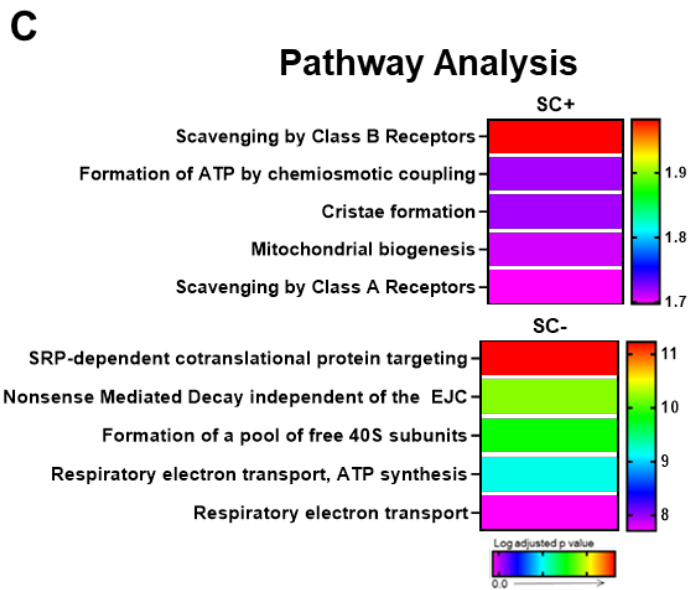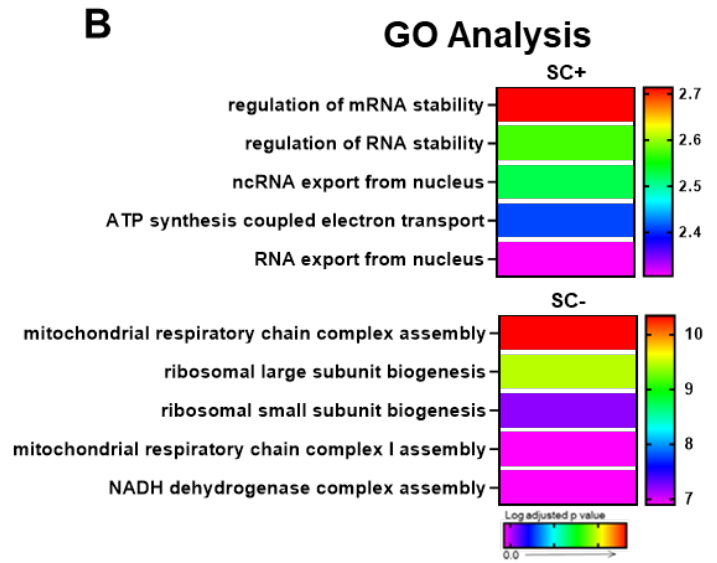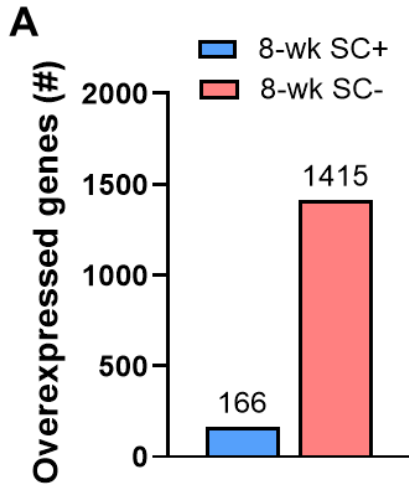
Satellite cell depletion led to aberrant collagen remodeling, capillarization and strength

adaptation to PoWeR. (A) Representative PSR-stained soleus images visualized with

bright field. (B) Relative area positive for PSR. (C) Representative PSR-stained soleus

images visualized with polarized light. (D) Relative area of red and green emitted light.

(E) Representative lectin (red) and laminin (blue) stained soleus images to identify

capillaries and fibers. (F) Relative number of Lectin+ events. (G) Force frequency curve.

Scale bar = 50 μm. Data are represented as mean ± SEM. Statistical analyses: (B), (D),

(E) unpaired two-tailed Student's t-test, (G) repeated measures one-way ANOVA with

Sidak's multiple comparisons test.

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Education

2010 – 2013	B.S. in Exercise Science with honors, 2013, University of Massachusetts Boston
2013 – 2015	M.S. in Exercise Physiology, 2015, Iowa State University Thesis: *Effects of high and low velocity muscle contraction on myosin heavy chain mRNA and protein expression in conjunction with muscle performance in the elderly*
2017 -	Doctoral Fellow, College of Health Sciences, University of Kentucky. Advisor: Charlotte A Peterson

Professional Experience

2011 – 2013	Physiology Tutor, Center for Clinical Education and Research, UMass Boston
2011 – 2012	Undergraduate Research Assistant, Go-Kids Boston, College of Nursing and Health Sciences, UMass Boston
2012 – 2013	Undergraduate Research Assistant, Exercise Science Laboratory, Bouve College of Health Sciences, Northeastern University
2013 – 2015	Graduate Research Assistant, Department of Kinesiology, Iowa State University
2015 – 2017	Senior Research Technician, the Nutrition, Exercise Physiology and Sarcopenia Lab, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University
2017 –	Research Doctoral Fellow, College of Health Sciences, University of Kentucky

Honors and Awards

2013	Awarded, Corcoran/Rody Scholarship, College of Nursing and Health Sciences, UMass Boston
2016	Awarded, Alumnus of the Year, College of Nursing and Health Sciences, UMass Boston
2017	Awarded, Doctoral Fellowship, Rehabilitation Sciences Doctoral Program, University of Kentucky
2018	Awarded, Rehabilitation Sciences Pilot Study Funding, College of Health Sciences, University of Kentucky
2018	Awarded, Rehabilitation Sciences Travel Funding, College of Health Sciences, University of Kentucky
2018	Awarded, the Donovan Scholarship in Gerontology, Office of Lifelong Learning, University of Kentucky

2018	Awarded, Research Enhancement Award, College of Health Sciences, University of Kentucky
2019	Awarded, Graduate Student Congress Travel Award, University of Kentucky
2019	Awarded, Nathan Shock Center Travel Award, University of Oklahoma
2019	Awarded, Rehabilitation Sciences Travel Funding, College of Health Sciences, University of Kentucky
2019	Awarded, Student Investigator Research Grant, College of Health Sciences, University of Kentucky
2020	Awarded, Supplemental Stipend - Graduate Student Incentive Program, The Graduate School, University of Kentucky
2020	Awarded, Robinson Graduate Award for Research Creativity, College of Health Sciences, University of Kentucky

Peer-Reviewed Publications

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