Skeletal muscle undergoes fiber type metabolic switch without myosin heavy chain switch in response to defective fatty acid oxidation.

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Highlights

- Fuel oxidation differences in glycolytic and oxidative muscles are intrinsic to the mitochondria.
- Muscle CPT2 loss elicits fiber-type dependent mitochondrial biogenesis
- Muscle CPT2 loss elicits an oxidative-to-glycolytic shift in metabolism.
- Muscle CPT2 loss does not alter myosin heavy chain isoform composition.
- CPT2 deficient muscles experience a metabolic-contractile apparatus mismatch.

Abstract

Objective: Skeletal muscle is a heterogeneous and dynamic tissue that adapts to functional demands and substrate availability by modulating muscle fiber size and type. The concept of muscle *fiber type* relates to its contractile (slow or fast) and metabolic (glycolytic or oxidative) properties. Here, we tested whether muscle-specific disruptions in oxidative catabolism are sufficient to prompt parallel adaptations in energetics and contractile composition.
Methods: Mice with impaired mitochondrial long-chain fatty acid oxidation (mLCFAO) in the skeletal muscle due to loss of carnitine palmitoyltransferase 2 (*Cpt2*$^{Sk-/-}$) modeled a shift in muscle macronutrient catabolism. Glycolytic and oxidative muscles of *Cpt2*$^{Sk-/-}$ mice and control littermates were compared for the expression of energy metabolism-related proteins, mitochondrial respiratory capacity, and myosin heavy chain isoform composition.
Results: Intrinsic distinctions in mitochondrial bioenergetics were observed between muscle types whereby oxidative, but not glycolytic, muscle mitochondria had the capacity to increase mLCFAO to meet energy demands. The loss of CPT2 ablated mLCFAO and resulted in mitochondrial biogenesis occurring most predominantly in oxidative muscle fibers. The metabolism-related proteomic signature of *Cpt2*$^{Sk-/-}$ oxidative muscle more closely resembled that of a glycolytic muscle than of a control oxidative muscle. However, CPT2 deletion did not result in contractile-based fiber type switching according to myosin heavy chain composition

analysis. Despite the lack of shift in contractile units, the intrinsic mitochondrial bioenergetic profile of CPT2 deficient oxidative muscles did shift to closely match that of glycolytic muscles. **Conclusion:** The loss of mitochondrial long-chain fatty acid oxidation elicits an adaptive response involving conversion of oxidative muscle towards a metabolic profile that resembles a glycolytic muscle but that is not accompanied by changes in myosin heavy chain isoform content. These data suggest that shifts in muscle catabolism are not sufficient to drive shifts in the contractile apparatus but are sufficient to drive adaptive alterations in metabolic properties.

Keywords: skeletal muscle, fatty acid oxidation, carnitine palmitoyltransferase 2, mitochondrial biogenesis, bioenergetics, fiber-typing

1.1 Introduction:

Skeletal muscles are composed of numerous distinct muscle fibers which exist in a diverse array within a given muscle. A muscle's fiber composition dictates the characteristics of its contractile dynamics, and this will, in turn, determine the muscle's contribution to whole body locomotion. The concept of *fiber type* relates to a combination of both contractile (slow or fast twitch) and metabolic (glycolytic or oxidative) properties (1). The metabolic properties of skeletal muscle encompass an important characteristic for the tissues' ability to cope with sudden and extreme changes in energy demands and consumption. Transitioning between rest and intense physical activity, for example, acutely increases muscle ATP requirements by 100-fold (1). In order to meet such versatile demands, muscle tissue has a unique set of resources to replenish its ATP reserves quickly and efficiently: creatine kinase (CK) activity, substrate level phosphorylation during glycolysis, and aerobic metabolism via mitochondrial oxidative phosphorylation. In different muscles, the relative contribution of these three processes to ATP production is highly distinct and linked to the contractile properties of the muscle. Thus, in slow twitch fibers, energy is provided mainly by mitochondrial oxidative metabolism, while in fast twitch fibers there is a greater contribution of glycolysis towards ATP generation. Based on these biochemical properties, fibers are further classified as oxidative (type 1), oxidative-glycolytic (type 2A), glycolytic (type 2B), and fourth type (2X) presenting as intermediate between 2A and 2B (2). Regarding the contractile-based properties of muscle, the expression of different myosins is a major characterizing component due to myosin's critical and diverse ability to act as a molecular motor. Myosin heavy chain (MyHC) isoforms are widely used as markers of muscle fiber types. Specifically, MyHC-based characterization constitutes the four principal fiber types in mammalian muscles as: type 1 composed of MyHC-β, type 2A composed of MyHC-2A, type 2B composed of MyHC-2B and type X composed of MyHC-2X. Myosin heavy chains contribute to contractile velocity and fatigue resistance in muscle fibers allowing for more broadly-based classification into two categories: slow twitch (type 1) and fast twitch fibers (2A, 2B and 2X). Across the distinct fiber-types classifications, the metabolic properties are thought to match the contractile protein properties of a given muscle to allow for form-fit-function based physiology.

Muscle fibers are highly dynamic, possessing the ability to adapt to functional demands and substrate availability. These adaptations are predicted to tightly couple contractile apparatus to energy metabolism. Indeed, damage to mitochondrial DNA results in changes in contractile apparatus suggesting that mitochondrial function is a regulator of skeletal muscle MyHC fiber type (3). Yet, in the absence of deteriorations in overall mitochondrial function, it is less well understood how primary shifts in muscle macronutrient catabolic activity impact adaptations of the contractile apparatus. In the present study we utilized a model of impaired mitochondrial long-chain fatty acid oxidation (mLCFAO) to investigate the consequences of macronutrient catabolic imbalances on the contraction-metabolism coupling of skeletal muscle. Prohibited mLCFAO was induced by the loss of carnitine palmitoyltransferase 2 in skeletal muscle of mice

(*Cpt2*$^{Sk-/-}$) (4, 5). CPT2 is a critical and irreplaceable enzyme for mLCFAO such that the loss of CPT2 effectively ablates flux of long-chain fatty acids through mitochondrial oxidative metabolism and results in the accumulation of its substrate, long-chain acylcarnitines (5). Impairments in fatty acid oxidative flux and accumulation of long-chain acylcarnitines are routinely observed in the skeletal muscle of individuals and animal models with obesity, insulin resistance, and diabetes (6-8).

Herein, we hypothesized that the halt of mLCFAO in muscle-specific CPT2 knockout mice would drive a shift in mitochondrial energy metabolism towards non-fatty acid substrates, similar to the metabolic profile of glycolytic muscles; and that this metabolic change would be accompanied by a coupled shift towards predominantly glycolytic myosin heavy chain isoforms. Thus, muscle plasticity during mLCFAO deficiency would drive an adaptive reduction of oxidative-prone fibers and increase of glycolytically-active fibers to allow for effective contraction-metabolism coupling. Therefore, we profiled the proteome, pathology, and bioenergetic metabolic capacity of muscle, distinguishing between predominantly glycolytic and predominantly oxidative muscles in control compared to *Cpt2*$^{Sk-/-}$ mice. In glycolytic muscles, CPT2KO had proteomic profiles and substrate-supported mitochondrial respiration capabilities that were similar to controls. However, in oxidative soleus muscle,*Cpt2*$^{Sk-/-}$ proteome and mitochondrial bioenergetics shifted towards that of the glycolytic muscles. Surprisingly, the metabolic shift of CPT2-deficient oxidative muscle was not accompanied by myosin heavy chain isoform switch. Together, these data suggest that mLCFAO deficient muscles adapt fiber type-specific energy metabolism but that this adaptation is not coupled to changes in fiber type contractile components.

paper1.2 Materials and Methods

Animal model

Skeletal muscle specific (*Cpt2*$^{Sk-/-}$) CPT2 conditionally deficient mice were generated using the human alpha-skeletal actin promoter (The Jackson Laboratory; Stock No: 006149) as described (5, 9). Littermates lacking the Cre gene were used as controls. Mice were given free access to water and standard chow (PicoLab 5053, Lab Diets), in pathogen-free housing under 12-hour light-dark cycles. All procedures were performed in 3-8 month old mice and were approved by the Purdue Animal Care and Use Committee, Assurance (Assurance A3231-01) and the Institutional Animal Care and Use Committee of East Carolina University (Assurance A3469-01).

Histology and immunohistochemistry

Skeletal muscle was collected immediately after euthanasia, covered by a thin layer of Optimal CuttingTemperature compound (O.C.T., Fisher Scientific), flash frozen in liquid nitrogen-chilled isopentane and stored at -80°C until further processing. 10μm thick serial crossections were obtained and immunohistochemical detection of myosin heavy chain (MyHC) isoforms was performed as previously described (10). Briefly, sections were fixed in 4% PFA ice-cold, permeabilized in 0.3% Triton X-100, blocked with 5% goat serum and incubated overnight with a primary antibody mix against MyHC-β (type I fibers;Developmental Studies Hybridoma Bank BA-F8), MyHC-2A (type IIa fibers; DSHB SC-71), MyHC-2B (type IIb fibers; DSHB BF-F3) and dystrophin (PA5-32388). MyHC-2X (type IIx fibers) were identified by absence of staining when all previous antibodies were used on a given muscle section. The following secondary antibodies were used to visualize the different MyHC isoforms: Alexa Fluor 350 IgG2b (A-21140, Life Technologies) for MyHC I, Alexa Fluor 488 IgG1 (A-21121) for MyHC IIa, Alexa Fluor 546

IgM (A-21045) for MyHC IIb and Alexa fluor 647 goat anti-mouse IgG1 (A-21236) for dystrophin. Gomori Trichrome stain (Engel-Cunningham modification) was performed in serial crossections as described elsewhere (11, 12) with modifications. Briefly, sections were fixed in 4% PFA, counterstained with Mayer's hematoxylin, immersed in modified Gomori Trichrome stain (Chromotrope 2R+ Fast Green+Phosphotungstic acid) for 20 minutes, differentiated in 0.2% acetic acid, dehydrated and mounted. Images were acquired using EVOS FL auto microscope andsoftware (Life Technologies, Inc.) and fiber type distribution was quantified using ImageJ software (National Institutes of Health). Images were coded and randomized for blinded analyses.

Mitochondrial oxidation assays

Oroboros high-resolution respirometry was performed on freshly isolated mitochondria from skeletal muscle similar to previous reports (13, 14). Specifically, mitochondria were isolated from white quadriceps, tibiales anterior and soleus muscles by differential centrifugation in KMEM buffer (KCl 100mM, MOPS 50mM, EGTA 1mM, MgSO4 5mM, BSA 0.2% pH 7.1) and yield was measured by protein concentration using the BCA method (15). Maximum respiration was measured in ATP containing buffer Z (K-MES 105mM, KCl 30mM, KH2PO4 10mM, MgCl2 5mM, EGTA 1mM, BSA 2.5g/L, pH 7.1) using 25-50µg of isolated mitochondria in each chamber. After baseline respiration was established, malate (2 mM, Sigma M1296) was added to maintain the TCA cycle. Substrates were added in the following concentrations: octanoylcarnitine 0.2mM (Sigma 50892), palmitoylcarnitine 0.02mM (Sigma P1645), pyruvate 5mM (Combi-Blocks, QA1116), or succinate 10mM (Fisher BP336) plus rotenone 0.005mM (Sigma R8875). A modified version of the creatine kinase clamp technique was used to determine oxygen consumption in response to changes in ATP free energy, as described before (13). Briefly, the respiration assay buffer Z was supplemented with 20U/mL of creatine kinase (CK), 5mM ATP and 1mM Tris-Phosphocreatine (PCr) to stimulate maximal demand for ATP sysnthesis. Sequential additions of PCr to 6, 9, 15, 21, 24 and 30mM allowed for gradual reduction of the ATP demand state. Calculation of the ΔGATP at each titration point was performed with the following online bioenergetics calculator tool described in reference (14). The relationship between ΔGATP and JO2 was plotted, and the slope of the linear portion was calculated to serve as the conductance measure of the system under the specified respiratory substrate conditions. The experiments were repeated at least three times with mitochondria isolated from separate animals

Acylcarnitine analysis

Acylcarnitine profiling of tissue was performed as previously described (5, 9). Briefly, lipids were extracted from tissues using Bligh and Dyer Method (16), dried, resuspended, and injected directly via a micro-autosampler (G1377A) into a QQQ6410 triple quadrupole mass spectrometer (Agilent Technologies, San Jose, CA) operated in the positive ion mode and equipped with ESI ion source. Data was collected in multiple reaction monitoring mode (9, 17, 18) and ion intensities acquired by an in-house script were further normalized to sample protein concentration and expressed as percent of total ion counts.

Mass spectrometry lipid imaging

Lipid imaging was performed by nanospray desorption electrospray ionization (nano-DESI) as previously described (19). Briefly, 10µm-thick muscle sections were mounted onto a glass slide and stored at -80°C prior to analysis. Nano-DESI MSI experiments were performed on an Q

Exactive HF Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a custom-designed nano-DESI source. A shear-force probe was used to maintain a constant distance between the sample and probe. A 9:1 (v/v) methanol:water mixture was used as solvent at a flow rate of 0.5μL/min. Additionally, the solvent contained 200nM LPC (19:0) and 5μM C5-carnitine internal standards. Ionization was achieved by applying a 3.5kV potential to the solvent syringe needle. The heated capillary inlet was held at 30V and 200°C. Mass spectra were acquired in positive mode in the range of m/z 150–1000 with a mass resolution of m/Δm=60,000 at 412m/z. Imaging data were acquired at a scan rate of 40μm/s and a step between the lines of 30μm. Each line scan was collected as an individual file. Data processing was performed using Peak-by-Peak software (Spectroswiss, Lausanne, Switzerland).

Gene expression analysis

For **real-time PCR**, RNA was isolated using Trizol (Invitrogen) and RNA was converted to cDNA (Applied Biosystems High Capacity cDNA RT Kit) and used for SYBR Green (Bio-Rad) based real-time PCR using an Applied Biosystems QuantStudio3 Real-time PCR system. Results of detection with specific target primers were normalized to housekeeping gene (Rpl22) and expressed as arbitrary units of $2^{-\Delta CT}$.

For **mitochondrial DNA** (mtDNA) analysis, total DNA was isolated from skeletal muscle using QiAmp DNA Mini kit (Quiagen, #51304) and 10μg per reaction were used as template for SYBR Green (Bio-Rad) based real-time PCR. Mitochondrial target genes were NADH Dehydrogenase 1 (Nd1) (20) and Mito1 (21) and H19 was used as nuclear DNA.Average C_T values from triplicate reactions were obtained and the mitochondrial DNA content relative to nuclear DNA was determined (22).

Protein analysis

For **immunoblotting**, skeletal muscle was harvested after 3-4 hours of fasting. Tissue homogenates were performed in sucrose media (10mM Tris, 1mM EDTA, 250mM sucrose) with protease and phosphatase inhibitors using a hand-held mechanic homogenizer (Tissue Tearor, Fisher Scientific). Protein concentration was measured by BCA assay, and samples were equally loaded (20μg protein/lane) onto 10 or 12% SDS-polyacrylamide gels. After electrophoresis, gel was transferred to nitrocellulose membrane and stained with Ponceau S for total protein visualization. Then it was blocked with 5% milk-TBST for 1h, incubated with primary antibody (1:1,000-1:2,000) against CPT2 (Millipore, ABS85), or Total OXPHOS cocktail (Abcam, MS604-300)), washed, and incubated with secondary antibodies (1:10000) conjugated to IR dye 800CW or 680LT (LiCor). Proteins were visualized with Odyssey and quantified using Image Studio (LiCor).

For **native gel electrophoresis**, 200μg of isolated mitochondria from white quadriceps and soleus muscle were lysed by mechanical trituration in 1X sample Buffer (Invitrogen, BN2003) supplemented with protease inhibitor (cOmplete mini, Roche) and 6% digitonin (Sigma, D141). Lysates were incubated on ice for 20 minutes and then centrifugated at 20,000xg. Clear supernatants were recovered, and protein concentration was measured again by BCA assay (Thermo, 23225). 30ug of the recovered mitochondrial protein was mixed with G-250 sample additive (Invitrogene, BN2004) at 1.5% final concentration and loaded into a 4-16% BisTris gel (BN1004BOX10). Electrophoresis was performed at 150V for 3.5 hours at 4°C. Upon completion, gel was fixed in 40% methanol (Fisher, 194449) + 10% acetic acid (Fisher A38-212) for 15 minutes, destain in 8% acetic acid until the desired background level was achieved and image using ChemiDoc™ Touch system (BioRad).

For **TMT quantitative proteomics**, soleus and EDL muscles 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 $CaCl_2$, 1x cOmplete ULTRA mini EDTA-free protease inhibitor tablet), as described previously (13). Three freeze-thaw cycles were performed, and samples were disrupted by sonication with a probe sonicator in three 5s bursts set at an amplitude of 30 (Q Sonica, Newtown, CT). Samples were centrifuged at 10,000×g for 10 min at 4°C. Protein concentration was determined by BCA, and equal amounts of protein (200µg, adjusted to 2.5mg/mL with Urea Lysis Buffer) were reduced with 5mM DTT at 32°C for 30 min, cooled to room temperature, and then alkylated with 15mM iodoacetamide for 30 min in the dark. Unreacted iodoacetamide was quenched by the addition of DTT up to 15mM. Initial digestion was performed with Lys C (Thermo Fisher) 1:100w-w; 2µg enzyme per 200µg protein) for 4 hr at 32°C. Following dilution to 1.5M urea with 50mM Tris (pH 8.0), 30mM NaCl, 5mM $CaCl_2$, the samples were digested overnight with trypsin (Promega, Madison, WI) 50:1 w/w, protein:enzyme at 32°C. Samples were acidified to 0.5% TFA and centrifuged at 10,000×g for 10 min at 4°C to pellet insoluble material. Supernatant containing soluble peptides was desalted on a 50mg tC18 SEP-PAK solid phase extraction column (Waters, Milford, MA) and eluted (500µL 25% acetonitrile/0.1% TFA and 2 × 500µL 50% acetonitrile/0.1% TFA). The 1.5mL eluate was frozen and dried down via SpeedVac.

TMT labeling was performed as previously described (23). Dried down peptides were re-suspended in 100µL of 200mM triethylammonium bicarbonate (TEAB), mixed with a unique 10-plex Tandem Mass Tag (TMT) reagent (0.8mg re-suspended in 50µL100% acetonitrile) and shaken for 4 hr at room temperature (Thermo Fisher). A total of 3 x 10-plex kits were used. Following quenching with 0.8µL 50% hydroxylamine samples were frozen and placed in SpeedVac. Samples were re-suspended in ~1mL of 0.5% TFA and again subjected to solid phase extraction, but with a 10 mg tC18 SEP-PAK SPE column (Waters). The multiplexed peptide sample was subjected to high pH reversed phase fractionation (Thermo Fisher; Catalog #84868). In this protocol, peptides (100µg) are loaded onto a pH-resistant resin and then desalted with water washing combined with low-speed centrifugation. A step-gradient of increasing acetonitrile concentration in a high-pH elution solution is then applied to columns to elute bound peptides into 8 fractions. Following elution, fractions were frozen and placed in SpeedVac.

nLC-MS/MS was performed as described previously (23). Peptide fractions were suspended in 0.1% formic acid at a concentration of 0.25 µg/µL, following peptide quantification (ThermoFisher). All samples were subjected to nanoLC-MS/MS analysis using an UltiMate 3000 RSLCnano system (Thermo Fisher) coupled to a Q Exactive PlusHybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher) via nanoelectrospray ionization source. For each injection of 4µL (1µg), the sample was first trapped on an Acclaim PepMap 100 20mm × 0.075mm trapping column (Thermo Fisher) 5µl/min at 98/2 v/v water/acetonitrile with 0.1% formic acid, after which the analytical separation was performed over a 90-min gradient (flow rate of 300nl/min) of 3 to 30% acetonitrile using a 2µm EASY-Spray PepMap RSLC C18 75µm × 250mm column (Thermo Fisher) with a column temperature of 55°C. MS1 was performed at 70,000 resolution, with an AGC target of 1×10^6 ions and a maximum IT of 60 ms. MS2 spectra were collected by data-dependent acquisition (DDA) of the top 20 most abundant precursor ions with a charge greater than 1 per MS1 scan, with dynamic exclusion enabled for 30s. Precursor ions were filtered with a 1.0 m/z isolation window and fragmented with a normalized collision energy of 30. MS2 scans were performed at 17,500 resolution, AGC target of 1×10^5 ions, and a maximum IT of 60 ms.

Proteome Discoverer 2.2 (PDv2.2) was used for **raw data analysis**, with default search parameters including oxidation (15.995Da on M) as a variable modification and carbamidomethyl (57.021Da on C) and TMT6plex (229.163Da on peptide N-term and K) as fixed modifications, and 2 missed cleavages (full trypsin specificity). Data were searched against both the full mouse proteome, as well as the Mito Carta 3.0 database (24) and

mitochondrial gene/protein lists from Stenton et al (25) and Murayama et al (26). PSMs were filtered to a 1% FDR. PSMs were grouped to unique peptides while maintaining a 1% FDR at the peptide level. Peptides were grouped to proteins using the rules of strict parsimony and proteins were filtered to 1% FDR using the Protein FDR Validator node of PD2.2. MS2 reporter ion intensities for all PSMs having co-isolation interference below 0.5 (50% of the ion current in the isolation window) and an average S/N > 10 for reporter ions were summed together at the peptide and protein level. Imputation was performed via low abundance resampling. The protein group tab in the PDv2.2 results was exported as tab delimited.txt. files, and **statistically analyzed** based on a previously described workflow (23). First, M2 reporter (TMT) intensities were summed together for each TMT channel, each channel's sum was divided by the average of all channels' sums, resulting in channel-specific loading control normalization factors to correct for any deviation from equal protein input in the 10-plex experiments. Reporter intensities for proteins were divided by the loading control normalization factors for each respective TMT channel. All loading control-normalized reporter intensities were converted to $\log_2$ space and the average value from the ten samples per kit was subtracted from each sample specific measurement to normalize the relative measurements to the mean of each kit. Data from each kit were then combined for statistical comparisons. For comparisons, condition average, standard deviation, p-value (p, two-tailed student's t-test, assuming equal variance), and adjusted p-value ($P_{adjusted}$, Benjamini Hochberg FDR correction) were calculated (27, 28). For protein-level quantification, only Master Proteins—or the most statistically significant protein representing a group of parsimonious proteins containing common peptides identified at 1% FDR—were used for quantitative comparison. Venn diagrams were generated using the free online tool *jvenn* (29). Pathway analysis was perfomed using Reactome (30).
Statistics: Data is presented as mean ± SEM, unless otherwise specified. Statistical analysis and figures were generated using Excel or GraphPad Prism version 8.0.0 for Windows (GraphPad Software). Data was compared using unpaired Student's *t*-test, 1-way or 2-way ANOVA followed by multiple comparison analysis. Significance level was set at P<0.05.

Results

Muscle fiber type composition is a major determinant in fatty-acid supported mitochondrial respiration and responsiveness to increased energetic demands.

Understanding the consequences of mitochondrial long chain fatty acid oxidation deficiency in skeletal muscle requires establishing first the physiological contribution of these lipid substrates to energy production across muscle types. Here, glycolytic (extensor digitorum longus (EDL)), mixed (tibialis anterior (TA)), and oxidative (soleus) muscles were assessed. These muscles were investigated due to their known distinct fiber type composition relative to one another. To confirm the co-existence of different fiber types within these muscles, the fluorescence-based immunohistochemical detection was used to evaluate the myosin heavy chain (MyHC) isoforms traditionally used to classify fiber types. The EDL muscle was rich in the expression of MyHC-2B hallmarking fast-twitch glycolytic type IIb fibers (Figure 1A). The TA muscle was composed of approximately equal combination of the glycolytic Type IIb and the metabolically intermediate MyHC-2X-expressing Type IIx fibers (Figure 1B). The soleus muscle was highly enriched with the expression of MyHC-β and MyHC-2A proteins representing slow, oxidative Type I and fast, oxidative-glycolytic Type IIa fibers, respectively (Figure 1C). Based upon MyHC expression patterns these data confirm that EDL and soleus muscles are at opposite ends of the fiber-type spectrum, from predominately fast-glycolytic to predominately slow-oxidative, respectively, with TA as an intermediate muscle.

Next, we compared mitochondrial oxidative capacity between these muscles when long-chain fatty acids are provided as energetic substrate. To do so, oxygen consumption rates (JO_2), as indicative of mitochondrial respiration, were measured in isolated mitochondria using high resolution respirometry in the presence of palmitoylcarnitine (PC), with a high concentration of malate (M; >2mM) to facilitate exchange of metabolites and TCA cycle intermediates between mitochondria and assay buffer. The assay was conducted under a creatine kinase (CK) clamp to facilitate the exploration of mitochondrial oxidative metabolism and responsiveness across a range of physiological energy demands (14). This clamp takes advantage of the physiological function of CK enzyme in muscle which converts extra-mitochondrial ADP to ATP when phosphocreatine (PCr) is available. Thus, by manipulating the levels of phosphocreatine in the respiration assay buffer it is possible to alter the ATP/ADP ratio and ATP free energy levels which in turn regulate mitochondrial respiration.

At resting energy demands, represented by DeltaG_ATP=-14.91 and generated by increasing the ATP/ADP ratio with high phosphocreatine, the rate of mitochondrial respiration on long-chain fatty acid palmitoylcarnitine was not different between glycolytic and mixed muscles as indicated by similar JO_2 (Figure 1D). However, respiratory rate at resting conditions of the oxidative soleus muscle with palmitoylcarnitine was significantly lower than for mixed muscle (Figure1D). This was surprising given that oxidative muscles are known to rely on fatty acid oxidation for energy production more than glycolytic or mixed counterparts (31).
To assess the intrinsic mitochondrial response to increased energy demands, respiration on palmitoylcarnitine was measured on a continuum of free energy, from low energy demand (DeltaG_ATP=-14.91) to high energy demand (DeltaG_ATP=-12.94) and the slope of the linear portion of the relationship between the two variables was calculated to generate a measure of respiratory conductance (Figure 1D,E). This calculated respiratory conductance serves as a measure of OXPHOS pathway sensitivity to changes in the system's free energy, such that a steeper slope reflects higher responsiveness of the mitochondria to a given substrate over the range of given free energy conditions. Here, neither glycolytic nor mixed muscles demonstrated a significant increase in respiration rates for palmitoylcarnitine between low- and high-energy demands (Figure 1D). Conversely, the oxidative soleus muscle energized with palmitoylcarnitine exhibited a ~5-fold increase in mitochondrial respiration (Figure 1D) and a ~3-fold increase in respiratory conductance (Figure 1E), in response to increased energy demands. These data suggest intrinsic differences in the composition and/or regulation of fatty acid oxidative metabolism between glycolytic and oxidative muscles. In agreement, significant enrichment of mLCFAO-related proteins was observed in oxidative compared to glycolytic muscle (Figure 1F). Specifically, medium chain acyl-CoA dehydrogenase (ACADM; 2-fold), long chain acyl-CoA dehydrogenase (ACADL;1.6-fold), long chain acyl-CoA synthetase family member 1 (ACSL1; 1.9-fold), short chain acyl-CoA synthetase family member 1 (ACSS1; 2.6-fold), carnitine palmitoyltransferase 1B (CPT1B; 1.7-fold), carnitine palmitoyltransferase 2 (CPT2; 1.6-fold) and hydroxyacyl-CoA dehydrogenase subunit B (HADHA-B; 2-fold) were all significantly more abundant in oxidative muscle (Figure 1F). Next, we sought to determine if differential reliance on lipid catabolism for energy production occurred in a fiber-type dependent manner by assessing the spatial distribution of long-chain acyl-carnitines (LCACs), which are indicators of mLCFAO flux. Mixed TA muscle demonstrated high relative abundance of LCACs particularly C16 and C18:2 (Figure 1G) which were subsequently targeted for visualization by mass spectroscopy-based imaging (nano-DESI). Here, presence, distribution, and abundance of LCACs in different fibers of TA muscle was determined based on the differential puncta-like signaling the lipid

imaging (Figure 1H,I). Comparing the distribution patterns of LCACs and of fiber types in serial sections revealed that oxidative fibers, marked by type IIa MyHC protein (Figure 1J,K) had higher intensity for LCAC signal compared to glycolytic fibers, marked by type IIb MyHC (Figure 1H,I). These data allowed visualization of the metabolic distinctions between the heterogeneous fibers within a given muscle. The differences in spatial distribution of long-chain acylcarnitines suggest that metabolic activity can be strikingly distinct between two adjacent muscle fibers. Furthermore, it can be used to distinguish muscle fiber types from a metabolic perspective. Taken together these data suggest that differences in fatty acid oxidative capabilities between glycolytic and oxidative muscles are intrinsic to the mitochondria. The significantly high conductance observed in oxidative-fiber enriched muscles when energized with palmitoylcarnitine and the spatial distribution of long-chain acylcarnitines suggests that long-chain fatty acids are a critical substrate for oxidative muscle fibers, perhaps more significantly under high energy demands.

Loss of carnitine palmitoyltransferase 2 in skeletal muscle halts mLCFAO and induces mitochondrial biogenesis.

Next, to determine how impairing muscle metabolic capacity might disproportionately impact muscles with different fiber type composition, we targeted CPT2, an irreplaceable enzyme in the mLCFAO pathway. In agreement with our proteomic data (Figure 1F), we found a differential amount of CPT2 protein between muscles with higher expression in oxidative (soleus) compared to mixed (TA) and glycolytic (EDL, white quadriceps) muscles (Figure 2A). Muscle-specific genetic targeting of Cpt2 using floxed-conditional knockout mice (*Cpt2*$^{Sk-/-}$) (4, 5) effectively depleted CPT2 protein in glycolytic, mixed and oxidative muscles (Figure 2A). To confirm that loss of CPT2 compromises mLCFAO in *Cpt2*$^{Sk-/-}$ muscles, fatty acid-supported oxygen consumption in isolated mitochondria was measured. Respiration rates for the long-chain fatty acid substrate, palmitoylcarnitine, were decreased in both glycolytic and oxidative muscles by 77% and 86%, respectively (Figure 2B), and in agreement with our previous reports (4, 5).

Mitochondrial biogenesis is a common response to limitations in mitochondrial metabolic capacity (32). Macroscopic inspection of muscles demonstrated increased red hues in *Cpt2*$^{Sk-/-}$ animals (Figure 2C). To determine if the increased muscle redness was related to changes in mitochondrial content, mitochondrial DNA was quantified to reveal a ~2.5-fold increase in *Cpt2*$^{Sk-/-}$ oxidative soleus compared to control but no changes were observed in glycolytic or mixed muscles (Figure 2D). In agreement, *Cpt2*$^{Sk-/-}$ oxidative, but not glycolytic, muscles showed a significant increase in the expression of PGC1α, a master regulator of mitochondrial biogenesis (Figure 2E). Specifically, mRNA levels of PGC1α isoforms 2 and 3, which are known to regulate fatty acid oxidation and mitochondrial biogenesis genes (33), were elevated in soleus muscle of *Cpt2*$^{Sk-/-}$ mice (Figure 2E). In further agreement, western blotting demonstrated increases in several individual proteins of the oxidative phosphorylation (OXPHOS) complexes I through V in mixed and oxidative muscles of *Cpt2*$^{Sk-/-}$ mice, but to a lesser extent in glycolytic muscles (Figure 2F).

An untargeted comprehensive inquiry of the muscle proteome was next performed via whole tissue proteomics. A total of 2581 proteins were identified and quantitated in glycolytic and oxidative muscles of both, control and *Cpt2*$^{Sk-/-}$ mice. Of those, 588 were identified as mitochondrial targets according to the MitoCarta 3.0 database (24). Comparison of the summed abundance of mitochondrial proteins relative to total protein abundance between muscles confirmed intrinsically higher mitochondrial enrichment in control oxidative soleus compared to control glycolytic EDL (Figure 2G). Upon muscle-specific CPT2 deletion, mitochondrial protein abundance was not different between genotypes in glycolytic muscles (Figure 2G). However, in Cpt2-deficient oxidative muscles, the abundance of mitochondrial proteins was ~30% higher compared to controls (Figure 2G). Furthermore, only 21% (122 of the 588) of mitochondrial proteins were differentially expressed (q value <0.1) in *Cpt2*$^{Sk-/-}$ glycolytic muscles, whereas in *Cpt2*$^{Sk-/-}$ oxidative muscle a vast majority, 82% (481 of the 588), were significantly different relative to control muscle (Figure 2H). Of the altered mitochondrial proteins in oxidative muscle, only 3% were downregulated and 97% were upregulated (Figure 2H).

Detailed assessment of the mitochondrial proteome revealed that, 93% of the identified individual subunits and assembly factors of the OXPHOS complexes I through V (111 out of 119 protein targets) were significantly increased in *Cpt2*$^{Sk-/-}$ oxidative muscle compared to control (Figure 3A-F). Contrastingly, in *Cpt2*$^{Sk-/-}$ glycolytic muscle, only 8% of these OXPHOS-related proteins were upregulated compared to control (Figure 3A-F) suggesting that the mitochondrial biogenesis response to CPT2 deletion is predominately muscle-type specific. Similar to the OXPHOS expression response, only oxidative Cpt2-deficient muscle presented with a significant increase in the expression of the majority of proteins needed to support mitochondrial biogenesis such as mtDNA maintenance and transcription (Figure 3G), mitochondrial dynamics and morphology, protein quality control and import, and synthesis of iron-sulphur clusters (Figure 3H). Together this data suggests that upon CPT2 deletion, mitochondrial biogenesis and upregulation of proteins directly involved in oxidative phosphorylation and mitochondrial function are muscle-specific, with a predominantly robust response in oxidative muscles.

Mitochondrial biogenesis in response to CPT2 deficiency occurs in a fiber type specific manner.

Next, we sought to assess the muscle fiber-type specific response to mitochondrial biogenesis by performing modified Gomori stain in conjunction with traditional myosin heavy chain immunostaining in glycolytic (EDL) and oxidative (Soleus) muscles. Typically, physiological abundance of muscle mitochondria is not visible by Gomori stain; however, when mitochondrial biogenesis is elevated, mitochondria can be detected as red aggregates throughout the myofiber cross section. In control muscles, mitochondria were not visible (Figure 4 A_1, A_2); contrarily, CPT2 deficient muscles presented with red-positive staining at regions pertaining to both the subsarcolemmal and intermyofibrillar compartments suggesting a high degree of mitochondrial proliferation (Figure 4 A_3, A_4). Although affected myofibers could be observed in both muscles, the number and extent of highly stained Gomori-positive fibers were far greater in oxidative Soleus compared to glycolytic EDL muscle. When comparing individual fibers of the same muscle, the *Cpt2*$^{Sk-/-}$ soleus presented with a high degree of differential staining, suggesting that the intensity of mitochondrial biogenesis occurred in a fiber-type specific manner. Indeed, the heavily Gomori stained fibers lined up with slow-oxidative type I and IIa on serial sections, whereas fast-glycolytic type IIx myofibers appeared relatively uncompromised (Figure 4B) indicating a fiber-type specific mitochondrial biogenic response.

Together, this data suggests that mLCFAO deficient muscles due to CPT2 deletion undergo a significant wave of mitochondrial proliferation in a fiber-type specific manner. Specifically, slow oxidative type I and small fast oxidative-glycolytic type IIa fibers become heavily loaded with mitochondria in the absence of CPT2. In the oxidative muscles of *Cpt2*$^{Sk-/-}$ mice, enhanced mitochondrial biogenesis was regulated, in part, by the master transcriptional regulator PGC1α, and was accompanied by upregulation of a majority of bioenergetic-related proteins, such as the mitochondrial OXPHOS complexes.

CPT2 deficiency shifts oxidative muscles towards a glycolytic-like metabolic proteome

Given the distinct mitochondrial biogenic response in oxidative compared to glycolytic muscles of *Cpt2*$^{Sk-/-}$ mice, we next assessed native conformation and assembly of OXPHOS complexes which are known to be unique between glycolytic and oxidative muscles (27). As expected, a distinctive pattern of band thickness and molecular weight-based migration, visualized by native gel electrophoresis, was evident between control glycolytic and oxidative muscle mitochondria (Figure 5A). For glycolytic muscle, the band separation was not different between control and *Cpt2*$^{Sk-/-}$ (Figure 5A). However, for oxidative muscle, the *Cpt2*$^{Sk-/-}$ muscle presented an electrophoretic pattern that was highly distinct from control oxidative muscle but was nearly identical to glycolytic muscle (Figure 5A). These data suggest that the absence of CPT2 in oxidative muscle not only promotes mitochondrial biogenesis but also alters the mitochondrial proteome and OXPHOS conformation towards a more glycolytic-like phenotype.

Out of the 30,000 genes present in the mouse genome, ≈1000 are differentially expressed between different muscle fiber types (34). Herein, we calculated the abundance of individual proteins relative to the tissue mean to identify the most abundant targets and established a top-500 most abundant protein signature for each sample. Comparison of the top 500 most abundant proteins between muscle types showed that glycolytic and oxidative muscles shared a total of 161 of these 500 proteins (32%) (Figure 5B). However, the *Cpt2*$^{Sk-/-}$ glycolytic and oxidative muscles shared a total of 303 proteins (61%), double that of the controls (Figure 5B). Furthermore, oxidative *Cpt2*$^{Sk-/-}$ and glycolytic control muscles had 384 (77%) proteins in common, which was 2.4-fold more than those common between control glycolytic and oxidative muscles. These data suggest that the oxidative *Cpt2*$^{Sk-/-}$ muscle protein signature was more similar to glycolytic muscle than it was to counterpart control oxidative muscle. Next, comparison between genotypes revealed that glycolytic muscles from control and *Cpt2*$^{Sk-/-}$ mice shared a majority, 310 (62%), of common proteins; however, oxidative control and *Cpt2*$^{Sk-/-}$ muscle had far less in common, 91 (18%) (Figure 5B). This data suggests that CPT2-deficiency in mitochondria of oxidative muscles impacts the overall tissue proteome, causing a shift towards a more glycolytic-like protein signature.

Functional analysis of these top 500 most abundant proteins provided further insight on how glycolytic and oxidative muscles are differentially affected by lack of mLCFAO due to CPT2 loss. The most significant biological pathways in glycolytic EDL muscle of both the control and the *Cpt2*$^{Sk-/-}$ mice included mitochondrial biogenesis, cristae formation, respiratory electron transport chain, and ATP synthesis (Figure 5C, D). This suggests that, in glycolytic muscles of both genotypes, the relative abundance of the mitochondrial machinery is significant and contributes largely to the whole-muscle proteome. On the contrary, highly abundant signature pathways in control Soleus muscle involved muscle contraction, cell-extracellular matrix interaction and cytoskeletal remodeling (Figure 5E), which highlights the relevance of sarcomere-related structures in oxidative muscles that work to produce long-lasting contractions. Once more, *Cpt2*$^{Sk-/-}$ oxidative muscle diverged from its control counterpart showing mitochondrial-centered pathways that were of high similarity to glycolytic muscles (Figure 5F). This data suggests that in CPT2 deficient oxidative muscles, relative abundance and pathway profile of the protein signature shifts towards the glycolytic muscle phenotype.

Next, we questioned if this shift in the oxidative muscle proteome included specific elements of the metabolic machinery necessary for energy production. Analysis of quantitative proteomics data revealed that the expression pattern of targets related to macronutrient catabolism and energy provision in oxidative *Cpt2*$^{Sk-/-}$ muscle did not resemble that of the control oxidative muscle, but rather was remarkably similar to that of control glycolytic muscle (Figure 5G-J). Specifically, the relative abundance of several enzymes involved in the oxidative metabolism of glucose, lactate, pyruvate (Figure 5G), ketone bodies and amino acids (Figure 5H) in *Cpt2*$^{Sk-/-}$ oxidative muscle resembled glycolytic muscle. Likewise, several mitochondrial matrix dehydrogenases, TCA cycle enzymes (Figure 5I) and metabolite transporters (Figure 5J) in *Cpt2*$^{Sk-/-}$ soleus muscle, showed relative abundance levels comparable with glycolytic muscle. This data demonstrates that upon CPT2 deletion, muscles experience an oxidative-to-glycolytic proteome shift, specifically increasing the abundance of targets involved in glucose, ketones and amino acid catabolism.

Shifts in glycolytic metabolism of Cpt2Sk-/- oxidative muscle were not accompanied by shifts in myosin heavy chain

The structure and molecular composition of a muscle fiber is shaped by the type of mechanical work the muscle is required to perform (2, 35). As such, training and fitness regulate both mitochondrial content and specific sarcomere characteristics. One of the major determinants of fiber contractile capacity is myosin, wherein myosin heavy chain (MyHC) isoforms differentially distributed across fiber types allow for a straightforward distinction of the contractile properties of individual myofibers. Here, we performed immunohistochemical detection of MyHC isoforms in the EDL, TA, and soleus muscles to determine if a deficit in fatty acid oxidation and a shift in proteome markers from oxidative towards glycolytic would also result in changes in the expression of the contractile proteins. Despite the loss of mLCFAO and the proteomic shifts observed in *Cpt2*$^{Sk-/-}$ oxidative muscles, the relative composition of the MyHC isoforms was not different compared to control in EDL or Soleus muscles (Figure 6). These results show that loss of mLCFAO capacity in skeletal muscle via deletion of CPT2 does not trigger fiber type switching in the sense of changes in myosin heavy chain isoform composition.

mLCFAO deficiency causes metabolic reprogramming of mitochondria in oxidative muscles towards a glycolytic phenotype

Next, we wanted to determine how the intrinsic differences in the proteome of glycolytic and oxidative muscles would reflect the capacity to utilize different substrates for energy production. To test so, we performed high-resolution respirometry on isolated mitochondria from glycolytic and oxidative muscles under an array of energy substrates at rest and at high energy demand states. Respiration rates on all substrates tested, i.e. pyruvate, medium-chain octanoylcarnitine, long-chain palmitoylcarnitine, and succinate, which was administered along the ETC Complex I inhibitor rotenone (Rot) to capture complex II metabolism independent of complex I, were significantly lower in oxidative muscle compared to glycolytic of control mice at rest conditions (Figure 7A). These data suggests that mitochondria from oxidative muscle are intrinsically built to cover low energy demands with less respiratory effort than mitochondria from glycolytic muscle which could be related to the lower amount of ATP required to produce tension in slow twitch, oxidative myofibers (36). Comparison between respiratory rates across different substrates revealed that succinate was the top contributing fuel for both glycolytic and oxidative control muscles under resting conditions (Figure 7A).

It has been proposed that transitioning from rest state to contraction increases ATP consumption in muscle by ≈100 fold (1). Thus, mitochondria were challenged with increased energy demands by keeping phosphocreatine levels low which in turn decreases ATP/ADP ratio which drives mitochondrial ATP synthesis. Oxygen consumption (JO_2) in this condition was considered the maximum mitochondrial respiration rate (JO_2 Max Rate). In mitochondria from glycolytic control muscle, maximum respiration rates were at its highest when energized with succinate, followed by pyruvate and the medium- and long-chain acylcarnitines which elicited respiratory rates 74% and 77% lower than succinate respectively (Figure 7B). In mitochondria from oxidative control muscle, succinate-supported respiration also provided the highest maximum rate (Figure 7B) but respiration on pyruvate and on acylcarnitines were equal, and only 30% lower than that for succinate (Figure 7B). Taken together this data suggests that glycolytic muscles are primed to utilize succinate and pyruvate to support mitochondrial respiration while medium- and long-chain fatty acids contribute to a lesser extent to energy production. On the contrary, while succinate was also the major contributor to respiration in oxidative muscles, acylcarnitines elicit remarkably higher respiration rates, ~1.6-fold higher, compared to glycolytic mitochondria. Pyruvate and acylcarnitines respired at similar levels in oxidative muscle mitochondria but were still 30% lower than in glycolytic control muscle (Figure 7B). Comparison of mitochondrial respiratory rates from low to high energy demands with any given substrate demonstrated that oxidative control muscle mitochondria have a greater response to high energy demands compared to glycolytic muscle mitochondria as shown by the 5 to 7-fold increase in oxygen consumption when ATP free energy decreased (Figure 7C). Taken together these data suggest that while glycolytic muscles are better equipped to utilize substrates more directly linked to carbohydrates and proteins, oxidative muscle mitochondria have a higher reliance on fatty acids as energetic substrates. These data also suggest a more robust capacity of oxidative muscles to respond to increasing energy demands.

Next, we questioned if the glycolytic-like proteome shift would better position the *Cpt2*$^{Sk-/-}$ oxidative muscle to utilize non-fatty acid fuel sources. At low and high energy demands, mitochondria from glycolytic control and *Cpt2*$^{Sk-/-}$ muscle had a similar substrate-supported respiration pattern, suggesting that loss of CPT2 in glycolytic muscles did not impact glycolytic mitochondrial respiration (Figure 7D,G). However, significant differences between genotypes were demonstrated in oxidative muscle mitochondria, particularly in response to high energy demands. Specifically, compared to controls, KO oxidative muscle mitochondria had higher maximum respiration rates for pyruvate and succinate by 50% and 80%, respectively (Figure 7E, H). This change in pyruvate utilization represented an 18-fold increase in oxygen consumption going from low to high energy demands in the *Cpt2*$^{Sk-/-}$, compared to a 3-fold increase in control oxidative soleus mitochondria (Figure 7F). In agreement with the proteomics data, *Cpt2*$^{Sk-/-}$ mitochondria from oxidative muscle had nearly identical max respiration rates to mitochondria from glycolytic muscle for pyruvate and succinate (figure 7E). Taken together, these data suggest that in *Cpt2*$^{Sk-/-}$ mice, unlike in control, the mitochondria from oxidative and glycolytic muscles have a high degree of metabolic similarity. Specifically, in the absence of mLCFAO, oxidative muscle mitochondria increased reliance on pyruvate and succinate as energetic substrates, to levels comparable with glycolytic muscle.

Taken together these data suggests that when substrate-supported max respiratory capacity (JO2) and sensitivity are analyzed as a whole, the overall metabolic profile of *Cpt2*$^{Sk-/-}$ mitochondria isolated from oxidative muscle reflects that of glycolytic mitochondria. Thus, impairments in fatty acid oxidation will shift energy metabolism without changing myosin heavy chain structure thereby resulting in a metabolic-contractile mismatch. Ultimately, these data suggest that shifts in substrate-supported metabolism are not sufficient to drive corresponding shifts in the contractile apparatus.

Discussion

The net energy production upon oxidation of palmitate, a common and abundant dietary fatty acid, is roughly 129 ATP equivalents, whereas complete glucose oxidation yields approximately 32 ATP equivalents. Thus, on a purely molecule-to-molecule comparison fatty acids have far greater capacity than glucose to provide energy for cellular processes. The loss of mLCFAO capacity in tissues that are highly dependent on fatty acids as substrates, such as the heart and oxidative skeletal muscles, is bound to have large metabolic consequences. As such, the loss of CPT2 in the heart drives the reversion of mature adult cardiomyocytes into their fetal genetic program (9), in which mLCFAO is low and glucose oxidation predominates. In a similar fashion, the loss of CPT2 in skeletal muscle might be expected to drive a shift in myofibers that would favor utilization of non-lipid substrates for energy production.

In order to understand the impact of mLCFAO deficiency in different muscles, we characterized glycolytic and oxidative muscles separately for whole-tissue proteome and mitochondrial metabolic profiles, and as a result identified significant distinctions between these muscle types. Notably, our dataset showed that among the whole-tissue proteome, oxidative muscle had relatively lower contribution of mitochondrial proteins compared to glycolytic. This was surprising because traditionally, oxidative muscles are described as red-colored tissue, heavily loaded with mitochondria, in contrast to white, glycolytic muscles which are perceived to be lower in mitochondrial content (37). We report here that oxidative muscles had a more contraction-centered proteome, while in glycolytic muscles the most abundant proteins relative to whole-muscle were related to mitochondrial biogenesis and mitochondrial function. This is in agreement with glycolytic muscles producing high power contractions in a short period of time, which puts them at risk of achieving ATP consumption rates higher than regeneration rates (1). Therefore, glycolytic muscles utilize a combination of mitochondrial metabolism and glycolysis to meet ATP demands (1). Although our data does not discredit the mitochondrial abundance in oxidative muscles, it does question whether a mitocentric view of red muscles is appropriate.

Mitochondrial biogenesis is a natural response to energetic stress, thus the robust mitochondrial biogenesis that took place in *Cpt2*$^{Sk-/-}$ muscles, particularly those of an oxidative nature, was likely compensatory to maintain functional capacity in an energetically compromised condition. While mitochondrial biogenesis is generally accepted as a positive training-based adaptation for optimal performance, it is possible that the sheer overload of mitochondrial reticulum in *Cpt2*$^{Sk-/-}$ oxidative fibers could interfere with normal muscle function and structure. Mechanisms governing the response of the contractile apparatus in relation to the degree of mitochondrial content have not been extensively investigated. In addition to greater mitochondrial content in *Cpt2*$^{Sk-/-}$ muscles, further adaptive processes occurred including increased mitochondrial oxidative capacity for non-fatty acid substrates. Particularly, CPT2 deletion in oxidative muscles forced a shift in metabolism towards a glycolytic phenotype demonstrated by a higher contribution of mitochondria to whole-muscle proteome and a substrate-supported respiration that favored carbohydrates and amino acids for energy production to levels comparable with glycolytic muscles. This oxidative-to-glycolytic metabolic shift was also evident in the distinctive OXPHOS complex assembly pattern on native gels.

Despite the metabolic adaptation of oxidative muscle towards a glycolytic phenotype in *Cpt2*$^{Sk-/-}$ mice, we did not observe a matched conversion of MyHC isoforms suggesting that mLCFAO deficiency due to CPT2 deletion is not sufficient to induce a concerted metabolic and contractile fiber type switching. This is contrary to what has been reported when mLCFAO is increased in skeletal muscle by overexpressing CPT1 which results in glycolytic muscles increasing reliance on fatty acids as fuel along with a matched switch towards oxidative MyHC isoforms (38). Furthermore, numerous physiological and pathological conditions have been shown to result in adaptations of both metabolic and contractile facets in a seemingly synchronous manner. Specifically, exercise training, the overexpression or loss of PGC1alpha or PPARdelta, and defects in mitochondrial DNA are all reported to alter levels of oxidative metabolism in concert with changes in MyHC isoforms (39). Consequently, MyHCs are considered the best marker to characterize fiber types with the assumption that metabolism is matched with MyHC isoforms to provide optimal function (40). However, there are conditions where metabolism and contractile units are not changedin concert. Specifically, variables in exercise training modalities (41), atrophy (42), high fat diet feeding (42), and genetic predispositions that impact mitochondrial metabolism such as presented herein, result in mismatches between metabolism-contractile coupling.

A direct consequence of CPT2 deletion in skeletal muscle is the significant accumulation of long-chain acylcarnitines (LCACs) as we have reported before (43). There is speculation that LCAC accumulation interferes with the muscle bioenergetics by eliciting direct mitochondrial dysfunction, independent of mtFAO-derived energetic deficit (31-33). However, in the case of *Cpt2*$^{Sk-/-}$ mice, long-chain acylcarnitine accumulation did not result in discernable defects in mitochondrial metabolism. We conclude this because LCACs accumulate to a similar degree in *Cpt2*$^{Sk-/-}$ oxidative and glycolytic muscles, yet *Cpt2*$^{Sk-/-}$ and control glycolytic mitochondria were metabolically indistinguishable from one another.

In summary, we report here clear distinctions in the mitochondrial metabolic profile across a range of substrates and energy demands between oxidative and glycolytic muscles that condition the response to mLCFAO deficiency. While mitochondrial function itself did not appear to be overtly impaired upon CPT2 deletion, the metabolic rates, proteome, and OXPHOS complex assembly of *Cpt2*$^{Sk-/-}$ oxidative soleus muscle matched tightly to the ones of glycolytic muscle and strikingly mismatched with those of control soleus muscle. These changes in energy metabolism were not accompanied with the corresponding MyHC-type switch alerting that standard fiber-type assessment by MyHCs alone could mask unappreciated differential metabolic underpinnings. The consequences of a mismatch between energy metabolism and MyHC isoform expression for muscle physiology remain to be explored.

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Figure Legends:

Figure 1. Muscle fiber type composition is a major determinant in fatty-acid supported mitochondrial respiration. (A), (B), (C) Representative imaging of muscle fiber typing via Myosin Heavy Chain (MyHC) immunohistol in predominately glycolytic (EDL), mixed (TA), and oxidative (Soleus) muscles. Magnification 20X. Scale bar 100µm. (D) Mitochondrial respiration (JO_2) across a continuum of ATP free energy (ΔG_{ATP}) and (E) respiratory conductance in predominantly glycolytic, mixed and oxidative muscles energized with palmitoylcarnitine/malate (PC/M). (F) Heatmap of abundance of proteins involved in mitochondrial fatty acid oxidation in glycolytic (EDL) and oxidative (SOL) muscles. (G) Relative abundance of long-chain acylcarnitines (LCACs) in mixed TA muscle. (H) Lipid imaging of C16 acylcarnitine and (I) C18:2 acylcarnitine by NanoDESI in mixed TA muscle (J) immunostaining for myosin heavy chain type 2A (green) and dystrophin (white) and (K) for MyHC-2A (green), 2B (red) and dystrophin (yellow) on consecutive muscle section to (H) and (I). Data is presented as Mean±SEM. n=3-6 and $^*P \leq 0.05$ by 2-way ANOVA. For (F) $^*P \leq 0.05$ adjusted p-value calculated by Benjamini Hochberg FDR correction.

Figure 2. Loss of carnitine palmitoyltransferase 2 in skeletal muscle halts mLCFAO and induces mitochondrial biogenesis. (A) Western blot for CPT2 on glycolytic (EDL and White Quadriceps), mixed (TA) and oxidative (Soleus) muscle homogenates (representative image and quantitation)(B) Mitochondrial respiration (JO_2) at minimum (standing icon) and maximum (running icon) energy demands in predominantly glycolytic and oxidative muscles energized with palmitoylcarnitine/malate (PC/M). (C) Representative image of Gastrocnemius (GA), Tibialis Anterior (TA), Soleus (Sol) and Extensor Digitorium Longus muscles from control (top row) and *Cpt2*$^{Sk-/-}$ (bottom row) mice. (D) Quantitation of mitochondrial DNA (mtDNA) by levels of NADH Dehydrogenase 1 (Nd1), and Mito1 relative to nuclear DNA (nDNA) in different muscles of control and *Cpt2*$^{Sk-/-}$ mice. (E) Relative mRNA levels of PCG1α total, variant 2 (v2 and variant 3 (v3) in glycolytic and oxidative muscles from control and *Cpt2*$^{Sk-/-}$ mice. (F) Western blot for individual subunits of the mitochondrial OXPHOS complexes. (G) Enrichment of MitoCarta 3.0 positive proteins relative to the muscle total proteome as determined by quantitative proteomics in glycolytic (EDL) and oxidative (Soleus) muscle. (H) Differential expression of MitoCarta 3.0 positive proteins between control and *Cpt2*$^{Sk-/-}$ in glycolytic (EDL) and oxidative (SOL) muscles. Data is presented as Mean±SEM. For (B), (D) and (E), n=4-6 and $^*P \leq 0.05$ by 2-way ANOVA. For (G), n=6 and $^*P \leq 0.05$ by 1-way ANOVA

Figure 3. CPT2 deficiency triggers major mitochondrial response in a muscle type specific manner. (A), (B), (C), (D), (E), (F) Heatmaps of expression abundance of proteins related to electron transport chain complexes subunits and assembly factors in glycolytic (EDL) and oxidative (Sol) muscles of control and *Cpt2*$^{Sk-/-}$ mice. (G), (H) Abundance of proteins involved in mitochondrial processes in control and *Cpt2*$^{Sk-/-}$ oxidative muscle. Data is presented as Mean±SEM. n=6 and $^*P \leq 0.05$ adjusted p-value calculated by Benjamini Hochberg FDR correction.

Figure 4. Differential muscle mitochondrial biogenesis in *Cpt2*$^{Sk-/-}$ mice is fiber type-specific. (A_1 to A_4) Representative Gomorii staining of mitochondria (red) in muscle fibers (light blue) of glycolytic (EDL) and oxidative (Soleus) muscles for both genotypes. Far-right column contains magnified images of A_2 and A_4 (B) Myosin heavy chain immunodetection (right)

matched to Gomorii stain (left) in same region of *Cpt2*$^{Sk-/-}$ soleus muscle. Orange triangle= MyHC type I positive fibers with severe mitochondrial biogenesis; White star= MyHC-type IIa fibers with moderate mitochondrial biogenesis; and Purple star= MyHC-2X (type IIx) positive fibers with not visible mitochondrial biogenesis. Data presented here are representative images of total n=3.

Figure 5. Loss of CPT2 shifts oxidative muscle proteome towards a glycolytic-like protein signature. (A) Representative image of Native-PAGE of mitochondria lysates to demonstrate OXPHOS complexes in control and *Cpt2*$^{Sk-/-}$ muscles. (B) Venn diagram demonstrating overlap among the top 500 most abundant proteins in glycolytic (EDL) and oxidative (Soleus) muscle from control and *Cpt2*$^{Sk-/-}$ mice. (C), (D), (E), (F) Pathway analysis for the top 500 most abundant proteins arranged by *-log p value*. (G), (H), (I), (J) Heatmaps of abundance of proteins relative to the tissue mean related to macronutrient catabolism and metabolite transport in glycolytic (EDL) and oxidative (Sol) muscles of control and *Cpt2*$^{Sk-/-}$ mice. N=6 and *P≤0.05 adjusted p-value calculated by Benjamini Hochberg FDR correction.

Figure 6. CPT2 deficiency does not induce myosin heavy chain isoform switching.
(A) Representative images and (B) quantification of control and *Cpt2*$^{Sk-/-}$ muscles by immunofluorescent detection of myosin heavy chain isoforms: MyHC-β (Type I, blue), MyHC-2B (Type IIb, red), MyHC-2A (Type IIa, green) or no stain (Type IIx, black). Myofiber perimeter is demonstrated with dystrophin (yellow and pink). EDL, extensor digitorum longus. Data is presented as Mean±SEM. N=3.

Figure 7. Bioenergetic adaptations in *Cpt2*$^{Sk-/-}$ oxidative muscles result in a glycolytic metabolic phenotype.
(A) Maximum rates of substrate-supported oxygen consumption (JO_2) in isolated mitochondria from control glycolytic and oxidative muscles under low energy demands; PC=palmitoylcarnitine, OCT=octanoylcarnitine, PYR=pyruvate, S=succinate, M=malate and ROT=rotenone. (B) same as A but under high energy demands. (C) Oxygen consumption fold-change from low to high energy demands in control glycolytic and oxidative muscle mitochondria. (D) Same as A for mitochondria isolated from *Cpt2*$^{Sk-/-}$ glycolytic and oxidative muscles. (E) Same as D under high energy demands. (F) Same as C for *Cpt2*$^{Sk-/-}$ mitochondria. (G) Comparisson of mitochondrial oxygen consumption rates at low energy demands between genotypes for glycolytic and oxidative muscles presented as *Cpt2*$^{Sk-/-}$ percentage change from control. (H) Same as G under high energy demands. Data is presented as Mean±SEM. N=3-6 For (A), (B), (D) and (E) *P≤0.05 by T-test between glycolytic and oxidative muscle mitochondria for each substrate. For (G) and (H) *P≤0.05 by T-test between control and *Cpt2*$^{Sk-/-}$ muscle mitochondria for each substrate.

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43. !!! INVALID CITATION !!! (5).