Progressive Weighted Wheel Running - PoWeR

**0-4 wks**
- **RNA-seq**
  - Collagen remodeling
  - Capillarization
  - Glycolysis
  - Hypertrophy

**SC+**
- scRNA-seq
- SC- vs SC+
  - Oxidative metabolism
  - Ribosome biogenesis
  - Myofibrillar assembly

**SC-**
- RNA-seq
  - Collagen remodeling
  - Capillarization
  - Glycolysis
  - Hypertrophy

**4-8 wks**
- **RNA-seq**
  - Transcriptional homeostasis maintained
  - Hypertrophy

**SC depletion** alters the whole muscle and myonuclear transcriptional response to PoWeR and attenuates skeletal muscle adaptation

**RNA-seq**
- Oxidative metabolism
- Ribosome biogenesis
- Hypertrophy
Satellite cell depletion disrupts transcriptional coordination and muscle adaptation to exercise

Davis A. Englund¹,², Vandré C. Figueiredo¹,², Cory M. Dungan¹,², Kevin A. Murach¹,² Bailey D. Peck¹,², Jennifer M. Petrosino⁵, Camille R. Brightwell²,⁴, Alec M. Dupont², Ally C. Neal², Christopher S. Fry²,⁴, Federica Accornero⁵, John J. McCarthy²,³, Charlotte A. Peterson¹,²

¹Department of Physical Therapy, College of Health Sciences, University of Kentucky, Lexington, Kentucky, USA. ²Center for Muscle Biology, University of Kentucky, Lexington, Kentucky, USA. ³Department of Physiology, College of Medicine, University of Kentucky, Lexington, Kentucky, USA. ⁴Department of Athletic Training and Clinical Nutrition, University of Kentucky, Lexington, Kentucky, USA. ⁵Department of Physiology and Cell Biology, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio, USA.

Corresponding Author: Davis A. Englund; englund.davis@mayo.edu
Abstract

Satellite cells are required for post-natal development, skeletal muscle regeneration across the lifespan and skeletal muscle hypertrophy prior to maturity. Our group has aimed to address whether satellite cells are required for hypertrophic growth in mature skeletal muscle. Here, we generated a comprehensive characterization and transcriptome-wide profiling of skeletal muscle during adaptation to exercise in the presence or absence of satellite cells in order to identify distinct phenotypes and gene networks influenced by satellite cell content. We administered vehicle or tamoxifen to adult Pax7-DTA mice and subjected them to progressive weighted wheel running (PoWeR). We then performed immunohistochemical analysis and whole-muscle RNA-seq of vehicle (SC+) and tamoxifen-treated (SC-) mice. Further, we performed single myonuclear RNA-seq to provide detailed 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 and adaptation are ultimately blunted. Transcriptional profiling reveals several gene networks key to muscle adaptation are altered in the absence of satellite cells.

Keywords: Satellite cell, muscle stem cell, Pax7, exercise, hypertrophy, adaptation, muscle function

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) 1-4. Certain groups, including our own, have aimed to address the role of satellite cells during hypertrophic growth in response to mechanical loading, anabolic agents, and exercise in mature skeletal muscle 2,5-9. Our laboratory has leveraged the Pax7CreER; R26DTA/+ (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 3,5,9-11. 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 synergist ablation or wheel running requires satellite cells for maximal adaptation 5,6,9.

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 12,13,14. We have developed 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 15,16. This new model provides an opportunity to perform rigorous studies to generate novel insight that further elucidates how satellite cells regulate skeletal muscle adaptation to exercise. Here, we aimed to generate a comprehensive characterization and transcriptome-wide profiling of skeletal muscle during growth and adaptation to PoWeR in the presence and
absence of satellite cells in order to identify distinct phenotypes and gene networks influenced by satellite cell content. We subjected Pax7-DTA mice to 4 and 8 weeks of PoWeR and performed immunohistochemistry (IHC) on the soleus (oxidative phenotype) and plantaris (mixed oxidative and glycolytic phenotype) of vehicle- (SC+) and tamoxifen-treated (SC-) mice and whole-muscle RNA-sequencing (RNA-seq) on the soleus. We further performed single myonuclear RNA-seq (smnRNA-seq) on the soleus muscle of SC+ and SC- mice after PoWeR to provide detailed information on how satellite cells/fusion affects myonuclear transcription. We show that while skeletal muscle can mount an adaptive response to PoWeR in the absence of satellite cells, hypertrophic growth and adaptation are attenuated in both the soleus and plantaris. Transcriptome-wide profiling of the soleus revealed several gene networks key to muscle adaptation are altered in the absence of satellite cells at the 4 and 8 week time points.

Results
**PoWeR-induced muscle hypertrophy is blunted in the absence of satellite cells**

The study design is shown in **Figure 1A**. Neither satellite cell depletion (SC-) nor PoWeR influenced body weight over the duration of the study (**Figure 1B**). Mice ran equal amounts over the 4-wk and 8-wk PoWeR protocols and this was not influenced by the presence of satellite cells (**Figure 1C**). PoWeR induced higher normalized soleus weight after 4-wks and 8-wks of PoWeR in SC+ and SC- groups; however, this effect was blunted at both time points in the absence of satellite cells (**Figure 1D**). PoWeR also induced higher normalized plantaris weight after 4-wks and 8-wks of PoWeR in SC+ and SC- groups which was blunted at 8-wks in the absence of satellite cells (**Figure 1E**). Tamoxifen administration did not influence body or muscle weights in sedentary controls, nor did it influence body weight or higher muscle weights after 8-wks of PoWeR in parental Pax7-CreER mice (**Supplemental Figures 1A-C**).

**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 IHC images from the soleus (**Figure 2A**) and quantitatively in the soleus and plantaris (**Figures 2B and C**). PoWeR training led to higher satellite cell density at 4-wks and 8-wks in the SC+ soleus and plantaris (**Figures 2B-C**).

**PoWeR promoted a shift in fiber type distribution in the plantaris, independent of satellite cell content**
Four-weeks of PoWeR training led to a myosin heavy chain (MyHC) 2b to MyHC 2a shift in fiber type distribution in the plantaris, which was unaffected by satellite cell depletion. This is shown qualitatively in representative IHC images (Supplemental Figure 2A) and quantitatively in Supplemental Figure 2B. Fiber type distribution did not differ between 4-wks and 8-wks of PoWeR in the plantaris (Supplemental Figure 2B). The soleus did not show any alterations in fiber type distribution in response to PoWeR (Supplemental Figure 2C).

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

PoWeR training led to higher myonuclear density at 4-wks in the soleus and plantaris in SC+ PoWeR trained mice, which did not increase further at 8-wks. No increase in myonuclear content was apparent in SC- muscle at either time point. This is shown qualitatively in representative IHC images from the soleus (Figure 3A) and quantitatively in the soleus and plantaris (Figures 3B and C). PoWeR training led to larger muscle fiber size at 4-wks and 8-wks in both the soleus and plantaris in SC+ and SC- groups; however, satellite cell depletion blunted muscle fiber hypertrophy at 4-wks and 8-wks in the soleus and at 8-wks in the plantaris (Figures 3D and E). To examine how satellite cell content transcriptionally influenced both short- and long-term muscle adaptation, RNA-seq was performed on the soleus as it displayed blunted muscle fiber hypertrophy at both the 4-wk and 8-wk time points.

Whole-muscle RNA-seq reveals altered gene expression during adaptation to 4-wks of PoWeR in satellite cell-depleted soleus muscle
To identify gene networks contributing to blunted muscle fiber hypertrophy in SC- soleus we performed whole-muscle RNA-seq on satellite cell replete and deplete soleus. After 4-wks of PoWeR training 1902 genes were overexpressed in SC+ soleus and 2600 were overexpressed in SC- soleus relative to their sham controls (Figure 4A). Gene Ontology (GO) and pathway analyses revealed substantial differences in gene expression profiles in response to 4-wks of PoWeR between SC+ and SC- soleus (Figures 4B and C). While both SC+ and SC- soleus displayed increased expression of genes promoting glycolysis, genes involved in extracellular matrix (ECM) remodeling and capillarization were significantly overexpressed only in the in SC+ soleus in response to PoWeR (Figures 4B and C). 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 5A expands the ECM remodeling network and Figure 5C 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 (Figure 5B) and capillarization (Figure 5D) networks, specific to SC+ soleus muscle, were further analyzed in GeneMANIA to visualize and provide information around the genes enriching 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+ soleus and 2502 were underexpressed in SC- soleus compared to their sham controls.
Enrichment analyses showed similarity in the pathways downregulated in response to 4-wks of PoWeR between SC+ and SC- soleus (Supplemental Figures 3B and C), including downregulation of oxidative metabolism and ribosome biogenesis. We also performed whole muscle RNA-seq on SC+ and SC- plantaris to examine if similar changes would occur in a different muscle undergoing adaptation to PoWeR. Supporting findings in the soleus, there is an upregulation in glycolytic gene networks and a downregulation in ribosome biogenesis gene networks in SC+ and SC- plantaris (Supplemental Figures 4A-C and 5A-C).

Analysis of the myonuclear transcriptome after 4-wks of PoWeR revealed downregulation of translation and oxidative metabolism genes specific to satellite cell deplete soleus

In order to examine the myonuclear transcriptome in relation to the whole-muscle transcriptome, we performed RNA-seq on individual isolated myonuclei (smnRNA-seq) from SC+ and SC- soleus muscles after 4-wks of PoWeR. A schematic of the workflow is shown in Figure 6A. The UMAP plot shows unbiased clustering of the combined data from SC+ and SC- soleus muscles, with the myonuclear cluster indicated (Figure 6B). Myonuclei within this cluster derived from SC+ muscle are shown in blue and from SC- muscle in pink in Figure 6C. Myonuclei from SC- soleus showed 964 underexpressed genes and 83 overexpressed genes when compared to SC+ myonuclei (Figure 6D).

GO and pathway analysis of the myonuclear transcriptome revealed lower expression of genes involved in ribosome biogenesis, oxidative metabolism and sarcomeric adaptation in SC- soleus, demonstrating a substantial suppression of these pathways.
after PoWeR in the absence of satellite cells (Figures 6E-G). An expanded list of the genes in these enriched pathways and their expression levels are shown in Figures 7A-C.

**SC- soleus displayed delayed transcriptional activity after 8-wks of PoWeR**

After 8-wks of PoWeR training only 166 genes were overexpressed in SC+ soleus while 1415 were overexpressed in SC- skeletal muscle relative to 4-wk PoWeR (Figure 8A). Consistent with a robust transcriptional response, GO and pathway analyses displayed a greater degree of pathway enrichment in SC- soleus in response to 8-wks of PoWeR (Figures 8B and C). In particular, translation and ribosome biogenesis networks were only enriched in the absence of satellite cells (Figures 8B and C and Supplemental Figures 6 A and B) and there was higher degree of enrichment in oxidative pathways in SC- soleus (Figures 8B and C). Pathways most highly enriched in the presence of satellite cells (e.g., RNA/mRNA stability) were also upregulated in SC- soleus at 8-wks compared to 4-wks (Supplemental Figures 6C and D).

After 8-wks of PoWeR training 157 genes were underexpressed in SC+ soleus and 1368 were underexpressed in SC- soleus (Figure 9A), consistent with the large difference in overexpressed genes seen between groups (see Figure 8A). GO and pathway analyses revealed few pathways to be heavily enriched in either group, but heterogeneity existed in enrichment analysis between SC+ and SC- soleus (Figures 9B 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+ soleus 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 soleus 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 not influenced by the presence of satellite cells in response to PoWeR. This is shown qualitatively in Picosirius red (PSR)-stained cross sections of the soleus imaged under bright field excitation (**Figure 10A**), quantified in **Figure 10B**. We found that only in the presence of satellite cells was there an increase in densely 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 10C**), quantified in **Figure 10D**. We also assessed capillarization and found a trend for reduced capillary density in response to PoWeR in SC- skeletal muscle. Representative images of lectin-stained cross sections of the soleus are
presented in Figure 10E and quantified in (Figure 10F). 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 were unable to maintain levels of peak torque when compared to SC+ muscles (Figure 10G). Neither PoWeR nor satellite cell depletion influenced gastrocnemius weight, suggesting the size of this muscle did not drive the noted differences in strength (Supplemental Figure 7).

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. What remains less clear is the extent to which satellite cells are necessary to mount a growth response to an exercise (or overload) stimulus. 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 responses are blunted when compared to satellite cell replete muscle. This is the first report to characterize how two metabolically distinct hindlimb skeletal muscles phenotypically adapt to short (4-wks) and sustained (8-wks) periods of muscle overload in the absence of satellite cells. Measures of strength, capillarization and collagen content in satellite cell deplete muscle during adaption are unique to this report. Further, the transcriptional profiling (RNA-seq) of skeletal muscle during different phases of adaptation in satellite cell deplete skeletal muscle is novel and our SMN-seq data set is if the first of its kind. Our data show, for the first time, that a hallmark of the response to exercise in the absence of satellite cells is an immense altering of the transcriptome that is highly ineffective—failing to influence biologically meaningful processes. Further, we identify the dysregulation of several exercise-induced gene networks in the absence of satellite cells that likely 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 appropriate
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 collagen networks and total collagen content. Analysis revealed lower proportions of densely 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.\textsuperscript{24,25} 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.\textsuperscript{24,26} Further research is needed to determine exactly how densely organized collagen networks directly contribute to force production.

Based on the different composition of collagens comprising 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 the level of peak force achieved by SC+ and SC- mice are not different, 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.\textsuperscript{5} 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 27 26.

While glycolytic pathways were enriched and shifts in MyHC composition occurred independent of satellite cell content, gene expression profiling showed that artery morphogenesis/capillarization pathways were preferentially activated in SC+ compared to SC- soleus, which was associated with a trend for lower capillary density in the absence of satellite cells. This suggests that the requirement for satellite cells in response to exercise is not ubiquitous and can vary between metabolic adaptations (e.g., shifts in MyHC isoform vs. capillarization). Capillaries are a critical component of muscle adaptation as they represent the points of exchange between the blood and surrounding tissues for oxygen, growth factors and nutrients 28. Low capillary density is associated with blunted skeletal muscle growth in humans, and muscle specific VEGF knockout attenuates angiogenesis and hypertrophy of the plantaris after functional overload 29 30 31 32. 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) 33-35. 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 33-35. Further, the report by Verma et al., showed ECM organization to be a highly enriched satellite cell to endothelial cell interaction pathway, which may in part explain our ECM findings 34. If attenuated capillarization manifests in lower maximal aerobic capacity was not addressed in the current study and is an exciting area of future exploration. Further, while we show phenotypic alterations that support the enrichment
of certain transcriptional networks, it should be acknowledged that a discordance between the proteomic and transcriptomic response to exercise has been reported 36.

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 show genes promoting ribosome biogenesis, sarcomeric adaptation, and oxidative phosphorylation are substantially lower in SC- compared to SC+ myonuclei, demonstrating a dysregulation of the myonuclear (muscle fiber) transcriptome in the absence of satellite cells. Downregulation of ribosomal genes in SC+ muscle corroborates what has been shown after chronic resistance training in humans and supports the idea that the downregulation of ribosome biogenesis plays a role in skeletal muscle adaptation 37. This is likely a form of energy partitioning through the stabilization of ribosomes (ribosome biogenesis is the most energy demanding process in cells) during exercise in order to meet the transcriptional and energy demands required for metabolic adaptation, sarcomeric remodeling and growth 38,39. However, the magnitude of transcriptional suppression in the absence of satellite cells that was detected at the myonuclear level is extreme and likely contributed to blunted growth. This transcriptional dysregulation may reflect a reliance on myonuclear addition.
via satellite cell fusion for the muscle fiber to effectively coordinate metabolic pathway
adaptation and muscle growth in response to PoWeR. This idea is supported by the
report from Omairi et al. who compared hypertrophic growth alone (Mtn+/− model) to
hypertrophic growth plus metabolic adaptation (Mtn+/−/ErryTg/+ model) and found
myonuclear accretion to occur only when the muscle was challenged with both growth
and metabolic stimuli.21.

These findings help to explain certain differences for the reliance on satellite cells based
on the overload stimulus utilized; e.g., synergist ablation, primarily a muscle growth
stimulus, vs PoWeR, a stimulus that drives muscle growth and substantial alterations in
metabolic pathways. The more granular approach taken to analyze the myonuclear
transcriptome and compare it to that of the whole muscle allows us to parse out
processes primarily regulated by myonuclei (ribosome biogenesis/muscle fiber
assembly/metabolic adaptation) vs processes potentially regulated by other cell types
which were present in our whole muscle sequencing, such as ECM remodeling via
fibroblasts and capillarization via endothelial cells. Although a recent publication using
single myonuclear RNA-seq demonstrated considerable heterogeneity in myonuclei,40
the extent to which the differences in myonuclear transcriptome can be attributed to
newly fused satellite cell-derived myonuclei remains to be determined.

The transcriptional signature of SC+ soleus 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. 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 over time. Interestingly, pathways downregulated at the 4-wk time point (oxidative phosphorylation and ribosome 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.

In response to both synergist ablation 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. 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 suggest that satellite cells are critical for a coordinated and effective transcriptional response to a novel stimulus, optimizing adaptations and
muscle hypertrophy. It seems possible that satellite cells and/or fusion are not anabolic 
per se, but that satellite cells are a prerequisite for a coordinated transcriptional 
response to exercise that will lead to maximal muscle adaptation.

Materials and Methods

Animals

The Pax7\textsuperscript{CreER}\textsuperscript{+/Rosa26\textsuperscript{DTA}\textsuperscript{+/}} strain, called Pax7-DTA, was generated by crossing the male 
Pax7\textsuperscript{CreER}\textsuperscript{+/CreER} mouse strain with the female Rosa26\textsuperscript{DTA}\textsuperscript{DTA} mouse strain \textsuperscript{41}. 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 \(^2\). 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 \textit{ad libitum} and were checked daily for health and wellness.
Running wheels were locked 24 hours before sacrifice and mice were fasted overnight.
Elevated mRNA expression following exercise peaks after 3-6 hours and returns to
baseline by 24 hours \(^42\). The gastrocnemius muscle was excised and weighed. The
soleus and plantaris muscles were excised, weighed and prepared for IHC analysis and the contralateral limb of the soleus for RNA extraction (a subset of plantaris muscles were prepared of RNA extraction), and then stored at −80 °C. As there were no within or between group differences in body or muscle weights in 4-wk Sedentary SC+/SC- or 8-wk Sedentary SC+/SC- mice, 4 and 8-wk time points were collapsed and treated as one group for each treatment (Sedentary SC+ and Sedentary SC-). For smnRNA-seq analysis, an additional 6 mice were randomly assigned to the 4-wk PoWeR SC+/SC- groups. Wheels were locked for 24 hours before sacrifice and mice were fasted overnight. The soleus muscle from one SC+ mouse and one SC- mouse was excised and prepared immediately for smnRNA-seq. Mice from each group (SC+/SC-) were selected based on similarity in body weight and running volume over the 4-wk protocol. Another group of 6 mice were randomly assigned to the 8-wk PoWeR SC+/SC- groups for in vivo muscle function testing. Wheels were locked 48 hours before testing. Adult (6-months old) female mice from the parental strain, Pax7^{CreER/CreER} (Pax7-CreER) were also randomized to vehicle (n=11) or tamoxifen (n=11) treatment and then sedentary or 8-wk PoWeR groups to determine the effects of tamoxifen, independent of satellite cell depletion.

**Immunohistochemistry (IHC)**

IHC analyses were performed as previously described. Briefly, hindlimb muscles were excised 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 myosin heavy chain (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. 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).

For Picrosirius red (PSR) and lectin staining soleus muscles were excised and 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. Muscle cross sections were stained with PSR for 1 hour per the manufacturer’s instructions (24901-500; Polysciences, Warrington, PA, USA). PSR 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 43. Fresh muscle sections were cut and allowed to air dry for 4 hours, rehydrated with PBS, blocked in 2.5% normal horse serum (NHS, Vector Laboratories, S-2012) and incubated in Texas Red conjugated Lectin from Griffonia Simplicifolia (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 44. PSR images were captured at 10x magnification and quantifications were
obtained with ImageJ software. 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. Images of lectin stained sections were taken at equal exposure times. Using the analyze feature in Zen, the images were thresholded for lectin positive areas and the analysis was applied across the entire cross section of muscle. Areas of excess background staining and auto florescence were excluded from the quantification. The number of capillaries detected by the software was summed to get the total number of capillaries in each section. Sections were measured for area using the spline contour feature in ZEN. Individual fibers were counted by hand to get the fiber number and the data expressed as Lectin+ events/fiber.

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. 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 micrograms 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 were 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 46.

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 were 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. FPKM (Reads Per Kilobase of exon model per Million mapped reads) of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM 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. Differential expression analysis between two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). DESeq2 provides 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 et al. was followed for isolation of myonuclei. 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, 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 FilteredBarcode_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 Data Monitor 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, 100 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.
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Disclosures

The authors declare no conflicts of interests
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Figure Legends

Figure 1. Satellite cell depletion blunted higher muscle weights induced by PoWeR. (A) Study design for Sedentary (Sed) and PoWeR trained satellite cell replete (SC+) and deplete (SC-) mice. The amount of weight in grams added to the wheel each week during PoWeR is indicated (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 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 both the soleus and 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.

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Figure 5. Enriched signaling networks and gene sets specific to SC+ soleus. (A) Collagen signaling network. (B) Gene set enriching the collagen activated tyrosine kinase receptor signaling pathway node. (C) Capillarization signaling network. (D) Gene set enriching the artery morphogenesis node.

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Figure 7. Muscle adaptation signaling networks are suppressed in the absence of satellite cells in soleus. (A-C) Gene list and expression levels of the gene sets enriching (A) SRP-dependent cotranslational protein targeting to membrane, (B) oxidative phosphorylation and (C) myofibril assembly.

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Figure 8. Oxidative phosphorylation and ribosome biogenesis are upregulated after 8-wks of PoWeR only in the absence of satellite cells in the soleus. (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 9. Collagen remodeling is suppressed after 8-wks of PoWeR only in the absence of satellite cells in the soleus. (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 10. Satellite cell depletion led to aberrant collagen remodeling, capillarization and strength adaptation to PoWeR. (A) Representative Picrosirius red (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 (n = 3/group). 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. *p<0.05, **p<0.01.