

Genetic deletion of skeletal muscle Cox6a2 (Cytochrome C Oxidase Subunit 6a polypeptide 2)
delays regeneration in preclinical model of Peripheral Artery Disease

By

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Abstract

Chronic limb threatening ischemia (CLTI) is the most severe clinical manifestation of peripheral artery disease (PAD). There are no current therapeutic options for CLTI outside of surgical intervention and it remains the leading cause of limb amputation. There is a dire need for therapeutic innovation for these patients. Mitochondrial insufficiency is a hallmark of the clinical CLTI presentation and an exciting avenue for therapeutic development. Previously, we identified unique CLTI patient deficits in Cox6a2 protein abundance, a muscle specific binding subunit in Cytochrome C Oxidase. We hypothesize that skeletal muscle *Cox6a2* is necessary for tissue regeneration after hindlimb ischemia (HLI) due to its integral role in the bioenergetics of skeletal muscle. To test this hypothesis, we generated Pax7-Cre⁺;Cox6a2^{fl/fl} mice, which genetically possess a non-inducible (lifelong) deletion of Cox6a2 in the muscle progenitor cells and mature skeletal muscle myofibers. At baseline, we observed similar oxygen consumption rates under physiologically relevant ranges of ATP free energy yet severe reductions in Complex IV-linked

respiration under maximal energetic demand in the Cox6a2 knockout (KO; n=10) group compared to the wildtype control group (Pax7-Cre⁻;Cox6a2^{fl/fl} or ^{fl/-} (WT); n=13). Proteomics profiling of mitochondria isolated from KO animals indicated significant decreases in the complex IV proteome. We then subjected these mice to hindlimb ischemia (HLI; n= 35 KO, n=31 WT). We observed similar blood flow and vascular recovery and no differences in muscle contractility between KO and WT ischemic limbs 7 days post-HLI. Decreased mitochondrial Complex IV-specific maximal respiration was observed in KO ischemic limbs, though no differences in respiration in a physiologically relevant range of ATP free energy occurred between groups. At d7, the KO ischemic mitochondrial proteome revealed major downregulations of complex IV proteins and upregulations of complex I and V, indicating a compensatory shift in expression due to the loss of cox6a2 in ischemic conditions. Extended (28-day) HLI (n=16 KO, n=19 WT) revealed delays in regeneration indicated by increased centrally located nuclei and decreased myofiber size in KO limbs. Fiber type, density of total and perfused vessels, and muscle contractility remained similar between KO and WT groups. Together, these data indicate that Cox6a2 is involved in efficient muscle regeneration during prolonged ischemic events.

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delays regeneration in preclinical model of Peripheral Artery Disease

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LIST OF ABBREVIATIONS

PAD	Peripheral Artery Disease
CLTI	Chronic Limb Threatening Ischemia
Cox6a2	Cytochrome C Oxidase Subunit 6a Polypeptide 2
QTL	Quantitative Trait Locus
KO	Knockout
WT	Wildtype
HET	Heterozygous
HLI	Hindlimb Ischemia
TA	Tibialis Anterior
EDL	Extensor Digitorum Longus
Gastroc	Gastrocnemius
FACS	Fluorescent-activated cell sorting
CSA	Cross-sectional area
OCT	Optimal cutting temperature
CK	Creatine Kinase
HK	Hexokinase
ΔG_{ATP}	ATP free energy

Introduction

It is estimated that over 200 million people have Peripheral Artery Disease (PAD) worldwide, which represents a 72% increase over the past 30 years^{1,2}. Chronic Limb Threatening Ischemia (CLTI) is the most severe form of PAD and is associated with increased mortality and amputation rates³. CLTI patients have zero interventional options outside of surgery and 42% of patients with CLTI still need amputation after surgery⁴. An additional 20% of CLTI patients are considered “no option” for surgical intervention^{5,6} due to the lack of adequate bypass and/or advanced occlusion of the pedal arteries⁷. These outcomes highlight the dire need for innovative approaches to therapeutic development in the CLTI population.

An association between skeletal muscle mitochondria and PAD presentation exists^{8,9}. Mitochondrial gene suppression, lower mitochondrial protein abundance, and lower mitochondrial capacity distinguish patients with CLTI from patients with intermittent claudication⁸. A causative role for mitochondria is not currently known, although targeting this organelle is of therapeutic benefit to the ischemic limb¹⁰⁻¹². Analysis of the CLTI mitochondrial transcriptome and proteome performed in our lab indicated a unique suppression of bioenergetic genes and proteins at the time of amputation^{8,13}. In conjunction with observed reductions in mitochondrial oxygen consumption and electron transport system enzyme function, the CLTI pathophysiology has been linked to mitochondrial deficiencies due to inadequate ATP production required for regeneration^{8,14}. The mitochondrial deficiencies observed in the CLTI population are a possible cause of the severity of the disease that warrants investigation. Identifying specific mitochondrial targets for interrogation remains an exciting area of therapeutic development for PAD.

Previous research identified a mouse quantitative trait locus (QTL) *lsq-1* on chromosome 7¹⁵ which included 37 genes linked to differences in necrosis and perfusion recovery between multiple mouse lineages (BALB/c, A/J, and C57Bl6/J). Cytochrome C Oxidase Subunit 6a polypeptide 2 (Cox6a2) was the only mitochondrial gene identified in this locus¹⁵. Cox6a2 is a nuclear encoded structural subunit of mitochondrial complex IV and plays a role in the assembly and stabilization of the COX enzyme¹⁶. Cox6a2, located on chromosome 16 in humans, is comprised of three exons and two introns¹⁷ and is predominantly expressed in cardiac and skeletal muscle^{18,19}. Cox6a2 mRNA and protein are decreased in CLTI patients supporting its' potential as a therapeutic target^{8,13}.

We generated a lifelong skeletal muscle progenitor cell and myofiber Cox6a2 knockout mouse utilizing a Pax7-Cre promoter and floxed Cox6a2 allele (Cox6a2^{fl/fl}). A benefit of using the non-inducible PAX7 is to avoid the reintroduction of Cox6a2 into muscle fibers via skeletal muscle progenitor cells during regeneration after ischemic injury. The aim of this study was to determine whether Cox6a2 is required for muscle regeneration and limb blood flow recovery after hindlimb ischemia (HLI). We hypothesized that the genetic deletion of Cox6a2 would alter mitochondrial bioenergetics at baseline and delay tissue recovery following ischemic injury. We discovered that the loss of Cox6a2 decreased the mitochondrial proteomic abundance of COX enzyme components and reduced maximal respiratory capacity of complex IV. After ischemia, delays in tissue recovery were observed despite perfusion and vessel recovery. Our data demonstrate that Cox6a2 plays an integral role in the timely regeneration of skeletal muscle after ischemic injury.

Materials and Methods

Animal Generation

The conditional Cox6a2 allele was created in collaboration with Transviragen (Chapel Hill, NC). A targeting vector flanking exons 1-3 of the Cox6a2 gene, including a region approximately 727bp upstream of Exon 1 and downstream in the 3' untranslated region just prior to the end point of the overlapping Itgad 3'UTR was transfected by electroporation into C57BL/6J ES cells. ES cell clones were screened for homologous targeting by long range PCR. Validated targeting clones were injected into C57BL/6J-Albino blastocysts for chimera production. Chimeras were then bred for germline transmission and selection cassette removal with C57BL/6-Albino Flpe females for removal of selectable marker and offspring were screened by PCR for targeting allele and selection cassette deletion. F1 floxed/+ offspring were backcrossed to C57BL/6J females to further establish Cox6a2^{fl} and then crossed to generate homozygous Cox6a2^{fl/fl} mice. Cox6a2^{fl/fl} mice were crossed to the commercially available Pax7-Cre⁺ mouse line (Jax Labs Strain #:010530). The resulting Pax7-Cre⁺; Cox6a2^{fl/-} male mice from this initial breeding scheme were then crossed with female Cox6a2^{fl/f} mice to generate Pax7-Cre;Cox6a2^{fl/f} mice. Mice were aged to 12-16 weeks before use.

Animal Genotyping

Animal genotyping was completed based on the presence of Pax7-Cre, and the heterozygote or homozygote Cox6a2^{fl} using genomic DNA. Primer sets for Pax7-Cre ck256- 5'-TCGGCCTTCTTCTAGGTTCTGCTC-3', ck118- 5'-GCTCTGGATACACCTGAGCT-3' and ck1172- 5'-GGATAGTGAAACAGGGGCAA-3' were used to verify Pax7-Cre animals, where Pax7-Cre is ~350 bp and the wildtype is ~450bp. Additionally, animals were genotyped for

Cox6a2^{fl} using primer sets F-5'-GAAGTCATTCCGTGCCACTGT-3' and R-5'-CCTGCTTGCTCCAGCCC-3', where the Cox6a2^{fl} amplification band is ~2038bp and the wild-type band is ~1891bp.

Experimental Approach

All animals were housed in a temperature (22°C) and light controlled (12-hour light/12-hour dark) room with free access to food and water. All animal experiments adhered to the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research, National Research Council, Washington, D.C., National Academy Press, 1996, and any updates. All procedures were approved by the Institutional Animal Care and Use Committee of East Carolina University

Hindlimb Ischemia

Acute unilateral hindlimb ischemia (HLI) of the left femoral artery was performed as previously described²⁰. Mice were sacrificed by cervical dislocation 7 or 28 days after HLI (d7 or d28) under a ketamine (90 mg/kg bodyweight) and xylazine (10 mg/kg) anesthetic. Limb blood flow scans were measured by Laser Doppler Perfusion Imaging (LDPI) using a LDI2-High Resolution (830 nm) System (Moor Instruments), as previously described²⁰. Blood flow scans were obtained at baseline (pre), immediately after HLI (d0) and at d7, d14, and d28 after HLI. 1.5 hours prior to sacrifice, 50 µL of 1 mg/mL Griffonia simplicifolia Isolectin-B4 (GSIB4) DyLight 594 conjugate (Vector Labs, #DL-1207) was injected into the left retroorbital sinus using a 31-gauge needle.

Histology

Transverse sections of the Tibialis Anterior (TA) frozen in Tissue-Tek optimal cutting temperature (OCT) medium in liquid nitrogen-cooled isopentane were cut at 10µm using a 3060S (Leica) cryostat and set on charged slides. Slides were stored at -20°C until staining.

Standard hematoxylin and eosin (H&E) was performed, as previously described^{20,21}.

Immunohistochemistry (IHC) staining was performed, as previously described^{20,21}. Briefly, sections were fixed in 1:1 acetone/methanol for 5min at -20°C, dried at room temperature for 5 min, and blocked in 5% goat serum in PBS for 1 hour. Sections were incubated overnight at 4 °C in primary antibodies for: Smooth Muscle Actin (1:100; ThermoScientific, # MA5-11547) and rat anti-mouse CD31 (platelet endothelial cell adhesion molecule 1; 1:100 dilution; BioRad, # MCA2388), or rabbit anti-mouse dystrophin (1:200 dilution; Thermo Rb-9024; targeted to the protein C-terminus), MyHC Type I (BA-F8, 1:50), Type IIa (SC71, 1:50) and Type IIb (BF-F3, 1:50; Developmental Studies Hybridoma Bank, University of Iowa). The following day, slides were washed with PBS and incubated for 1 hour with: Alexa-Fluor 488-conjugated anti-rat and 647-conjugated goat anti-mouse IgG secondary antibodies (1:250, Invitrogen, # A-11006 and #A32728) or Alexa-Fluor 647-conjugated anti-rabbit (1:250, Invitrogen, #A-21245), 350 goat anti-mouse IgG2b (1:250, Invitrogen, #A21140), 488 goat anti-mouse IgG1 (1:250, Invitrogen, #A21121), and 546 goat anti-mouse IgM (1:250, Invitrogen, # A21045). Samples were mounted using Vectashield hard mount medium (Vector Labs) and imaged with an Evos FL auto microscope (Thermo Fisher) with a fluorite 20x objective lens. Images were taken across each muscle section. Images were analyzed by a blinded investigator using ImageJ version 1.53a. Technical replicates were averaged, and data is represented by mean fluorescence positive area.

Immunoblot Analysis

Isolated mitochondria pellets were resuspended in RIPA buffer supplemented with protease inhibitors and a BCA assay was utilized to measure protein content as previously described⁸, with some modifications. Mitochondrial protein was separated via electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Trans-Blot Turbo Transfer System; Bio-Rad) and imaged for total protein using the stain-free labeling on a Bio-Rad Chemidoc Imaging system. After blocking, membranes were incubated overnight with primary polyclonal antibodies against Cox6a2 (1:1000, Invitrogen, #PA5-21922) and VDAC (1:1000, Invitrogen, #PA1-954A) at 4°C. The next day, membranes were incubated at room temperature for 1h with goat-anti rabbit HRP-conjugated secondary antibodies (1:12,500 Invitrogen). Proteins were visualized via chemiluminescence (Clarity Western ECL Substrate, Bio-Rad). The images were quantified using Image Lab and normalized to total protein content.

Muscle Contractile Function

Muscle contractile function was assessed in vitro for EDL muscles as previously described²², using an Aurora 300B-LR system (Aurora Scientific, Aurora, ON, Canada). Resting tension and muscle length were adjusted for each muscle to obtain the optimal twitch contraction force prior to force frequency and fatigue resistance protocols. The length and mass of each muscle were recorded for calculation of physiological CSA and specific force, expressed as (N/cm²). Force frequency curves from each stimulation frequency were integrated over time and then added together to calculate force capacity (N*s/cm²).

Skeletal Muscle Mitochondrial Isolation

Skeletal muscle mitochondrial isolation of the gastrocnemius complex (both heads of the gastrocnemius, plantaris, and soleus) was conducted using differential centrifugation as previously described²³, with some modifications. The following buffers were utilized: buffer A (PBS and 10 mM EDTA at pH 7.4); buffer B (50 mM MOPS, 100 mM KCl, 1 mM EGTA, 5 mM MgSO₄); and buffer C (buffer B and 2 g/liter BSA). Freshly dissected gastrocnemius complex was immediately placed in cold Buffer A, thoroughly minced, and subjected to a 5-minute 5% trypsin incubation on ice. The samples were centrifuged at 600xg for 5 minutes at 4°C. The tissue pellet was resuspended in 14 mL of Buffer C and homogenized via a drill-driven Teflon pestle and borosilicate glass vessel. Homogenates were centrifuged at 800xg for 10 min at 4°C. The supernatant was filtered through two layers of thin gauze and centrifuged at 10,000xg for 10 min at 4°C. Pellets were washed in 1.4 mL of Buffer B, transferred to 1.7 mL microcentrifuge tubes and centrifuged at 10,000xg for 10 min at 4°C. Final mitochondrial pellets were resuspended in 150 µL of Buffer B. Protein content was determined using a BCA protein assay. Freshly isolated mitochondria were immediately used for functional experiments. The remaining sample was centrifuged at 10,000xg for 10 min and the pellet was stored at -80 to be used for enzyme activity assays and Western blot analyses.

High Resolution Respirometry

High-resolution respirometry (Oroboros Oxygraph-2K) was used to measure O₂ consumption rate from isolated mitochondria. O₂ measurements were conducted at 37°C in a 1 mL reaction volume as previously described²³. The base assay buffer used for all experiments was: Buffer D (105 mM potassium-MES, 30 mM KCl, 10 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EGTA, 2.5 g/L BSA and 5 mM creatine monohydrate; pH = 7.2).

Creatine Kinase Clamp Experiments

The Creatine Kinase (CK) clamp was used to determine steady state JO_2 across a physiologically relevant range of ATP free energy (ΔG_{ATP}), as previously described²³. 20ug of fresh mitochondria were added to Buffer D followed by 3 different respiratory substrate conditions: 1) 5mM Pyruvate and 2mM Malate (P/M); 2) 5uM Rotenone and 10mM Succinate (R/S); and 3) 5mM Pyruvate, 2mM Malate, 5mM Glutamate, 0.2mM Octanoyl-carnitine and 5mM Succinate (Multi). The CK clamp was initiated by the addition of 5mM ATP, 1mM PCr and 20U/mL CK, simulating a high energy demand for ATP re-synthesis. Phosphocreatine (PCr) was then sequentially added to reach 6, 15 and 21mM concentrations to create a low energy demand state. Maximal uncoupled respiration was stimulated via carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a respiratory uncoupler that depolarizes mitochondrial membrane potential.

Hexokinase Clamp Experiments

The Hexokinase (HK) clamp was utilized with freeze fractured mitochondria to measure the respiration under saturating ADP conditions, as previously described²³. Buffer D supplemented with cytochrome C and 5mM ADP was loaded into the chambers, followed by 10ug of freeze thawed mitochondria. Hexokinase (1U/mL) and glucose (5mM) were then added to the chamber, followed by NADH to directly measure NADH-linked respiration (Complexes I-III-IV). This respiration was then inhibited by rotenone (0.002mM), and succinate-linked respiration was stimulated by the addition of succinate (10mM) (Complexes II-III-IV). Antimycin A (0.0025uM) was then added, inhibiting succinate-linked respiration, and complex IV was stimulated through the addition of the cytochrome C-specific electron donor N-tetramethyl- p-phenylenediamine (TMPD; 0.5 mM) and ascorbate (2 mM).

Proteomics- Isolated mitochondria and whole tissue

Label Free proteomics was conducted, as previously described^{24,25}. Mitochondrial samples were isolated without the addition of trypsin. Whole tissue gastrocnemius complexes were lysed in ice-cold 8 M Urea Lysis Buffer (8 M urea in 50 mM Tris, pH 8.0, 40 mM NaCl, 2 mM MgCl₂, 1x complete ULTRA mini EDTA-free protease inhibitor tablet) and homogenized using a drill-driven Teflon pestle and borosilicate glass vessel. The mitochondrial samples were resuspended in Urea Lysis Buffer and then all samples (both mitochondria and whole tissue) were frozen in liquid nitrogen, thawed for three freeze-thaw cycles, and then sonicated in three 5 second bursts (Q Sonica #CL-188; amplitude of 30). Samples were centrifuged at $10,000 \times g$ for 10 min at 4 °C. Protein concentration was determined by BCA, and equal amounts of protein were reduced with 5 mM DTT at 37 °C for 30 min and alkylated with 15 mM iodoacetamide (IAM) at room temperature for 30 min in the dark. Unreacted IAM was quenched by the addition of DTT to reach final concentration of 15 mM. Lys C (ThermoFisher Cat# 90307; 1:100 w-w) was added and sample was digested for 4 hours at 32 °C. Samples were diluted to 1.5 M urea with 50 mM Tris (pH 8.0), 5 mM CaCl₂, and then digested overnight with seq grade trypsin (Promega; Cat# V5113; 50:1 w/w) at 37 °C. Samples were acidified to 0.5% TFA and centrifuged at $10000 \times g$ for 10 min at 4 °C to pellet insoluble material. Supernatant containing soluble peptide was desalted, as previously described²⁴, and the 1.5 mL eluate was frozen and lyophilized.

FACS - PAX7+ cell isolation

Limb skeletal muscle was homogenized, washed, digested, and filtered to isolated cell stock for fluorescent-activated cell sorting (FACS) sorting. Briefly, tissue is minced and centrifuged in DMEM supplemented with antibiotics before digestion for 30 minutes at 37* in 10 mL Collagenase B/ Dispase solution. Digested slurry is filtered through a 100uM strainer and centrifuged to clear before debris removal and subsequent resuspension in PBS supplemented with FBS. Pelleted/resuspended cellular material is then transferred to separate FACS tubes for preparing unlabeled and labeled samples. Labeled sample aliquot is incubated with anti-Alpha 7 Integrin (R&D Systems #FAB3518P), CD34 (eBiosciences, #11-0341-82), CD45 (BD Biosciences, #559864), CD11b (BD Biosciences, #557686), CD31 (BD Biosciences, #563608), and Ly/6AE (BD Biosciences, #565355). 4',6-diamidino-2-phenylindole (DAPI; D1306, Thermo Fisher) was added to the sample to remove dead cells. Fluorochrome controls using antibody capture beads are used as compensation controls. Pax7+ satellite cells were identified in the heterogenous cell mixture using a set around live cells positive for alpha 7 integrin and CD34.

Statistics

Data are presented as mean $\pm$ SEM. Comparisons of data with more than 2 groups were performed using ANOVA with Tukey's post hoc test for multiple comparisons. In all cases, $P < 0.05$ was considered statistically significant. Figures were generated using GraphPad Prism (Version 10.0.2). Unless otherwise specified, the number of mice per experiment is represented by "N".

Results

Cox6a2 Deletion is Pax7⁺ Cell and Skeletal Muscle Specific

Non-inducible Pax7-Cre initiates Cre-mediated recombination in Pax7⁺ cells during development and maturation, which results in a Pax7⁺ cell population with recombined genetic material as well as developed myofibers with recombined genetic material^{26,27}. Pax7-Cre;Cox6a2^{fl} mice were bred to generate heterozygous and homozygous deletion littermates (Figure 1A). WT mice include background matched Cre⁻ mice heterozygous or homozygous for Cox6a2^{fl}. “HET” mice are representative of Pax7-Cre⁺;Cox6a2^{fl/-} and knockout (KO) representative of Pax7-Cre⁺;Cox6a2^{fl/fl}. To verify deletion of Cox6a2, genetic recombination was first performed on heart and skeletal muscle (Diaphragm and Quadriceps) samples collected from adult mice (Figure 1B). KO and HET groups displayed a deletion/recombination band (~295 bp) for quadricep muscles, partial recombination in diaphragm, and no recombination in the heart whereas the WT group displayed full length (~2038 bp) Cox6a2 in all three tissues tested. qRT-PCR was utilized to quantify Cox6a2 mRNA from adult EDL limb muscle (Figure 1C). Reductions of Cox6a2 message in the KO group averaged 98.4% (p=0.0007) in comparison to the WT group. There were no observed differences in mRNA expression (p=0.39) between HET and WT. To verify recombination specific to the Pax7⁺ cell population, adult skeletal muscle tissues were collected and FACS sorted prior to recombination analyses. FACS sorting of heterogenous cell samples of WT or KO mice were gated using different antibodies to remove dead and PAX7⁻ cells from the sample (Figure 1D). This resulted in a pure, live PAX7⁺ cell population we then used to confirm recombined Cox6a2 in the KO and full Cox6a2 in WT animals (Figure 1E). Lastly, Cox6a2 protein abundance was reduced in KO isolated limb muscle mitochondria compared with WT, and HET by Western blotting (Figure 1F). VDAC was

assessed as a mitochondrial loading control. There were no quantified differences between the HET and WT groups in protein abundance of Cox6a2. Together, these data confirm that Cox6a2 was deleted in the skeletal muscle progenitor cells and in mature skeletal muscle of the KO animals, but not in the HET or WT animals.

Cox6a2 KO Mice Do Not Display a Skeletal Muscle Phenotype at Baseline

KO mice displayed no observed outward anatomic phenotype compared to HET and WT groups (Figure 2A). No differences were observed in body weights (Figure 2B) or EDL muscle weights (Figure 2C) between genotypes. To visualize the skeletal muscle of the KO groups compared to WT and HETs, transverse sections of the TA were H&E stained and imaged (Figure 2D). There were no observed qualitative differences in myofiber size and nuclei distribution in the TA of KO compared to HET and WT groups at baseline. Contractility was measured using twitch protocols to achieve optimal length of the EDL and then a force frequency protocol to achieve maximal contractile force of the muscle. Absolute force production (Figure 2E) revealed no significant differences in total force production between groups. When values were normalized to muscle weight and length, there continued to be no differences between groups (Figure 2F).

Cox6a2 KO Mice Display Baseline Mitochondrial Deficiencies

To further investigate the impact of Cox6a2 deletion at baseline, label-free quantitative nLC-MS/MS was performed on isolated mitochondria isolated from KO and WT animals (Figure 3A-C). Cox6a2 was confirmed to be the most significantly decreased protein of the KO mitochondrial proteome (Figure 3A). The most severe mitochondrial protein disturbances were located specifically in complex IV (Cytochrome C Oxidase) of the KO group (Figure 3B). Mitochondrial enrichment factor (MEF), which indicates a ratio of mitochondrial protein content

per unit of isolated mitochondrial protein in the sample²⁸, revealed no differences between WT and KO mitochondrial protein of the isolated mitochondrial proteome, and served as a quality control of the mitochondrial isolation (Figure 3C). A heatmap of the significantly altered mitochondrial proteins demonstrated that the entire COX enzyme was dysregulated by the deletion of Cox6a2 in the KO group, indicated by the 9 most downregulated proteins in the KO mitochondria: Cox6a2, Cox6c, Cox5b, Cox6b1, Cox5a, Cox7a1, Cox7c, Cox4i1, and mt-Co2 (Figure 3D).

Using the creatine kinase (CK) clamp, high resolution respirometry was next used to determine whether decreased Complex IV protein abundances reduced mitochondrial respiration within a physiologically relevant range of ΔG_{ATP} (Figure 3E-G). At baseline, WT, HET, and KO animals showed no differences in oxygen consumption rates when measuring only complex I-induced respiration (Figure 3E), only complex II-induced respiration (Figure 3F), or when measuring respiration via both complexes I and II (Figure 3G). Electron transport system capacity with the CK clamp, assessed by additions of FCCP to uncouple the mitochondria, revealed no significant differences between groups under any of the substrate conditions. We next utilized the hexokinase clamp (HK) under saturating ADP conditions to measure maximal oxygen consumption rates of complexes I-III-IV, II-III-IV, and IV in freeze fractured mitochondria (Figure 3H). Cox6a2 KO reduced complex I-III-IV linked respiration by 67.2% and complex IV linked respiration by 58.6% at baseline. There were no observed effects of heterozygous deletion on enzymatic activity at baseline.

Effects of Cox6a2 deletion on vascular recovery at d7 HLI.

We next performed HLI on KO mice to determine the impact of mitochondrial alterations on the tissue ischemic response. mRNA expression of Cox6a2 remained significantly reduced in the

KO control limb (Figure 4A). Decreases of Cox6a2 expression were observed in both WT and KO ischemic limbs. There were no effects of KO on blood flow recovery at HLI d7. Non-invasive Laser Doppler Perfusion Imaging (LDPI) was utilized to analyze limb blood flow prior to HLI (pre), immediately post HLI (d0), and 7 days after HLI (d7) (Figure 4B). HLI resulted in decreased paw blood flow post-surgery (Figure 4C).

To better understand capillary density and perfusion recovery of ischemic skeletal muscle, immunofluorescence was performed on transverse sections of the TA (Figure 4D). There were no observed effects of Cox6a2 KO on the relative smooth muscle actin (Figure 4E), endothelial (CD31+; Figure 4F), or perfused vessel area (GSA-isolectin+; (Figure 4G). The ratio of perfused vessels to total vessels also revealed no differences between groups (Figure 4H), confirming that limb blood flow and muscle capillary perfusion recovery was not altered in the ischemic limb of the KO animals.

Effects of Cox6a2 deletion on muscle recovery at d7 HLI.

Qualitative analysis of H&E's at d7 revealed similar levels of ischemic lesion damage between groups (Figure 5A). After HLI, trending decreases in EDL weights of the ischemic limb of both WT and KO animals were observed, as expected, however no differences as a result of KO were observed (Figure 5B). EDL absolute (Figure 5C) and specific (Figure 5D) force production was reduced similarly in WT and KO muscles. The KO animals had significantly lower force production in the ischemic limb compared to the contralateral control limb at 10, 20, 80, 100 and 120 Hz ($p < 0.05$) while no significant reductions were observed in the WT ischemic to control limbs. When subjected to a 5-minute fatigue protocol, no differences were observed between KO and WT ischemic or controls limbs (Figure 5E).

Ischemia stimulates a distinct whole-tissue protein response and in the KO group

To investigate the proteomic impact of Cox6a2 deletion after ischemia, label-free quantitative nLC-MS/MS was conducted between KO and WT whole tissue gastrocnemius muscles (d7 ischemic and control). A volcano plot of WT ischemic to control limb revealed many upregulated proteins with a high significance in response to ischemia (Figure 6A). In WT animal limb tissues, ischemia downregulated 45 and upregulated 415 proteins (Figure 6B). Panther-go analysis revealed significantly downregulated “molecular function” targets within the ATP-dependent, binding, catalytic, cytoskeletal motor, molecular function, and structural molecule activity classifications. Significantly upregulated “molecular function” targets contained all classifications of the downregulated proteins as well as additional classifications of molecular function regulator, molecular transducer, transcription and translation regulator, and transporter activity. The top 15 up-regulated and down-regulated proteins (Log2FC) in the WT ischemic limb are provided in Table 1. The 5 most down regulated WT ischemic proteins were: ECSIT (Evolutionarily conserved signaling intermediate in Toll Pathway), Prkcsh (Isoform 2 of Glucosidase 2 subunit beta), Krt15 (Keratin 15), Mrps5 (28S ribosomal protein S5, mitochondrial), and Krt10 (Keratin, type I cytoskeletal 10). The 5 most upregulated proteins in the WT ischemic limb were: Lamp2 (Isoform LAMP-2B of Lysosome-associated membrane glycoprotein 2), Hmgb2 (High mobility group protein B2), Mustn1 (Musculoskeletal embryonic nuclear protein 1), Hnrnpdl (Heterogeneous nuclear ribonucleoprotein D-like), and Khshp (Far upstream element-binding protein 2).

A volcano plot of the KO ischemic to control limb also showed an abundance of upregulated proteins, though most with a reduced p value compared to the WT ischemic response (Figure 6C). In KO tissues, ischemia downregulated 25 and upregulated 354 proteins in the KO tissue

proteome (Figure 6D). The top 15 up-regulated and down-regulated proteins (Log2FC) in the KO ischemic limb are provided in Table 2. The 5 most down regulated KO proteins were: Ube2d3 (ubiquitin-conjugating enzyme E2D), Cops4 (COP9 signalosome complex subunit 4), Map2k3 (Dual specificity mitogen-activated protein kinase kinase 3), Myom1 (Myomesin-1), and Rtn4ip1 (Reticulon 4 interacting protein 1). The 5 most upregulated proteins in the KO ischemic limb were: Pkm (Pyruvate kinase PKM), Tubb2b (Tubulin beta-2B chain), Tubb6 (Tubulin beta-6 chain), Sarnp (SAP domain-containing ribonucleoprotein), and Rab6a (Ras-related protein Rab-6A). Panther-go analysis revealed significantly downregulated “molecular function” targets within ATP-dependent, antioxidant, binding, catalytic, cytoskeletal motor, and transporter activity classifications (Figure 6D). Significantly upregulated “molecular function” targets contained all classifications of the downregulated proteins as well as additional classifications of molecular adaptor, molecular function regulator, molecular transducer, structural molecule, transcription and translation regulator activities. Overlap of 6 of the 45 and 25 downregulated proteins in the WT and KO (ischemic: control limb), respectively, were observed (Figure 6E). Despite vast similarities in the upregulated protein molecular functions, only 218 of upregulated proteins occur in both genotypes (Figure 6F). The top 10 up-regulated and down-regulated proteins (Log2FC) in the KO v WT ischemic limb are provided in Table 3. Of the 10 most-downregulated proteins, Ube2d3 (Ubiquitin-conjugating enzyme E2D), Map2k3 (Dual specificity mitogen-activated protein kinase kinase 3), Tln2 (Talin 2), and Sun2 (SUN domain-containing protein 2) were the only proteins not affiliated with complex IV subunits. The 5 most-upregulated proteins in the KO ischemic proteome compared to WT ischemic limb were: Ak2 (adenylate kinase 2, mitochondrial), Glrx5 (Glutaredoxin-related protein 5, mitochondrial),

Ndufb5 (NADH dehydrogenase 1 beta subcomplex subunit 5), Hspb2 (Heat shock protein beta-2) and Trim72 (Tripartite motif-containing protein 72).

KO mitochondria have dysregulated protein expression and reduced Complex IV-linked maximal respiration in response to ischemia at d7 HLI

Mitochondrial enrichment factor (MEF) from the whole tissue proteome revealed a significant increase in mitochondrial content of the KO control limb compared to the WT control limb ($p=0.0017$) (Figure 7A). The ischemic KO limb revealed a reduction in overall mitochondrial enrichment in the tissue compared to its control limb, resulting in similar mitochondrial content compared to the WT. A heatmap of the WT ischemic to control limb revealed the most severely downregulated protein to be Ndufb5 (Complex Ia) and most upregulated protein to be Ndufa1 (Complex Ib) (Figure 7B). Meanwhile, the KO ischemic proteome revealed ECSIT (Complex Ia) to be the most severely downregulated protein and mt-Co2 (Complex IVa) to be the most upregulated compared to the KO control proteome (Figure 7C). A heatmap of the significantly altered mitochondrial proteins in the ischemic limbs (KO/WT) demonstrated that the entire COX enzyme was dysregulated by the deletion of Cox6a2 in the KO group, indicated by the 9 most downregulated proteins in the KO mitochondria: Cox6a2, Cox5b, Cox6b1, Cox7a1, Cox7a2, Cox5a, and mt-Co2 (Figure 7D). While the majority of downregulated mitochondrial proteins in whole tissue proteomics (ischemic KO/WT) were affiliated with the COX enzyme, mitochondrial protein upregulations were mostly affiliated with complex I and V (ATP-synthase): Ndufa10, Ndufb5, Ndufv3, Ndufs7, ATP5k, and ATP5j2.

Using the creatine kinase (CK) clamp, high resolution respirometry was next used to determine how decreased complex IV and increased complex I and V protein abundances in the ischemic KO limb would impact mitochondrial respiration within a physiologically relevant range of

ΔG_{ATP} (Figure 7 E-G). The ischemic KO and WT limbs revealed no differences in oxygen consumption rates when measuring only complex I-induced respiration (Figure 7E), only complex II-induced respiration (Figure 7F), and when measuring respiration via both complexes I and II (Figure 7G). Electron transport system capacity with the CK clamp, assessed by additions of FCCP to uncouple the mitochondria, revealed no significant differences between groups or treatments under any of the substrate conditions. We next utilized the hexokinase clamp (HK) under saturating ADP conditions to measure maximal oxygen consumption rates of complexes I-III-IV, II-III-IV, and IV in freeze fractured mitochondria (Figure 7H). Freeze fractured ischemic KO mitochondria displayed 74.7%, 45.7%, and 71.0% reductions in oxygen consumption rates of complexes I-III-IV, II-III-IV, and IV respectively compared to the WT ischemic limbs.

Effects of Cox6a2 deletion on prolonged vascular recovery from HLI

To understand the sustained impact of Cox6a2 deletion after ischemic injury, WT and KO mice were subjected to 28d HLI. Cox6a2 mRNA remained significantly reduced in the KO ischemic limb ($p=0.0164$) (Figure 8A). Both ischemic and control limbs averaged a 97.94% and 98.7% reduction in expression of Cox6a2 message compared to the WT control limb at d28. Non-invasive Laser Doppler Perfusion Imaging (LDPI) was utilized to analyze limb blood flow prior to HLI (pre), immediately post HLI (d0), 14 days (d14), and 28 days after HLI (d28) (Figure 8B). HLI resulted in decreased paw blood flow post-surgery however there were no effect of the Cox6a2 KO on blood flow recovery across the 28d timeline (Figure 8C). When quantified and compared to the control limb, both KO and WT ischemic limbs had similar blood flow recovery of approximately 41.4% in the WT group and 45.8% in the KO group. To better understand capillary density and reperfusion at a prolonged recovery of hypoxic skeletal muscle of the KO

and WT animals, immunofluorescence imaging was performed on transverse sections of the TA (Figure 8D). There were no observed effects of Cox6a2 KO on the relative smooth muscle actin (Figure 8E), endothelial (CD31+; Figure 8F), or perfused vessel area (GSA-isolectin+; (Figure 8G). The ratio of perfused vessels to total vessels also revealed no differences between groups (Figure 8H), confirming that limb blood flow and tissue capillary perfusion recovery was not altered in the Cox6a2 KO ischemic limb.

Effects of Cox6a2 deletion on prolonged muscle recovery from HLI.

Qualitative analysis of H&E-stained transverse sections of WT and KO control and ischemic TA's revealed the sustained presence of tissue regeneration in the KO limb, indicating a delay in recovery compared to the WT ischemic limb at this timepoint (Figure 9A). Arrows indicate centralized nuclei, lipid deposits, and regions of small, undefined fibers. MHC fiber typing revealed a similar fiber distribution in both the control and ischemic TA's of the KO and WT animals (Figure 9BC). The average size of the myofibers (cross sectional area; μm^2) in the KO limb trended lower than the WT ($p=0.0646$) (Figure 9D). The ischemic limb of the KO had significantly more fibers in the smallest (0-500 μm) range than the WT ischemic limb (Figure 9E). Both genotypes exhibited smaller fibers in the ischemic limb compared to their contralateral control limb. Additionally, the presence of centralized myofiber nuclei was increased approximately 130%, indicating a delayed regeneration in the KO group (Figure 9F). Reductions in ischemic limb weight were no longer observed between groups, and no differences in EDL weight were recorded (Figure 9G). Force frequency curves of isolated KO and WT EDL muscles revealed no differences in absolute force production (Figure 9H) nor specific force production (Figure 9I). Despite evidence of a delayed regeneration and recovery of the KO group, these deficits were not detrimental to the contractability or force production of the muscle.

Effects of Cox6a2 deletion on mitochondria of prolonged recovery from HLI

To determine if the lack of Cox6a2 in the mitochondria was driving the prolonged regenerative myopathy observed in the morphology of the skeletal muscle, we assessed mitochondrial function using high resolution respirometry. Oxygen consumption of freshly isolated mitochondria continued to display no differences in mitochondrial respiration under all three CK clamp conditions within a physiologically relevant range of ΔG_{ATP} between WT and KO ischemic limbs (Figure 10A-C). Electron transport system capacity with the CK clamp, assessed by additions of FCCP to uncouple the mitochondria, revealed no significant differences between groups or treatments under any of the substrate conditions. Interestingly, oxygen consumption of the ischemic KO limb consistently trended higher than that of the KO control limb in all substrate conditions. We next utilized the hexokinase clamp (HK) under saturating ADP conditions to measure maximal oxygen consumption rates of complexes I-III-IV, II-III-IV, and IV in freeze fractured mitochondria (Figure 10D). Oxygen consumption rates of the KO ischemic limb displayed significantly lower Complex I-III-IV linked respiration ($p= 0.0004$) than the WT ischemic limb, but differences were no longer observed in Complex II-III-IV or IV linked respiration of the control or ischemic limbs of both groups (Figure 10D).

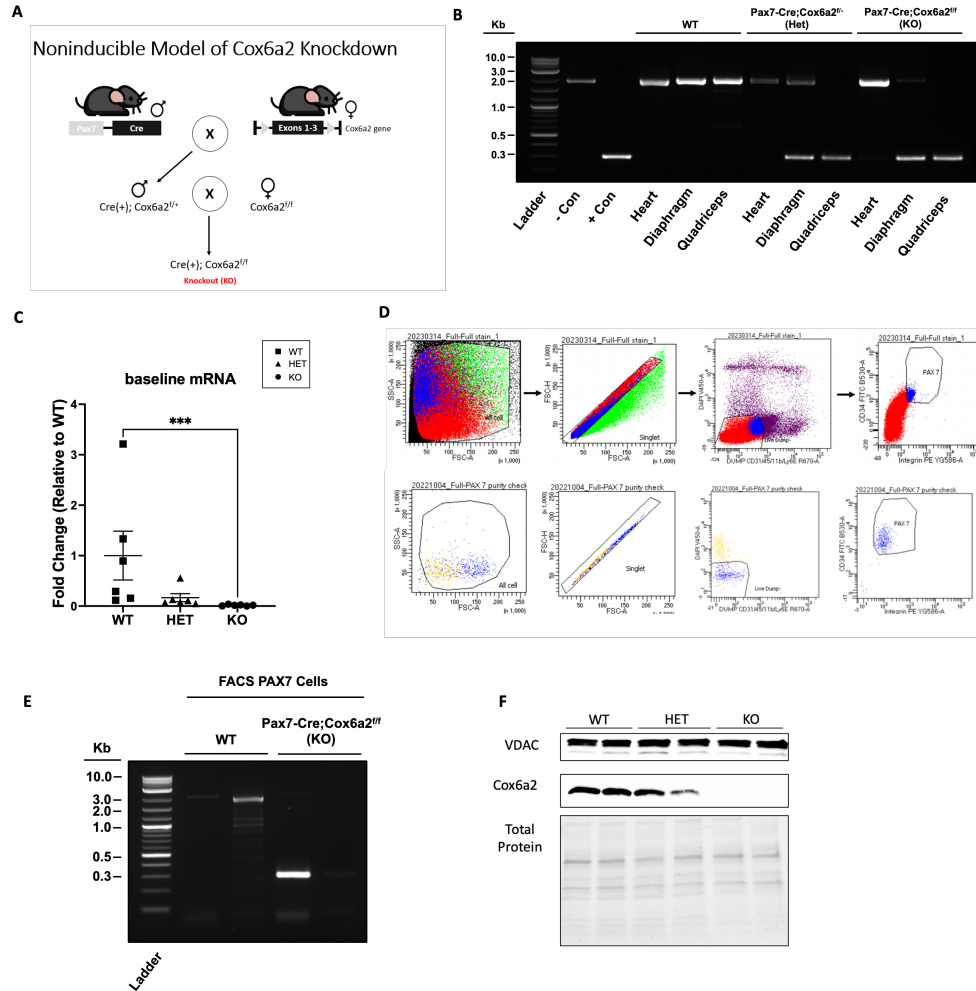


Figure 1: Cox6a2 Deletion is Pax7⁺ Cell and Skeletal Muscle Specific. (A) Breeding schematic to generate a satellite cell and mature skeletal muscle specific Cox6a2 knockout model. (B) Recombination of the Cox6a2 gene was observed in the KO and HET quadriceps, partial recombination in the KO and HET diaphragms, and no recombination was observed in the KO and HET hearts. No recombination was observed in the WT heart, diaphragm, and quadricep muscle. (C) Cox6a2 mRNA expression was measured in WT, HET, and KO groups at baseline and values were normalized relative to WT mRNA expression. Significant reductions in Cox6a2 mRNA expression were observed in the KO group but not in the HET group. (D) Fluorescent-activated cell sorting (FACS) was used to isolate live Pax7⁺ cells from the heterogenous skeletal muscle cell sample. (E) Recombination of the FACS Pax7 cells is observed in the KO group but not in the WT group. (F) Cox6a2 protein abundance was qualitatively observed using western blot analysis. VDAC was used as a mitochondrial protein control. Cox6a2 protein bands were observed in WT and HET groups, while no band was apparent in the KO animals. Data are presented as mean $\pm$ SEM. ***P<0.001.

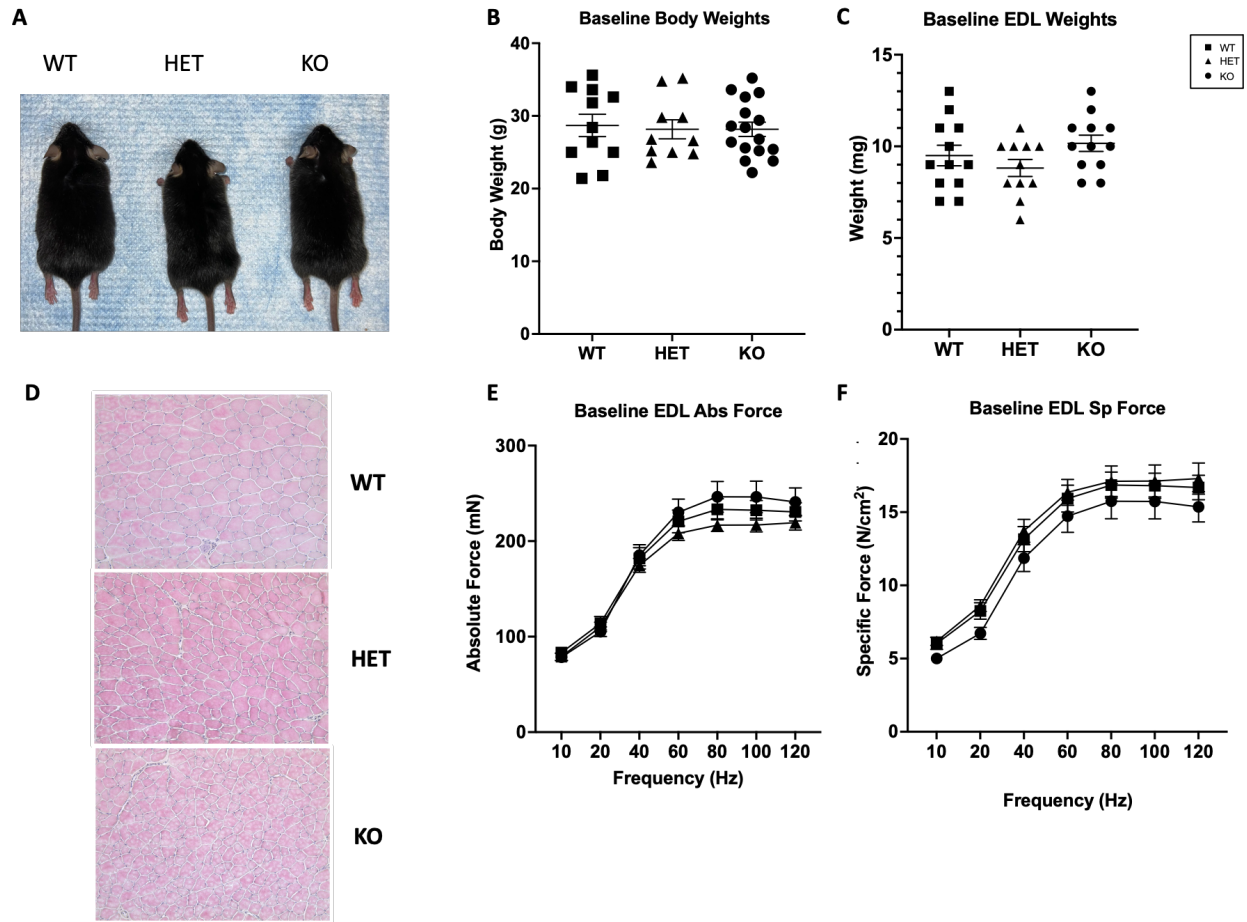


Figure 2: Cox6a2 Deletion Does Not Display a Skeletal Muscle Phenotype at Baseline. (A) Representative images of WT, HET, and KO mice. (B) Bodyweights are similar between groups. (C) Extensor digitorum longus (EDL) weights are similar between groups. (D) Representative images of 10 um transverse sections of Tibialis Anterior (TA) muscles from WT, HET, and KO groups with hematoxylin and eosin (H&E) staining. (E, F) Force frequency curves were generated between WT, HET, and KO groups. Data are presented as mean $\pm$ SEM.

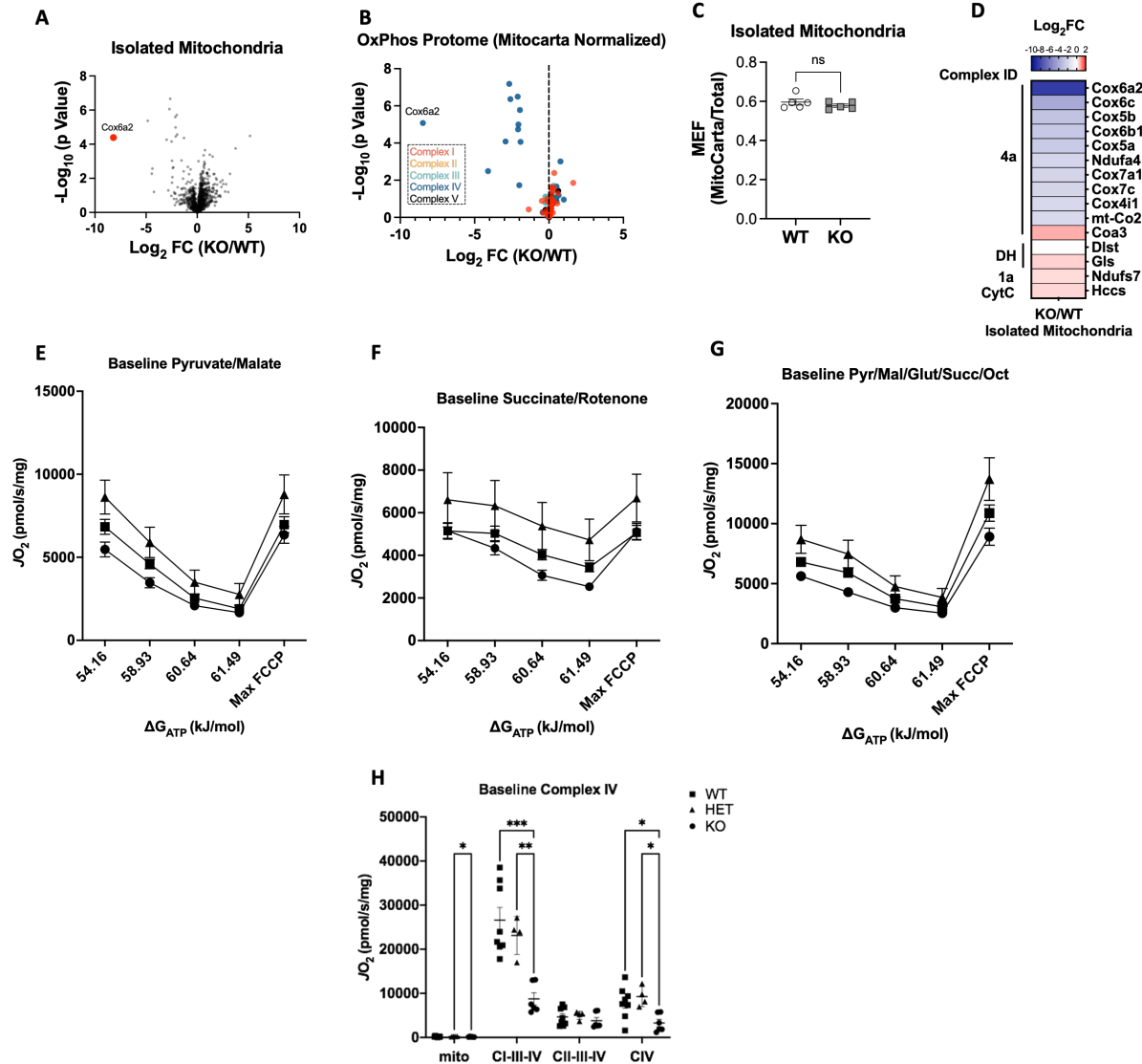


Figure 3: Cox6a2 Deletion Displays Baseline Mitochondrial Deficiencies. (A-D) LC/MS analysis of isolated mitochondria of WT and KO groups at baseline reveal a unique mitochondrial proteome of the KO group. (A) Cox6a2 is the most downregulated protein in the KO proteome. (B) Complex IV-specific proteins are the most significantly downregulated proteins in the KO proteome. (C) Mitochondrial enrichment factor (MEF) reveals no differences in mitochondrial protein of sample. (D) Heatmap of most significantly down- and upregulated proteins in the KO proteome. (E-G) high resolution respirometry was to measure respiration (JO_2) with a creatine kinase clamp. (E) Pyruvate/Malate substrate additions activate complex I-linked respiration (F) Succinate/Rotenone substrate additions activate complex II linked respiration (G) Multi-substrate (Pyr/Mal/Glut/Succ/Oct) condition was used. (H) High resolution respirometry was utilized on freeze fractured mitochondria with a hexokinase clamp. KO mitochondria displayed significantly reduced complex I-III-IV (NADH) and complex IV (Ascorbate/TMPD) linked respiration compared to the WT and the HET groups. No differences between WT and HET respiration were observed. Data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

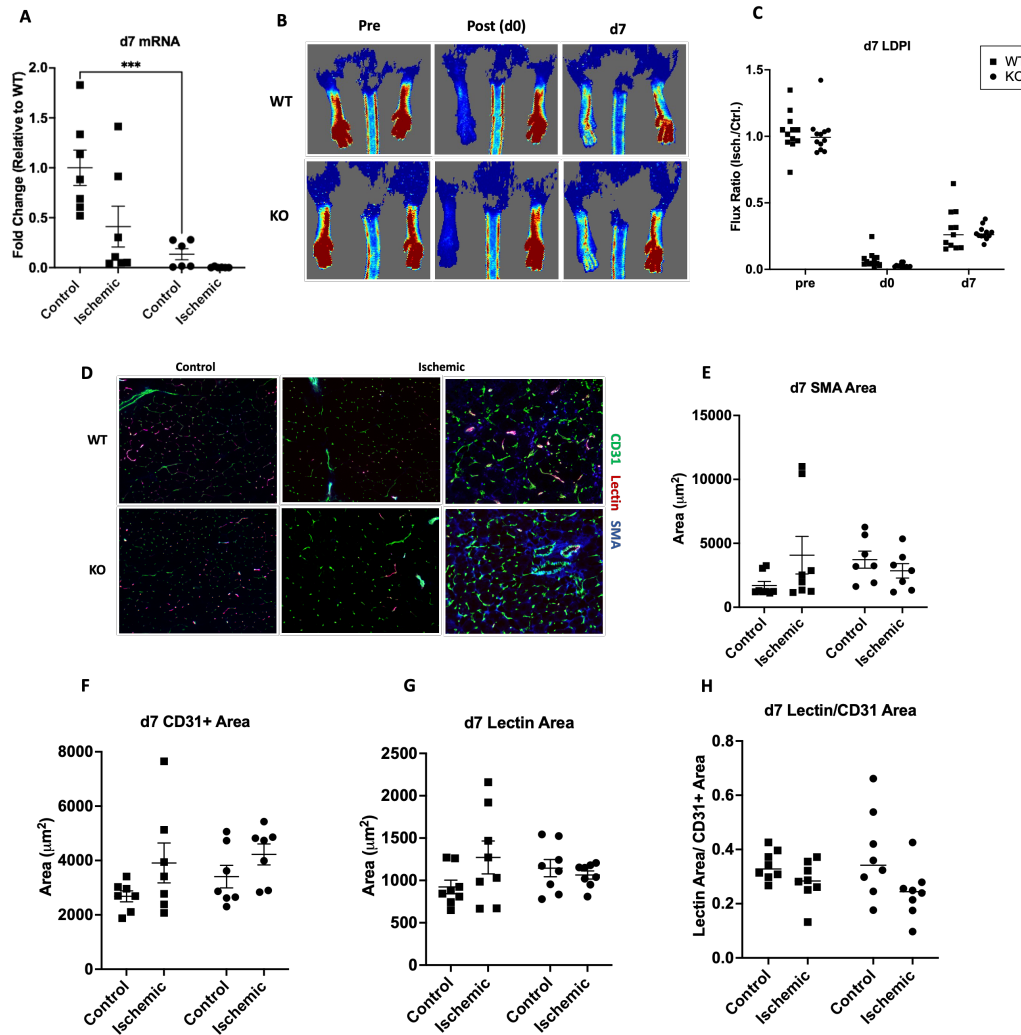


Figure 4: Effects of *Cox6a2* deletion on vascular recovery at d7 HLI. (A) *Cox6a2* mRNA expression was measured in WT and KO groups 7 days after HLI. Values were normalized relative to WT control limb mRNA expression. Significant reductions in *Cox6a2* mRNA expression were observed in the KO control limb. (B) Representative Laser Doppler Perfusion Images (LDPI) of the ischemic (left) and control (right) plantar blood flow in the prone position at baseline (pre), immediately post-HLI, and d7 post-HLI of both WT and KO groups. (C) Quantification of LDPI flux measurements at pre-, d0, and d7 represented as a ratio of ischemic over control limbs. (D) Representative 20 $\times$ immunofluorescent images of systemically DyLight 594 conjugated GS-IB4 lectin, indicating actively perfused vessels at the time of sacrifice (red), and cluster of differentiation 31 (CD31+, PECAM-1; green), indicating total endothelial cells, and smooth muscle actin, indicating smooth muscle in arterial and venous walls (blue) of transverse sections of TA of WT and KO ischemic and control limbs. (E) Average smooth muscle actin positive area in d7 WT and KO control and ischemic limbs. (F) Average cluster of differentiation 31 (CD31) positive area in d7 groups. (G) Average lectin positive area representing perfused vessels between groups at d7. (H) Ratio of lectin positive area (perfused vessels): CD31+ area (total endothelial cells) of WT and KO ischemic and control limb. Data are presented as mean $\pm$ SEM. ***P<0.001.

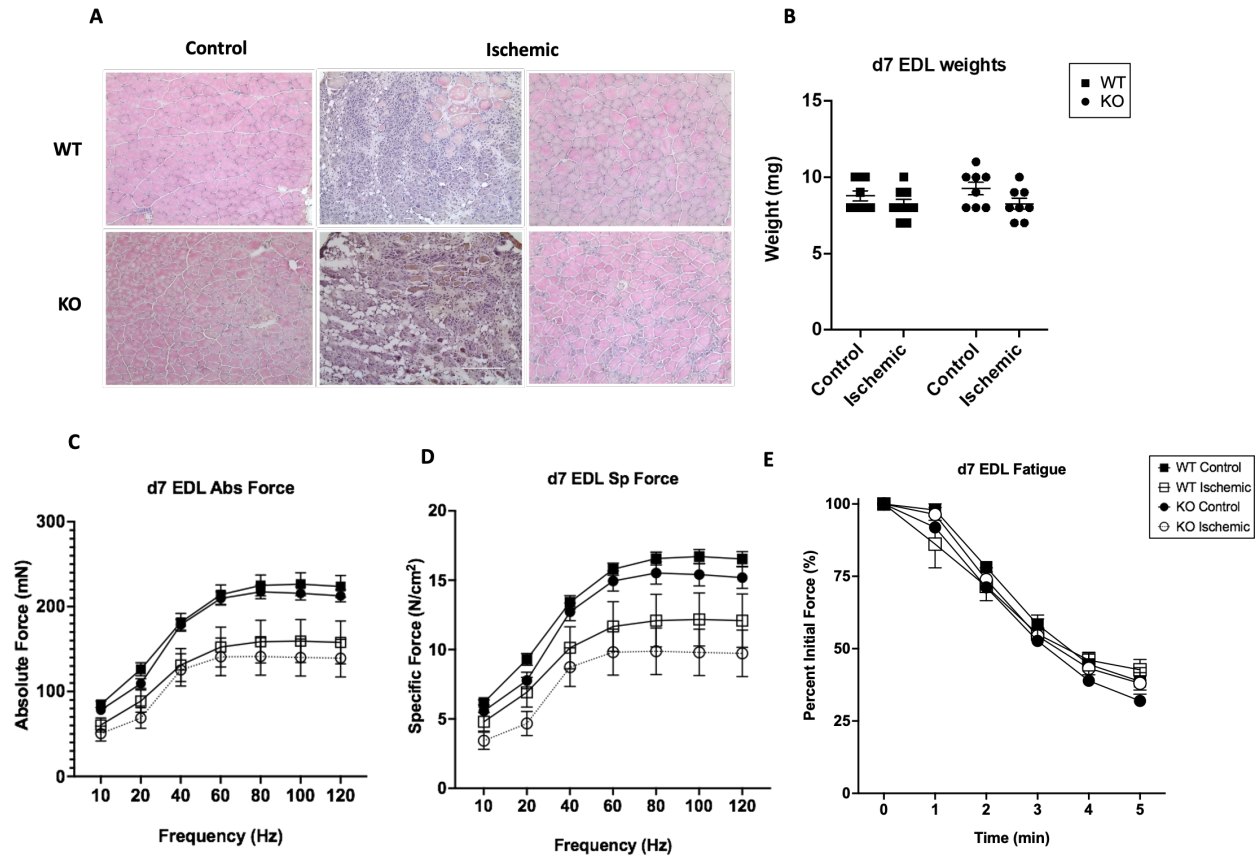


Figure 5: Effects of Cox6a2 deletion on muscle recovery at d7 HLI. (A) Representative images of 10 μ m transverse sections of Tibialis Anterior (TA) muscles from WT and KO ischemic and control limbs with hematoxylin and eosin (H&E) staining. (B) EDL weights are similar between WT and KO control limbs and between ischemic limbs. (CD) Force frequency curves were generated for WT and KO ischemic and control limbs. (E) 5-minute fatigue protocols were conducted for WT and KO limbs. Data are presented as mean $\pm$ SEM.

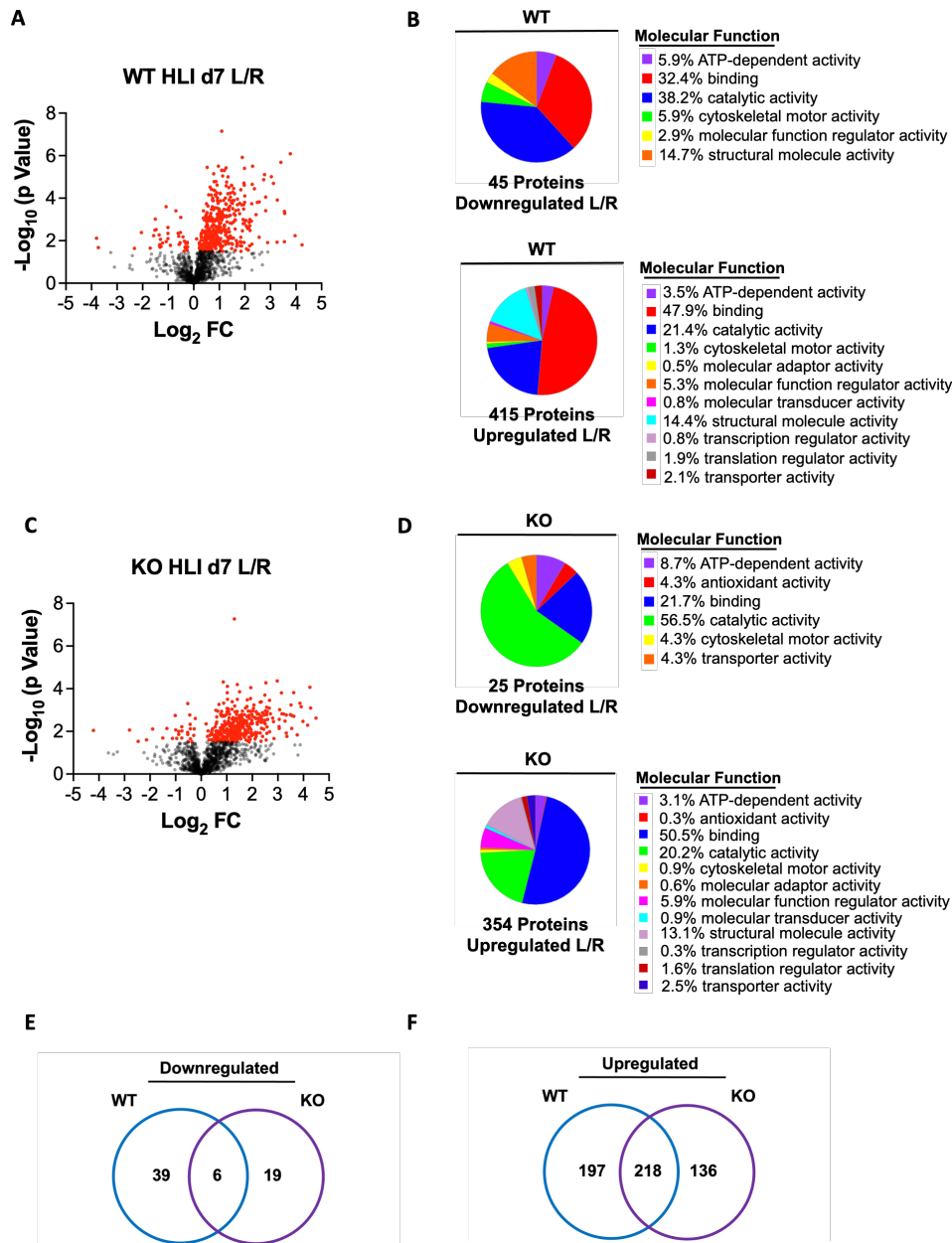


Figure 6: Ischemia stimulates a unique whole-tissue protein response in the KO group. (A) Volcano plot of proteins identified in ischemic to control WT limbs. (B) Pie charts depicting the proportions of differentially expressed proteins (downregulated and upregulated) by molecular function in WT ischemic to control limbs. (C) Volcano plot of proteins identified in ischemic to control KO limbs. (D) Pie charts depicting the proportions of differentially expressed proteins (downregulated and upregulated) by molecular function in KO ischemic to control limbs. (EF) Venn diagrams showing similarities and differences in differentially expressed proteins of KO and WT ischemic limbs.

Table 1. Alterations in the limb muscle proteome of WT Mice.

Downregulated			
Gene_Symbol	Name	Log2FC L/R	Adj <i>p</i> (<i>q</i><0.1)
Ecsit	Evolutionarily conserved signaling intermediate in Toll pathway	-3.80208	0.036914
Prkesh	Isoform 2 of Glucosidase 2 subunit beta	-3.73204	0.071872
Krt15	Keratin 15	-2.31815	0.075827
Mrps5	28S ribosomal protein S5, mitochondrial	-2.0483	0.023412
Krt10	Keratin, type I cytoskeletal 10	-1.70062	0.075099
Opa3	Optic atrophy 3 protein homolog	-1.56734	0.030111
Ndufa11	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	-1.5211	0.020135
Myl3	Myosin light chain 3	-1.50077	0.008943
Tpm3	Tropomyosin alpha-3 chain	-1.46859	0.019414
Tnnt1	Troponin T, slow skeletal muscle	-1.35469	0.028486
Bdh1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial	-1.33692	0.062752
Nlr1	NLR family member X1	-1.32884	0.057692
Msr3	Methionine-R-sulfoxide reductase B3, mitochondrial	-1.32316	0.059342
Myh7	Myosin-7	-1.32192	0.069389
Myl2	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	-1.30291	0.039857
Upregulated			
Gene_Symbol	Name	Log2FC L/R	Adj <i>p</i> (<i>q</i><0.1)
Lamp2	Isoform LAMP-2B of Lysosome-associated membrane glycoprotein 2	4.234803	0.058525
Hmgb2	High mobility group protein B2	3.962408	0.030111
Mustn1	Musculoskeletal embryonic nuclear protein 1	3.782435	0.000555
Hnrnpdl	Heterogeneous nuclear ribonucleoprotein D-like	3.56081	0.006599
Khsrp	Far upstream element-binding protein 2	3.549733	0.005609
Sec23a	Protein transport protein Sec23A	3.545372	0.048332
Tubb6	Tubulin beta-6 chain	3.4096	0.000569
Tubb2b	Tubulin beta-2B chain	3.2682	0.002848
Pkm	Pyruvate kinase PKM	3.261042	0.04974
Myh8	Myosin-8	3.121529	0.001198
Fscn1	Fascin	3.038602	0.0008
Arpc5	Actin-related protein 2/3 complex subunit 5	2.889281	0.050991
Galectin-3	Galectin-3	2.815167	0.002414
Ctsc	Dipeptidyl peptidase 1	2.815092	0.005356
Eef1a1	Elongation factor 1-alpha 1	2.769819	0.000762

Criteria for inclusion: *q*<0.1, top 15 Log2FC L/R provided.

Table 2. Alterations in the limb muscle proteome of KO Mice.

Downregulated			
Gene_Symbol	Name	Log2FC L/R	Adj <i>p</i> (<i>q</i><0.1)
Ube2d3	Ubiquitin-conjugating enzyme E2D	-4.21113	0.053688
Cops4	COP9 signalosome complex subunit 4	-2.80289	0.052804
Map2k3	Dual specificity mitogen-activated protein kinase kinase 3	-2.13432	0.094051
Myom1	Myomesin-1	-1.89965	0.050439
Rtn4ip1	Reticulon 4 interacting protein 1	-1.50013	0.082768
Aldh1a1	Aldehyde dehydrogenase 1A1	-1.34409	0.049112
Myh2	Myosin, heavy polypeptide 2, skeletal muscle, adult	-1.12506	0.085291
Bdh1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial	-1.013	0.031473
Bph1	Valacyclovir hydrolase	-0.99849	0.052804
Gstk1	Glutathione S-transferase kappa 1	-0.96032	0.085238
Myoz2	Myozenin-2	-0.88253	0.06546
Echs1	Enoyl-CoA hydratase, mitochondrial	-0.88222	0.055152
Ndufa2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	-0.75827	0.045396
Dglucy	D-glutamate cyclase	-0.75597	0.082866
Idh2	Isocitrate dehydrogenase [NADP], mitochondrial	-0.75328	0.053688
Upregulated			
Gene_Symbol	Name	Log2FC L/R	Adj <i>p</i> (<i>q</i><0.1)
Pkm	Pyruvate kinase PKM	4.488973	0.033284
Tubb2b	Tubulin beta-2B chain	4.281158	0.031473
Tubb6	Tubulin beta-6 chain	4.251205	0.015326
Sarnp	SAP domain-containing ribonucleoprotein	4.163302	0.041687
Rab6a	Ras-related protein Rab-6A	3.978802	0.037232
Snx5	Sorting nexin-5	3.91509	0.031473
Fubp1	Far upstream element (FUSE) binding protein 1	3.863894	0.028661
Jpt1	Jupiter microtubule associated homolog 1	3.760496	0.066924
Lgals3	Galectin-3	3.627602	0.031606
Camk2b	calcium/calmodulin-dependent protein kinase	3.620403	0.018078
Myl4	Myosin, light polypeptide 4	3.553826	0.031473
Tardbp	TAR DNA-binding protein 43	3.541704	0.049135
Eef1a1	Elongation factor 1-alpha 1	3.512142	0.029647
Myh8	Myosin-8	3.493092	0.031473
Cotl1	Coactosin-like protein	3.350199	0.07397

Criteria for inclusion: $q < 0.1$, top 15 Log2FC L/R provided.

Table 3. Differential alterations in the ischemic limb muscle proteome of KO Mice v WT.

Downregulated			
Gene_Symbol	Name	Log2FC KO/WT	Adj <i>p</i> (<i>q</i><0.1)
Cox6a2	Cytochrome c oxidase subunit 6A2, mitochondrial	-6.62065	0.036435
Ube2d3	Ubiquitin-conjugating enzyme E2D	-6.35854	0.01085
Map2k3	Dual specificity mitogen-activated protein kinase kinase 3	-4.27034	0.036435
Cox7c	Cytochrome c oxidase subunit 7C, mitochondrial	-3.25072	0.095179
Tln2	Talin 2	-2.54933	0.095303
Cox5b	Cytochrome c oxidase subunit 5B, mitochondrial	-1.89457	0.026495
Cox6b1	Cytochrome c oxidase subunit 6B1	-1.8188	0.009889
Sun2	SUN domain-containing protein 2	-1.79304	0.003684
Cox7a2	Cytochrome c oxidase subunit 7A2, mitochondrial	-1.68057	0.070284
Cox7a1	Cytochrome c oxidase subunit 7A1	-1.60895	0.065176
Upregulated			
Gene_Symbol	Name	Log2FC KO/WT	Adj <i>p</i> (<i>q</i><0.1)
Ak2	Adenylate kinase 2, mitochondrial	3.8436	0.040477
Glx5	Glutaredoxin-related protein 5, mitochondrial	1.392328	0.054238
Ndufb5	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	1.341817	0.014539
Hspb2	Heat shock protein beta-2	0.945749	0.007964
Trim72	Tripartite motif-containing protein 72	0.859422	0.045554
Usp5	Ubiquitin carboxyl-terminal hydrolase 5	0.810999	0.080455
Mccc2	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	0.697173	0.060727
Atp5j2	ATP synthase subunit f, mitochondrial	0.4602	0.080455
Prdx5	Peroxiredoxin-5, mitochondrial	0.42913	0.080455
Atp5b	ATP synthase subunit beta, mitochondrial	0.290567	0.069803

Criteria for inclusion: $q < 0.1$, top 10 Log2FC KO/WT, ischemic limb (L) only provided.

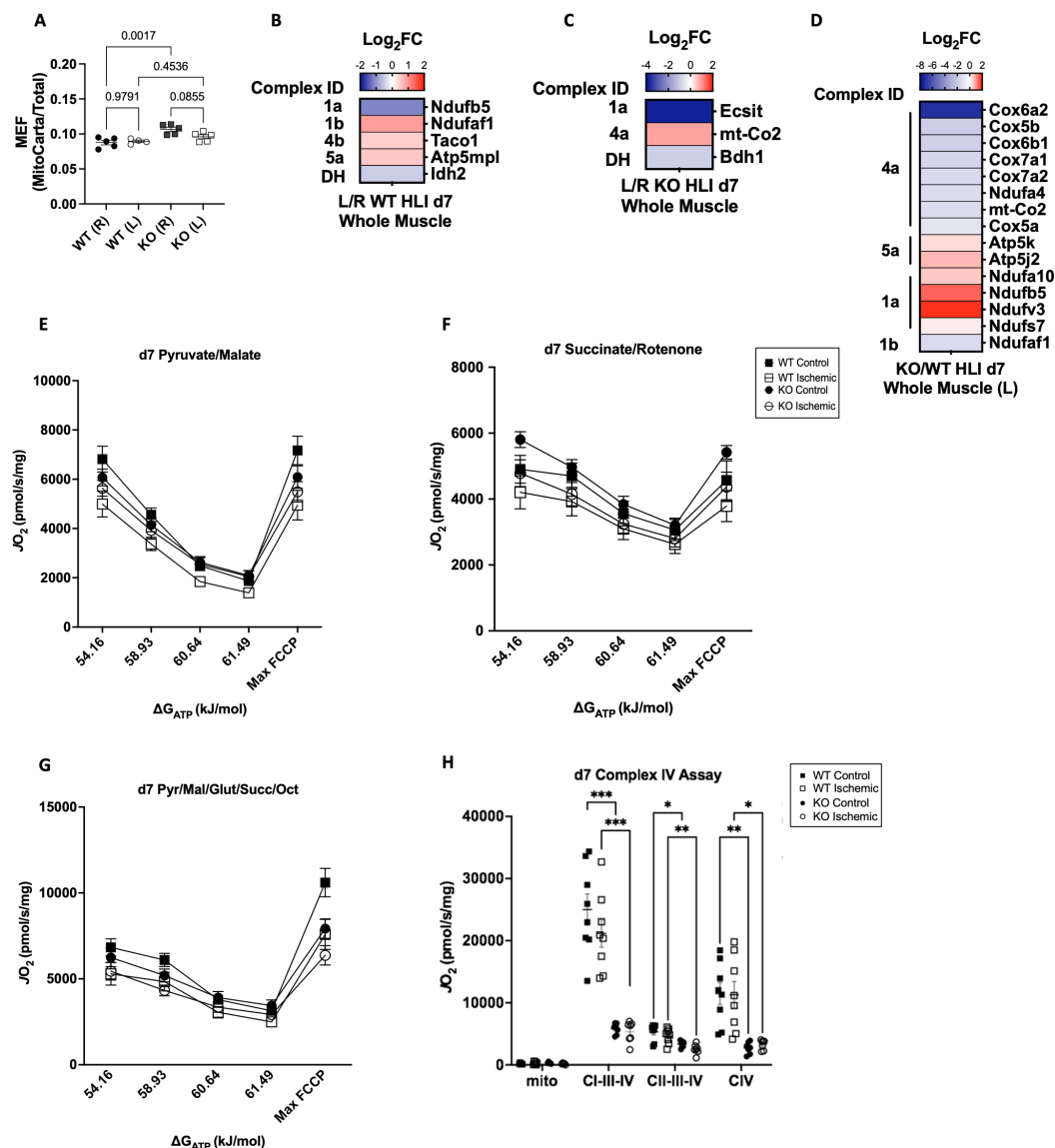


Figure 7- KO mitochondria have dysregulated protein expression and reduced Complex IV-linked maximal respiration in response to ischemia at d7 HLI (A) Mitochondrial enrichment factor (MEF) measuring mitochondrial protein to total protein of WT and KO left (ischemic) and right (control) limbs. (B-D) Heat maps of mitochondrial proteome differences (Log₂ fold change) for WT ischemic to control (B), KO ischemic to control (C), and KO ischemic to WT ischemic (D). (E-G) high resolution respirometry was utilized on isolated mitochondria from the gastrocnemius complex of WT and KO d7 ischemic and control limbs to measure respiration with a CK clamp. (E) Pyruvate/Malate substrate additions activated complex I-linked respiration (F) Succinate/Rotenone substrate additions activated complex II linked respiration while inhibiting complex I linked respiration (G) Multi-substrate (Pyruvate/Malate/Glutamate/Succinate/Octenoyl-Carnitine) condition was used. H) High resolution respirometry of freeze fractured mitochondria with a HK clamp and substrate additions of NADH (CI-III-IV), Succinate (CII-III-IV), and ASC/TMPD (CIV). Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

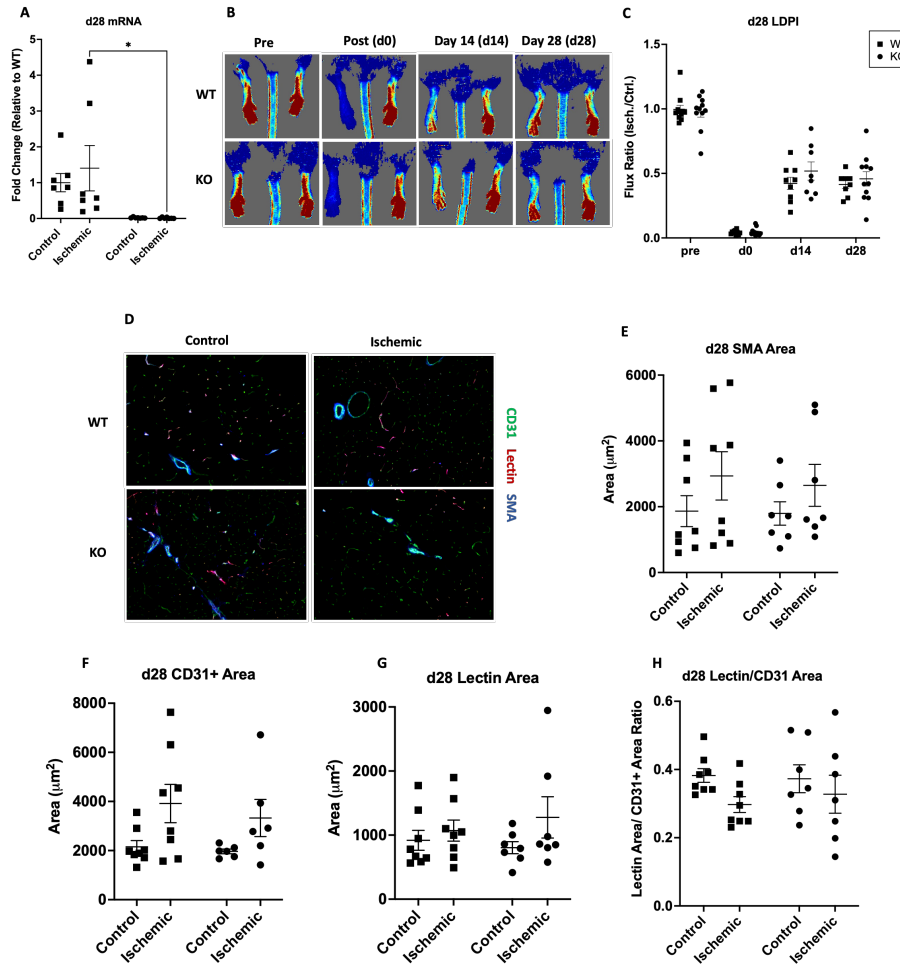


Figure 8: Effects of Cox6a2 deletion on prolonged vascular recovery from HLI. (A) Cox6a2 mRNA expression was measured in WT and KO groups 28 days after HLI. Values were normalized relative to WT control limb mRNA expression. (B) Representative Laser Doppler Perfusion Images (LDPI) of the ischemic (left) and control (right) plantar blood flow in the prone position at baseline (pre), immediately post-HLI, d14, and d28 post-HLI of both WT and KO groups. (C) Quantification of LDPI flux measurements at pre-, d0, d14, and d28 represented as a ratio of ischemic over control limbs. (D) Representative 20 $\times$ immunofluorescent images of systemically DyLight 594 conjugated GS-IB4 lectin, indicating actively perfused vessels at the time of sacrifice (red), and cluster of differentiation 31 (CD31+, PECAM-1; green), indicating total endothelial cells, and smooth muscle actin, indicating smooth muscle in arterial and venous walls (blue) of transverse sections of TA of WT and KO d28 ischemic and control limbs. (E) Average smooth muscle actin positive area in d28 WT and KO control and ischemic limbs. (F) Average cluster of differentiation 31 (CD31) positive area in d7 groups. (G) Average lectin positive area representing perfused vessels between groups at d7. (H) Ratio of lectin positive area (perfused vessels): CD31+ area (total endothelial cells) of WT and KO ischemic and control limb. Data are presented as mean $\pm$ SEM. *P<0.05.

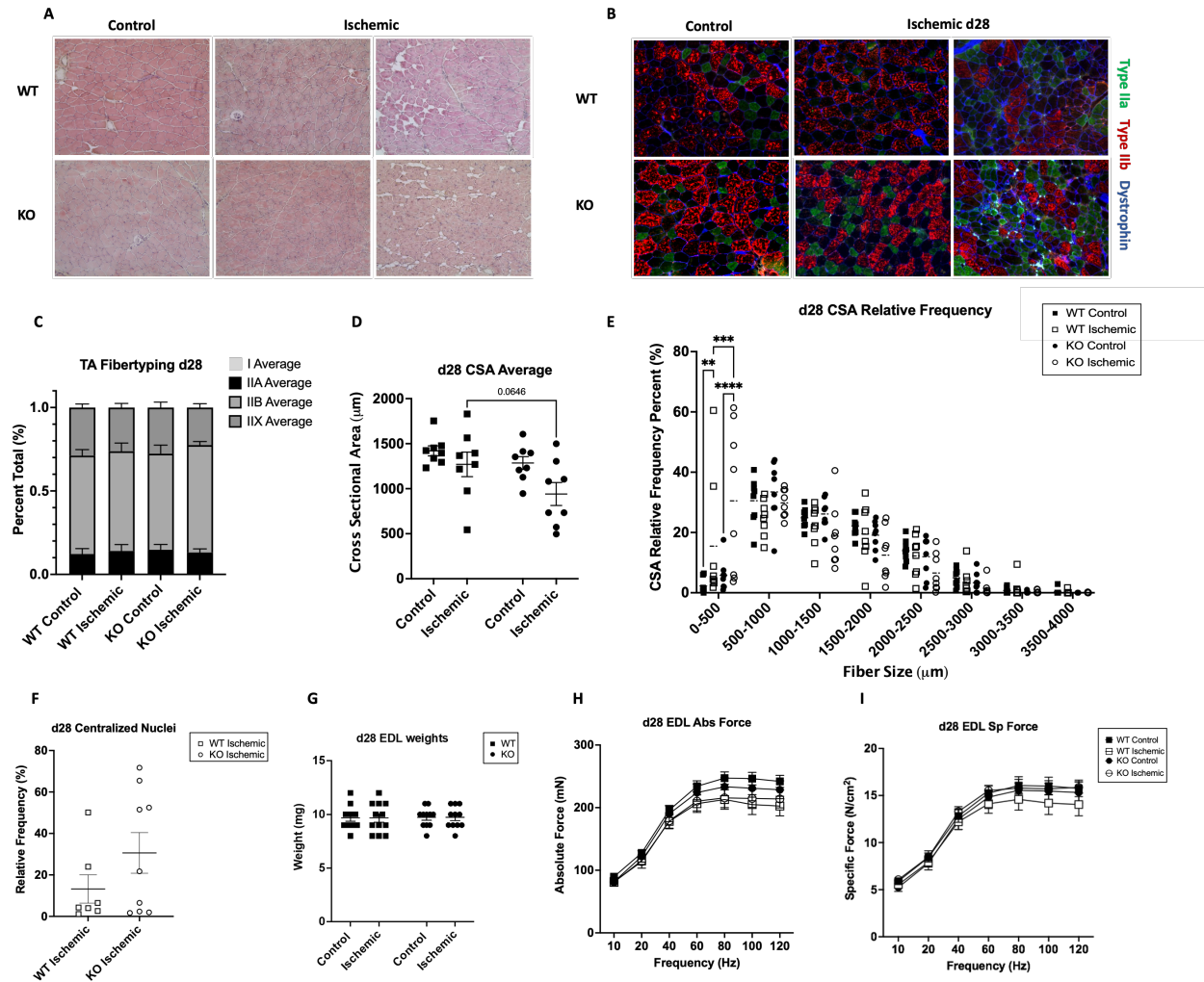


Figure 9: Effects of Cox6a2 deletion on prolonged muscle recovery from HLI. (A)

Representative images of 10 μm transverse sections of Tibialis Anterior (TA) muscles from WT and KO d28 ischemic and control limbs with hematoxylin and eosin (H&E) staining. Arrows indicate lipid deposits (top), centralized nuclei (left), and regions of small, undefined fibers (right). (B) Representative fiber type images of 10 μm transverse sections of Tibialis Anterior (TA) muscles from WT and KO d28 ischemic and control limbs with type IIa fibers (green), type IIb fibers (red), type IIx fibers (black) and dystrophin (blue). (C) Fiber type distribution in both ischemic and control limbs of WT and KO animals. (D) Myofiber size (cross-sectional area) of the TA muscles in both limbs. (E) Relative frequency of fiber size ranging from <500 μm to 4000 μm of both limbs. (F) Relative frequency of centralized nuclei per total fiber count in section of WT and KO ischemic limbs. (G) EDL weights are similar between WT and KO control limbs and ischemic limbs. (H) Force frequency curves were generated following a twitch protocol for WT and KO ischemic and control limbs. Data are presented as mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

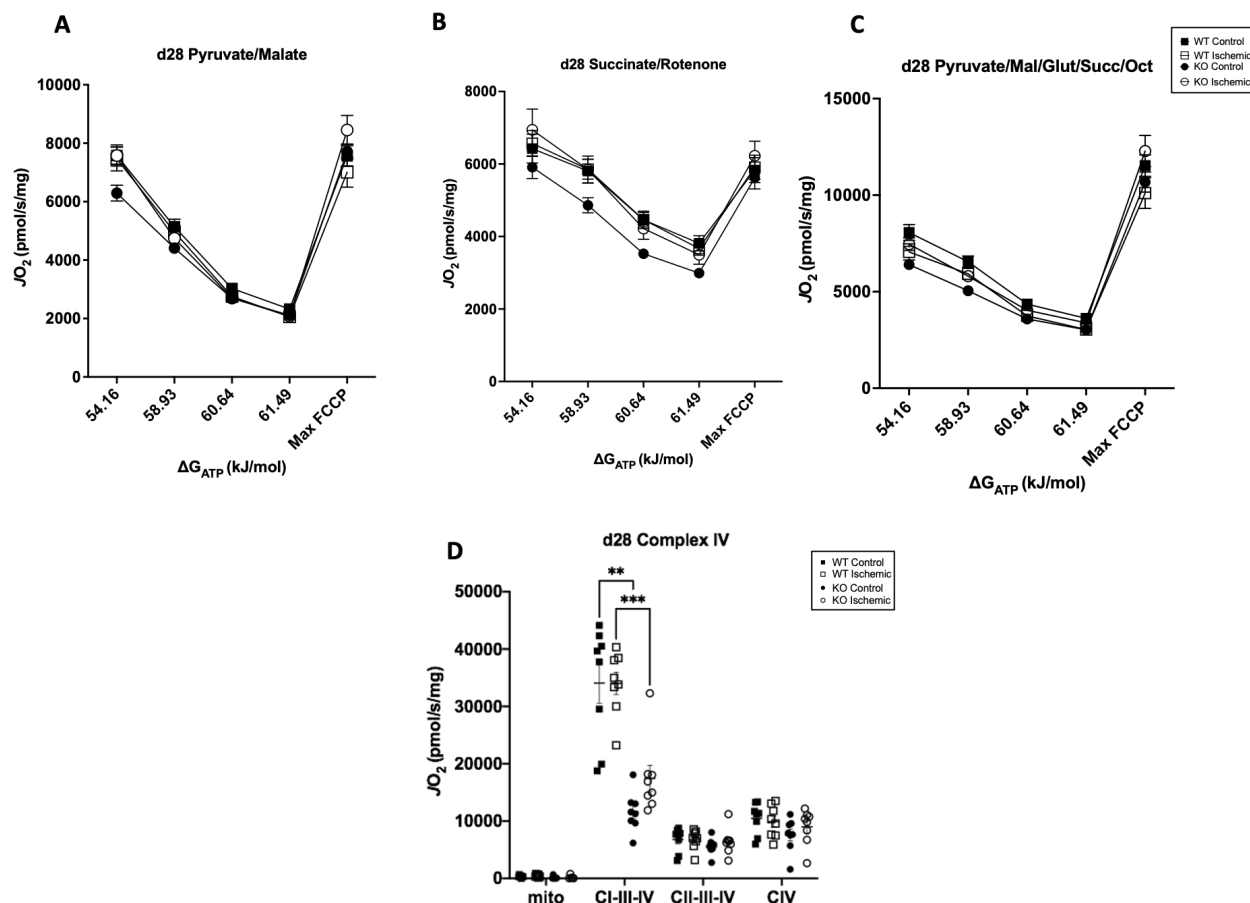


Figure 10: Effects of Cox6a2 deletion on mitochondria of prolonged recovery from *HLI*.

(A-C) high resolution respirometry was utilized on freshly isolated mitochondria from the gastrocnemius complex of WT and KO ischemic and control limbs to measure respiration with a CK clamp. (A) Pyruvate/Malate substrate additions activated complex I-linked respiration (B) Succinate/Rotenone substrate additions activated complex II linked respiration while inhibiting complex I linked respiration (C) Multi-substrate (Pyr/Mal/Glut/Succ/Oct) condition was used. D) High resolution respirometry of freeze fractured mitochondria with a HK clamp and substrate additions of NADH (CI-III-IV), Succinate (CII-III-IV), and ASC/TMPD (CIV). Data are presented as mean $\pm$ SEM. ** $P < 0.01$, *** $P < 0.001$.

Discussion

CLTI is uniquely defined by severe reductions in mitochondrial oxidative capacity, electron transport system enzyme function, and downregulation of both the transcriptome and proteome linked to mitochondria, including Complex IV^{8,13}. Cox6a2 was originally identified in a pre-clinical QTL Lsq-1 on chromosome 7 associated with perfusion recovery and tissue necrosis¹⁵ and we later revealed Cox6a2 mRNA and protein to be decreased in CLTI patient limbs^{8,13}. To understand the biologic and metabolic importance of Cox6a2 to the ischemic limb, we developed a murine model of Pax7⁺ and mature skeletal muscle-specific genetic deletion of Cox6a2. The deletion of Cox6a2 altered the mitochondrial proteomic abundance of COX enzyme components and reduced Complex IV-linked maximal respiration. It did not, however, negatively impact body morphology or development postnatally. After HLI, maximal oxygen consumption rates remained reduced in all complexes (I-III-IV, II-III-IV, and IV) at d7 and only complexes I-III-IV by d28. Cox6a2 KO also delayed complete tissue recovery from ischemia.

A whole body, lifelong knockout model of Cox6a2 has been previously described²⁹. These mice displayed no differences in oxygen consumption rates of isolated diaphragm yet significant reductions in complex IV enzyme activity between KO and WT mice aged 12-16 weeks. While we did not directly measure enzyme activity of complex IV, we observed significant reductions in oxygen consumption rates of complex IV under maximal energetic demand and no differences in oxygen consumption rates under physiologically relevant range of ΔG_{ATP} . In concordance with our model, there were no observed differences in force production between the KO and WT mice in the absence of ischemia. Whole body deletion of Cox6a2 in response to a HFD resulted in increased thermogenesis and a HFD-resistant phenotype, including smaller gastroc myofibers and a fiber type switch to more type IIa fibers. Our study did not observe a difference in TA

fiber size and did not observe a fiber type difference between WT and KO groups without inducing ischemia, which might be a result of the inherent differences in muscle function between the TA and gastrocnemius. While the TA is a dorsiflexor consisting of primarily type II (a, b, and x) fibers, the gastroc is a plantar flexor and is weight bearing with a greater distribution of all fiber types throughout different regions (red, white, and mixed) of the muscle³⁰. It is possible that the gastroc required a fiber type switch due to the deletion of Cox6a2, while the TA did not. In another study, Radford et al. (2002) examined Cox6a2 deletion in the heart³¹. They revealed significant reductions in Complex IV protein content and enzyme activity in the KO hearts under standard conditions. When stressed under maximal load, the hearts displayed impaired left ventricular filling. Neither of the previous studies induced ischemia to analyze the role of Cox6a2 in regeneration of muscle. Both studies suggest that Cox6a2 deletion in skeletal and cardiac muscle dysregulated Complex IV proteins and enzyme activity. While these models both have results that relate to our model, Cox6a2's involvement in the regenerative response of skeletal muscle after ischemic injury has not, to our knowledge, been previously investigated.

We employed a non-inducible Pax7-Cre to delete Cox6a2 from progenitor cells and mature skeletal muscle. The loss of Cox6a2 alone did not result in a baseline functional phenotype, despite a unique mitochondrial proteome and lower enzymatic respiration in the KO animals. Mitochondrial plasticity enables metabolic homeostasis despite the presence of functional mutations³². It is possible that regardless of complex IV deficits, the KO mitochondria have adapted to produce enough energy required for survival. Proteomics does not support a compensatory change in the abundance of the other Cox6a isoform (Cox6a1) in the KO either at baseline or after HLI. This is especially important given the partial restoration of mitochondrial function at d28 of HLI in the KO mice, which can't be attributed to a replacement of protein

isoforms. The KO mitochondrial enrichment factor, a ratio of mitochondrial protein in comparison to total protein content in the sample, indicated increased mitochondria in the KO control limb compared to the WT control limb without ischemia. An increase in mitochondrial proteins could be indicative of mitochondrial compensation due to lower mitochondrial complex enzymatic activity in the KO group. This effect may also be a result of increased load on the control limb considering the ischemic limb is often not able to bear weight after HLI. Measuring citrate synthase activity, a widely used biomarker of mitochondrial content³³, at baseline and after HLI would enable us to determine if the differences observed in the mitochondrial enrichment factor are a result of the deletion of Cox6a2 or by an adaptation to the increased load on the control (right) limb after ischemia of the left limb.

Limb ischemia increases skeletal muscle progenitor cell fusion into recovering myofibers³⁴. We chose to utilize the Pax7-Cre as a driver of Cox6a2 recombination to prevent redistribution of Cox6a2 into regenerating ischemic skeletal muscle by the Pax7+ population. A previous study that used Pax7-Cre as the driver to the genetic deletion of clock-interacting protein circadian (CIPC) in satellite cells of mdx mice found that there was an increased abundance of satellite cells in their KO group. The effect of the deletion of Cox6a2 on progenitor cells in our model is unknown, however previous research implies Cox6a2 may play a role in the differentiation of Pax7+ progenitors due to an increase in Cox6a2 expression in C2C12 myoblasts within the first two days of differentiation³⁵. The loss of Cox6a2 in C2C12 cells reduces differentiation of the myoblasts³⁵. In this study, the KO d28 ischemic limbs display a morphological phenotype indicative of delays of the regenerative response. At this timepoint, the KO group had more centralized nuclei and a decrease in myofiber size compared to the WT group, regardless of similar vascular and perfusion recovery in the limbs. These delays could be a result of the

Cox6a2 deficits in the progenitor cells. Considering rapid increases of Cox6a2 mRNA within the first two days of differentiation were observed in a previous study³⁵, it is possible that the lack of Cox6a2 in Pax7+ cells is triggering a decrease in differentiation and fusion of progenitors to existing myofibers, thus resulting in a recovery delay. In this study, our lab did not measure Pax7+ cell quantity in the muscle. The presence of smaller myofibers and centralized nuclei at HLI d28 in the KO animals supports a deficit in progenitor cell fusion and myofiber repair, however. A measurement of Pax7+ cells in the WT and KO mice from our model may be essential to properly assess if the delays in regeneration are due to inhibition of the progenitor cell fusion with myofibers or donation of myonuclei.

A significantly upregulated protein in the ischemic response of the KO compared to the WT limb was heat shock protein beta-2 (Hspb2). Expression of Hspb2 increases during C2C12 differentiation into myotubes^{36,37} and an observed upregulation in the KO ischemic limb could indicate an increased stress response to mitigate the deficits of Cox6a2 loss in the progenitors. Additionally, type-II pericytes, which can function myogenically and aid in the regeneration of skeletal muscle³⁸, have been recently associated with contributing to the satellite cell pool in postnatal murine development³⁹. While type-II pericyte contribution is usually limited in the TA (<1%), there has been an observed enhancement of type-II pericyte involvement in regeneration after acute and chronic regeneration of the muscle³⁹. In contrast, Type I pericytes are often responsible for the distribution of ectopic adipocyte deposition in the skeletal muscle³⁸. In the d28 ischemic TAs from both groups, increased distribution of fat deposits in the ischemic limb was observed, though the KO ischemic limb had a more severe distribution of lipids in the muscle. We did not analyze type I or II pericyte activity in the skeletal muscle of our model,

though it may be a valuable analysis to help uncover what is happening cellularly in the regeneration of the KO ischemic limbs.

Hindlimb ischemia disrupts homeostasis of the skeletal muscle and results in quiescent satellite cell activation and either division or merging of existing fibers to initiate regeneration³⁴. Our Pax7-Cre⁺;Cox6a2^{ff} mouse model utilizes parental strains from a C57Bl/6 strain background. Previous studies have determined C57bl/6 mice to have a more robust recovery response to ischemia compared to other mouse strains, regardless of genetic manipulation²⁰. After inducing ischemia in a C57bl/6 double ligation mouse model, fusion of newly-differentiated myonuclei from progenitors is increasing by day 7 of regeneration and a substantial resolution of injury has begun³⁴. By 28 days, regeneration of the muscle should largely be complete. The use of these two time points allows us to gauge the role of Cox6a2 in regeneration and have insight on the long-term recovery of the muscle after HLI. The proteome of the whole tissue ischemic response of the WT group compared to the KO group was vastly different, with minimal overlap of upregulated and downregulated proteins. When looking at overall molecular classification, however, the ischemic responses were quite similar. Interestingly, the mitochondrial differences caused KO after HLI were decreased complex IV proteins and increased complex I and V proteins, suggesting the mitochondria are adapting to the deficits in complex IV. Complex I and V specific proteins (ATP5J2, ndufa10, ndufb5, and ndufv3) were upregulated in the ischemic KO. Nevertheless, complexes I-III-IV, II-III-IV, and IV oxygen consumption under maximal energetic demand was significantly reduced in both the control and ischemic limbs in the KO at d7, indicating that the increased protein content in Complexes I and V didn't rescue the mitochondrial respiratory capacity.

Cox6a2 is primarily a stabilization and binding subunit to Cytochrome C Oxidase and is essential to the assembly of complex IV due to Cox6a2's transmembrane domain¹⁶. Cox6a2 also plays an important role in respirasome formation (a supercomplex of CI, CIII₂, and CIV) in the electron transport system⁴⁰. Respirasomes are supramolecular structures of the electron transport system enzymes that carry out cellular respiration as a large molecular machine, ultimately increasing efficiency of electron transfer, catalytic activity of the enzymes, and decreasing ROS formation⁴¹. Cox6a2's involvement in the stability of Complex IV dimers due to its location at the interface of the two CIV monomers⁴⁰ promotes overall stability and assembly of supercomplexes. In the absence of Cox6a2, however, Complex IV dimer and supercomplex formation will likely be altered, potentially decreasing the efficiency and catalytic activity of the complex. Evidence that supercomplexes are not essential to normal bioenergetics of mice has recently been established⁴². Despite evidence that complex IV-specific maximal respiration is decreased in the knockout, the whole mitochondria respire similarly to the WT, further supporting the possibility of impaired supercomplex formation in the KO group. Additionally, the deletion of Cox6a2 in our model led to significant decreases in protein expression of 8 other COX enzyme proteins. Decreases in complex IV protein expression due to the deletion of Cox6a2 likely interrupts the formation of respirasomes even further, potentially explaining the reductions of complex IV-specific oxygen consumption rates.

A common concern with the deletion of a mitochondrial binding subunit is the resulting free radical leak and oxidative stress it may induce on the mitochondria. Mitochondrial mutations have been previously established to increase Reactive Oxygen Species (ROS) production⁴³. Under normal physiological conditions, ROS emission is considered to account for ~2% of the total oxygen consumed by mitochondria⁴⁴. We did not directly measure ROS production in our

mouse model; however, a previous Cox6a2 KO study observed an increased ROS production in the whole body Cox6a2 knockout mouse as well as reduced enzyme activity, similar oxygen consumption rates and force production between their Cox6a2 KO and WT groups²⁹. This previous model was not subjected to HLI, however, thus the level of ROS after ischemia in the Cox6a2 deleted mouse has not been measured. Previous research from our lab observed that ischemia in the C57Bl/6 mouse does not increase ROS production unless subjected to a HFD⁴⁵. Additionally, proteomics profiling of the most upregulated proteins in the KO ischemic limb in comparison to the WT reveals a significant increase in Peroxiredoxin-5 (Prdx5) proteins. Prdx5 is an antioxidant enzyme that catalyzes the breakdown of a broad range of reactive oxygen species⁴⁶. A significant upregulation of this protein in the KO in comparison to the WT ischemic limbs suggests adaptations have occurred to negate the effects of oxidative stress in the KO mice. For these reasons, we would theorize, in our Cox6a2 KO model, that ROS will likely not impact the ischemic limb and thus is likely not the reason behind the delayed regeneration we have observed.

In this study, we revealed Cox6a2 KO reduced the mitochondrial COX enzyme proteome and reduce the oxygen consumption of complex IV, delaying complete tissue recovery from HLI. Previous data collected by our lab indicates downregulation of all electron transport complexes in the CLTI proteome in response to ischemia¹³. Our work reveals dysfunctional Complex IV as a mechanism, in part, for mitochondrial inefficiencies during HLI. Aging also results in a significant downregulation of complexes I and IV in skeletal muscle mitochondria⁴⁷⁻⁴⁹ and may provide an additional mechanism for the myopathy that plagues the CLTI population.

Summary & Future Directions

The primary purpose of this thesis was to determine the role the specific mitochondrial protein, *Cox6a2* plays in the survival and regeneration of skeletal muscle after ischemic injury. This thesis used a preclinical model of PAD to analyze the effects of a *Cox6a2* deletion *in vivo* using a lifelong *Cox6a2* knockout (KO) model. To develop this murine model in skeletal muscle progenitor cells, we used a PAX7-Cre promoter. We hypothesized that skeletal muscle *Cox6a2* is necessary for tissue regeneration after hindlimb ischemia (HLI) due to its integral role in the bioenergetics of skeletal muscle and thus is a key descriptor of the mitochondrial dysfunction observed in the CLTI population. Results from our studies indicated that the genetic deletion of skeletal muscle progenitor and myofiber *Cox6a2* altered the mitochondrial proteomic abundance of COX enzyme components and reduced complex IV-specific oxygen consumption rates. Further, we determined that *Cox6a2* deletion did not have deleterious effects on body morphology or development. After hindlimb ischemia, enzyme-linked maximal respiration remained reduced in all complexes at d7, and only complexes I-III-IV by d28. Prolonged recovery after HLI (d28) of *Cox6a2* deletion displayed increased centralized nuclei and smaller myofibers indicative of delays in skeletal muscle regeneration, though these delays were not translated into muscle function deficits.

While the results from this study are promising, the model used has some limitations. HLI causes one significant ischemic event while CLTI patients may suffer from nonhealing wounds (indicating an arterial occlusion) on their peripheral limbs for months to years before seeking surgical intervention. Based on the evidence collected from one ischemic event in the KO model, repetitive events of muscular atrophy and hypoxia would likely cause significant declines

in mitochondrial functioning of the KO animals. Future experiments on the Pax7-Cre;Cox6a2^{ff} mice should be conducted to look at progenitor cell quantity, citrate synthase activity, H₂O₂ emissions, and analysis of the d28 proteome. Additionally, type I and II pericyte analysis in the tissues and lipid staining may help explain the d28 delayed regeneration in the KO animals.

References:

1. Shu J, Santulli G. Update on peripheral artery disease: Epidemiology and evidence-based facts. *Atherosclerosis*. 2018;275:379-381. doi:10.1016/j.atherosclerosis.2018.05.033
2. Eid MA, Mehta K, Barnes JA, et al. The global burden of peripheral artery disease. *J Vasc Surg*. 2023;77(4):1119-1126.e1. doi:10.1016/j.jvs.2022.12.015
3. Conte MS, Bradbury AW, Kolh P, et al. Global vascular guidelines on the management of chronic limb-threatening ischemia. *J Vasc Surg*. 2019;69(6):3S-12S.e40. doi:10.1016/j.jvs.2019.02.016
4. Reinecke H, Unrath M, Freisinger E, et al. Peripheral arterial disease and critical limb ischaemia: still poor outcomes and lack of guideline adherence. *Eur Heart J*. 2015;36(15):932-938. doi:10.1093/eurheartj/ehv006
5. Taylor R, Belli AM, Jacob S. Distal venous arterialisation for salvage of critically ischaemic inoperable limbs. *The Lancet*. 1999;354(9194):1962-1965. doi:10.1016/S0140-6736(99)03164-5
6. Beck K, Howard D. Distal venous arterialisation for 'no-option' chronic limb-threatening ischaemia. *J Vasc Soc G B Irel*. 2022;1. doi:10.54522/jvsgbi.2022.016
7. Van Gils CC, Wheeler LA, Mellstrom M, Brinton EA, Mason S, Wheeler CG. Amputation prevention by vascular surgery and podiatry collaboration in high-risk diabetic and nondiabetic patients. The Operation Desert Foot experience. *Diabetes Care*. 1999;22(5):678-683. doi:10.2337/diacare.22.5.678
8. Ryan TE, Yamaguchi DJ, Schmidt CA, et al. Extensive skeletal muscle cell mitochondriopathy distinguishes critical limb ischemia patients from claudicants. *JCI Insight*. 2018;3(21):e123235. doi:10.1172/jci.insight.123235
9. Rontoyanni VG, Nunez Lopez O, Fankhauser GT, Cheema ZF, Rasmussen BB, Porter C. Mitochondrial Bioenergetics in the Metabolic Myopathy Accompanying Peripheral Artery Disease. *Front Physiol*. 2017;8. doi:10.3389/fphys.2017.00141
10. Ryan TE, Schmidt CA, Alleman RJ, et al. Mitochondrial therapy improves limb perfusion and myopathy following hindlimb ischemia. *J Mol Cell Cardiol*. 2016;97:191-196. doi:10.1016/j.yjmcc.2016.05.015
11. Pizzimenti M, Riou M, Charles AL, et al. The Rise of Mitochondria in Peripheral Arterial Disease Physiopathology: Experimental and Clinical Data. *J Clin Med*. 2019;8(12):2125. doi:10.3390/jcm8122125
12. Koutakis P, Ismaeel A, Farmer P, et al. Oxidative stress and antioxidant treatment in patients with peripheral artery disease. *Physiol Rep*. 2018;6(7):e13650. doi:10.14814/phy2.13650

13. Ryan TE, Kim K, Scali ST, et al. Interventional- and amputation-stage muscle proteomes in the chronically threatened ischemic limb. *Clin Transl Med.* 2022;12(1):e658. doi:10.1002/ctm2.658
14. Ryan TE, Schmidt CA, Green TD, Brown DA, Neufer PD, McClung JM. Mitochondrial Regulation of the Muscle Microenvironment in Critical Limb Ischemia. *Front Physiol.* 2015;6. doi:10.3389/fphys.2015.00336
15. Dokun AO, Keum S, Hazarika S, et al. A Quantitative Trait Locus (LSq-1) on Mouse Chromosome 7 Is Linked to the Absence of Tissue Loss After Surgical Hindlimb Ischemia. *Circulation.* 2008;117(9):1207-1215. doi:10.1161/CIRCULATIONAHA.107.736447
16. Inoue M, Uchino S, Iida A, et al. *COX6A2* variants cause a muscle-specific cytochrome *c* oxidase deficiency. *Ann Neurol.* 2019;86(2):193-202. doi:10.1002/ana.25517
17. Bachman NJ, Riggs PK, Siddiqui N, Makris GJ, Womack JE, Lomax MI. Structure of the Human Gene (*COX6A2*) for the Heart/Muscle Isoform of Cytochrome Oxidase Subunit VIa and Its Chromosomal Location in Humans, Mice, and Cattle. *Genomics.* 1997;42(1):146-151. doi:10.1006/geno.1997.4687
18. Taanaman JW, Hall RE, Tang C, Marusich MF, Kennaway NG, Capaldi RA. Tissue distribution of cytochrome *c* oxidase isoforms in mammals. Characterization with monoclonal and polyclonal antibodies. *Biochim Biophys Acta BBA - Mol Basis Dis.* 1993;1225(1):95-100. doi:10.1016/0925-4439(93)90128-N
19. Fabrizi GM, Sadlock J, Hirano M, et al. Differential expression of genes specifying two isoforms of subunit VIa of human cytochrome *c* oxidase. *Gene.* 1992;119(2):307-312. doi:10.1016/0378-1119(92)90288-Z
20. Schmidt CA, Amorese AJ, Ryan TE, et al. Strain-Dependent Variation in Acute Ischemic Muscle Injury. *Am J Pathol.* 2018;188(5):1246-1262. doi:10.1016/j.ajpath.2018.01.008
21. Schmidt CA, Ryan TE, Lin CT, et al. Diminished force production and mitochondrial respiratory deficits are strain-dependent myopathies of subacute limb ischemia. *J Vasc Surg.* 2017;65(5):1504-1514.e11. doi:10.1016/j.jvs.2016.04.041
22. Spangenburg EE, Le Roith D, Ward CW, Bodine SC. A functional insulin-like growth factor receptor is not necessary for load-induced skeletal muscle hypertrophy: Skeletal muscle hypertrophy. *J Physiol.* 2008;586(1):283-291. doi:10.1113/jphysiol.2007.141507
23. Fisher-Wellman KH, Davidson MT, Narowski TM, Lin CT, Koves TR, Muoio DM. Mitochondrial Diagnostics: A Multiplexed Assay Platform for Comprehensive Assessment of Mitochondrial Energy Fluxes. *Cell Rep.* 2018;24(13):3593-3606.e10. doi:10.1016/j.celrep.2018.08.091
24. McLaughlin KL, Kew KA, McClung JM, Fisher-Wellman KH. Subcellular proteomics combined with bioenergetic phenotyping reveals protein biomarkers of respiratory

- insufficiency in the setting of proofreading-deficient mitochondrial polymerase. *Sci Rep.* 2020;10(1):3603. doi:10.1038/s41598-020-60536-y
25. Fisher-Wellman KH, Draper JA, Davidson MT, et al. Respiratory Phenomics across Multiple Models of Protein Hyperacylation in Cardiac Mitochondria Reveals a Marginal Impact on Bioenergetics. *Cell Rep.* 2019;26(6):1557-1572.e8. doi:10.1016/j.celrep.2019.01.057
 26. Yin H, Price F, Rudnicki MA. Satellite Cells and the Muscle Stem Cell Niche. *Physiol Rev.* 2013;93(1):23-67. doi:10.1152/physrev.00043.2011
 27. Rahman NIA, Lam CL, Sulaiman N, et al. PAX7, a Key for Myogenesis Modulation in Muscular Dystrophies through Multiple Signaling Pathways: A Systematic Review. *Int J Mol Sci.* 2023;24(17):13051. doi:10.3390/ijms241713051
 28. McLaughlin KL, Hagen JT, Coalson HS, et al. Novel approach to quantify mitochondrial content and intrinsic bioenergetic efficiency across organs. *Sci Rep.* 2020;10(1):17599. doi:10.1038/s41598-020-74718-1
 29. Quintens R, Singh S, Lemaire K, et al. Mice Deficient in the Respiratory Chain Gene Cox6a2 Are Protected against High-Fat Diet-Induced Obesity and Insulin Resistance. Dzeja P, ed. *PLoS ONE.* 2013;8(2):e56719. doi:10.1371/journal.pone.0056719
 30. Bloemberg D, Quadrilatero J. Rapid Determination of Myosin Heavy Chain Expression in Rat, Mouse, and Human Skeletal Muscle Using Multicolor Immunofluorescence Analysis. Berdeaux R, ed. *PLoS ONE.* 2012;7(4):e35273. doi:10.1371/journal.pone.0035273
 31. Radford NB, Wan B, Richman A, et al. Cardiac dysfunction in mice lacking cytochrome- *c* oxidase subunit VIaH. *Am J Physiol-Heart Circ Physiol.* 2002;282(2):H726-H733. doi:10.1152/ajpheart.00308.2001
 32. Bahat A, Gross A. Mitochondrial plasticity in cell fate regulation. *J Biol Chem.* 2019;294(38):13852-13863. doi:10.1074/jbc.REV118.000828
 33. Larsen S, Nielsen J, Hansen CN, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J Physiol.* 2012;590(14):3349-3360. doi:10.1113/jphysiol.2012.230185
 34. Mohiuddin M, Lee NH, Moon JY, et al. Critical Limb Ischemia Induces Remodeling of Skeletal Muscle Motor Unit, Myonuclear-, and Mitochondrial-Domains. *Sci Rep.* 2019;9(1):9551. doi:10.1038/s41598-019-45923-4
 35. Ye X, Xie Y, Shi Y, et al. Switching ubiquitous and muscle-specific isoforms of mitochondrial respiratory complex IV in skeletal muscle fine-tunes complex IV activity. *FASEB J.* 2023;37(4):e22891. doi:10.1096/fj.202201223RR
 36. Sugiyama Y, Suzuki A, Kishikawa M, et al. Muscle Develops a Specific Form of Small Heat Shock Protein Complex Composed of MKBP/HSPB2 and HSPB3 during Myogenic Differentiation. *J Biol Chem.* 2000;275(2):1095-1104. doi:10.1074/jbc.275.2.1095

37. Nakagawa M, Tsujimoto N, Nakagawa H, Iwaki T, Fukumaki Y, Iwaki A. Association of HSPB2, a Member of the Small Heat Shock Protein Family, with Mitochondria. *Exp Cell Res.* 2001;271(1):161-168. doi:10.1006/excr.2001.5362
38. Birbrair A, Zhang T, Wang ZM, et al. Role of Pericytes in Skeletal Muscle Regeneration and Fat Accumulation. *Stem Cells Dev.* 2013;22(16):2298-2314. doi:10.1089/scd.2012.0647
39. Cappellari O, Cossu G. Pericytes in Development and Pathology of Skeletal Muscle. *Circ Res.* 2013;113(3):341-347. doi:10.1161/CIRCRESAHA.113.300203
40. Cogliati S, Calvo E, Loureiro M, et al. Mechanism of super-assembly of respiratory complexes III and IV. *Nature.* 2016;539(7630):579-582. doi:10.1038/nature20157
41. Guo R, Gu J, Wu M, Yang M. Amazing structure of respirasome: unveiling the secrets of cell respiration. *Protein Cell.* 2016;7(12):854-865. doi:10.1007/s13238-016-0329-7
42. Milenkovic D, Misic J, Hevler JF, et al. Preserved respiratory chain capacity and physiology in mice with profoundly reduced levels of mitochondrial respirasomes. *Cell Metab.* 2023;35(10):1799-1813.e7. doi:10.1016/j.cmet.2023.07.015
43. Srinivasan S, Avadhani NG. Cytochrome c oxidase dysfunction in oxidative stress. *Free Radic Biol Med.* 2012;53(6):1252-1263. doi:10.1016/j.freeradbiomed.2012.07.021
44. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. *Physiol Rev.* 2014;94(3):909-950. doi:10.1152/physrev.00026.2013
45. Ryan TE, Schmidt CA, Green TD, Spangenburg EE, Neufer PD, McClung JM. Targeted Expression of Catalase to Mitochondria Protects Against Ischemic Myopathy in High-Fat Diet–Fed Mice. *Diabetes.* 2016;65(9):2553-2568. doi:10.2337/db16-0387
46. Baković J, Yu BYK, Silva D, et al. A key metabolic integrator, coenzyme A, modulates the activity of peroxiredoxin 5 via covalent modification. *Mol Cell Biochem.* 2019;461(1-2):91-102. doi:10.1007/s11010-019-03593-w
47. Short KR, Bigelow ML, Kahl J, et al. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci.* 2005;102(15):5618-5623. doi:10.1073/pnas.0501559102
48. Lanza IR, Sreekumaran Nair K. Regulation of skeletal muscle mitochondrial function: genes to proteins. *Acta Physiol.* 2010;199(4):529-547. doi:10.1111/j.1748-1716.2010.02124.x
49. Desler C, Hansen TL, Frederiksen JB, Marcker ML, Singh KK, Juel Rasmussen L. Is There a Link between Mitochondrial Reserve Respiratory Capacity and Aging? *J Aging Res.* 2012;2012:1-9. doi:10.1155/2012/192503

50. Ceja Rodriguez M, Mark JR, Gosdin M, Humphries MD. Perceptions of patients with wounds due to chronic limb-threatening ischemia. *Vasc Med*. 2021;26(2):200-206. doi:10.1177/1358863X20987896

APPENDIX: IACUC APPROVAL FORM



003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 - 4354
Animal Care and Use Committee
252-744-2436 office | 252-744-2355 fax

March 16, 2023

Joseph McClung, Ph.D.
Department of Physiology, ECU

Subject: Protocol Q323c, original approval date 10/04/2022.

Dear Dr. McClung:

The amendment#2 to your Animal Use Protocol entitled, "Peripheral Endothelial and Muscle Cell Pathology in Cardiovascular Disease" (AUP#Q323c) was reviewed by this institution's Animal Care and Use Committee on 03/16/2023. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.

Sincerely yours,

A handwritten signature in black ink that reads "S McRae".

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure

www.ecu.edu

