Investigation of ATP Citrate Lyase as a regulator of skeletal muscle nutrient metabolism

by

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Abstract

As global pressures for livestock production grow, establishing innovative ways to improve livestock growth efficiency is imperative to meet the growing global demand for meat. Understanding mechanisms regulating skeletal muscle growth in livestock holds potential to accomplish this feat. This work aims to uncover mechanisms that modulate skeletal muscle metabolism to exploit during postnatal muscle growth of livestock animals. The enzyme ATP Citrate Lyase (ACLY) has been identified as a metabolic regulator in other cell types but has largely been unexplored in skeletal muscle. Our hypothesis is that ACLY dictates nutrient utilization and drives a unique metabolic fingerprint in skeletal muscle. Using an *in vitro* system, C2C12 myoblasts were transfected with GFP (control) or ACLY plasmids to investigate nutrient utilization in ACLY overexpressing myoblasts. Transfected cells were pulsed with $^{13}C_6$-glucose or $^{13}C_{16}$-palmitate to identify ACLY-mediated patterns of glucose utilization and nutrient preference. Myoblasts and culture media were collected and analyzed using gas chromatography-mass spectrometry. Glucose-pulsed myoblasts overexpressing ACLY exhibit a decrease in isotopic enrichment of the glycolytic substrate lactate and of TCA cycle intermediates oxaloacetate, citrate, succinate, α-ketoglutarate, and fumarate compared to controls ($P<0.05$). However, glucose uptake and isotopic enrichment of pyruvate were similar in ACLY myoblasts compared to control myoblasts ($P>0.05$). ACLY-overexpressing palmitate-pulsed myoblasts had decreased isotopic enrichment in lactate, oxaloacetate, citrate, succinate, α-ketoglutarate, and fumarate ($P<0.05$) with no change in glucose uptake or isotopic enrichment of pyruvate ($P>0.05$). These data depict an ACLY-mediated mechanism that regulates nutrient flux through glycolysis and the TCA cycle in skeletal muscle cells without a nutrient shift towards palmitate. Although additional investigation is needed to elucidate this mechanism, ACLY appears to alter

substrate allocation and utilization between the two major metabolic pathways and warrants further exploration as a target to improve economic nutrient utilization in growing muscle.

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Dedication

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Chapter 1 - Literature Review

Introduction

Current projections anticipate a global population increase of 1.5 billion people over the next 25 years (United Nations, 2024), increasing the demand for beef, pork and poultry meat and decreasing acreage of arable land available for food production (Dohlman et al., 2025). To meet this rise in global meat consumption with fewer inputs, livestock producers must utilize innovative strategies to increase production efficiency. Skeletal muscle, the tissue converted into meat postmortem, uptakes up to 80% of ingested glucose (Merz & Thurmond, 2020). Skeletal muscle metabolism impacts overall efficiency performance of livestock (Fu et al., 2017), making muscle an important target for efficiency improvements to meet consumer demand. A deeper understanding of the mechanisms regulating muscle cell physiology could elucidate cellular intervention points to improve production efficiency.

Skeletal muscle is a post-mitotic tissue consisting of many individual muscle cells, myofibers, which can be generally characterized by speed of contraction and type of inherent metabolism. Slow contracting fibers, also known as slow-twitch fibers, have a greater reliance on oxidative pathways whereas fast-twitch fibers can range from oxidative to glycolytic metabolism (Bourdeau Julien et al., 2018). Postnatal muscle growth occurs via myofibrillar hypertrophy (Felicioni et al., 2020). Efficient hypertrophy of skeletal muscle is critical to the growth of livestock animals and their use as a food source. From a livestock production standpoint, a major driver of growth efficiency is how muscle uptakes and utilizes nutrients. For example, an increase in oxidative enzymes is associated with a decrease in feed efficiency (FE) (Fu et al., 2017). Contrarily, greater glycolytic enzyme content is associated with improved

FE (Fu et al., 2017; Le Naou et al., 2012). However, the regulatory processes that modulate metabolic phenotype and efficient lean accretion remain vague.

While pathways that drive specific metabolic adaptations in response to nutritional changes have been elucidated, such as but not limited to nutrient sensing post-translational medications, signals of energy status, and insulin sensitivity, a regulatory point that dictates the interplay between the two major metabolic pathways in skeletal muscle has not been defined. Given the impact the metabolic phenotype has on efficient nutrient utilization, a deeper understanding of the regulatory processes that modulate cellular nutrient utilization between glycolytic and oxidative metabolic processes is important. One promising enzyme that has been established as a regulator of metabolism in cancer cells is ATP citrate lyase (ACLY), which catalyzes a reaction that generates acetyl-CoA from mitochondrial citrate to be used for epigenetic regulation and fatty acid synthesis. In skeletal muscle, Das et al. (2015) demonstrated that ACLY promotes muscle hypertrophy, but a fundamental gap in our knowledge of how ACLY regulates nutrient utilization and modulates gene expression remains. The objective of this literature review is to summarize existing literature on ACLY as a metabolic regulator and define the knowledge gap in its role in the biochemistry of skeletal muscle growth.

Glycolysis

Glycolysis is a central pathway in the cellular metabolism of living organisms, beginning with one molecule of 6-carbon glucose which is converted into two molecules of 3-carbon pyruvate through a series of ten reactions. In this series of stepwise reactions, two adenosine triphosphate (ATP) and two nicotinamide adenine dinucleotide (NAD^+) molecules are used to net yield two ATP and two nicotinamide adenine dinucleotide + hydrogen (NADH) molecules. The pentose phosphate pathway (PPP), hexosamine biosynthesis pathway (HBP), and serine

biosynthesis pathway (SBP) are anabolic pathways that branch off of glycolysis using glycolytic intermediates (Chandel, 2021a). These pathways are all avenues that cells can employ to divert intermediates from catabolic to anabolic processes, which are often upregulated in highly aggressive malignancies depicting their capacity to support unfettered rates of biosynthesis (Tong et al., 2009).

Glycolytic intermediates can enter the PPP following the conversion of glucose into glucose-6-phosphate (G6P) to generate 5-carbon precursors for nucleic acid synthesis and the reducing equivalent NADPH to support several biosynthetic processes and to combat oxidative stress. Alternatively, in glycolysis, G6P can be converted to fructose-6-phosphate (F6P) which can then enter the HBP for nutrient sensing (Chandel, 2021a; Hussain, 1998). Another glycolytic substrate, 3-phosphoglycerate, serves as an entry point to the SBP which generates serine for protein synthesis (Mantyselka et al., 2024). Glycolysis provides energy in the form of ATP, precursors for biosynthetic processes, and substrates to fuel mitochondrial metabolism, all of which are crucial for skeletal muscle physiology and growth (Chandel, 2021a).

High glycolytic flux, termed the Warburg effect, has been demonstrated to support cancer growth through accommodating energy demands while providing precursors for biosynthetic processes. This effective use of substrates is a hallmark characteristic of malignant cell types to support efficient growth (Faubert et al., 2013). For example, glycolysis starts with 6 carbons and results in two 3 carbon molecules and a net of 2 ATP molecules, thus carbons in glycolysis are conserved while generating ATP. In fact, glycolysis has the capacity to accommodate high energy demands, albeit for a short period of time. Contrarily, subsequent oxidative steps in the tricarboxylic acid (TCA) cycle result in the loss of carbons as CO_2. Preservation of these carbon

backbones facilitates greater nutrient utilization efficiency, which is necessary for economic muscle hypertrophy (Bogorad et al., 2013).

The final glycolytic end-product, pyruvate, can be converted to lactate through lactate dehydrogenase alpha (LDHα). This reaction regenerates NAD^+ to support sustained glycolytic flux (Spriet et al., 2000). Lactate can be shuttled out of skeletal muscle to be used for gluconeogenesis in the liver, termed the Cori cycle. Alternatively, lactate can serve as a substrate for metabolic pathways within skeletal muscle including the TCA cycle (Zhang et al., 2024). The indirect use of lactate in the TCA cycle begins with the translocation to the mitochondrial intermembrane space and subsequent conversion to pyruvate via LDHβ (Hashimoto et al., 2006). Alternatively, pyruvate can be shuttled into the mitochondrial matrix through the mitochondrial pyruvate carrier to be used in the TCA cycle (Bricker et al., 2012). In these ways, pyruvate and lactate are central to glycolysis and serve as a connection to other metabolic pathways.

Mitochondrial metabolism

Mitochondrial pyruvate can be converted to acetyl-CoA, which can then enter the TCA cycle through the mitochondrial pyruvate dehydrogenase (PDH) complex (Saunier et al., 2016) or pyruvate carboxylase (PC). Although pyruvate largely enters the TCA cycle through PDH resulting in acetyl-CoA and CO_2, PC serves as an alternative entry point that sequesters a carbon molecule from sodium bicarbonate to synthesize oxaloacetate (OAA) (Patel et al., 2012; Stacpoole, 2017). In addition to intermediates of carbohydrate metabolism entering the TCA cycle, fatty acid and amino acids can also be used. Skeletal muscle cells are metabolically plastic, characterized by the ability to shift and preferentially utilize nutrients based upon what is available (Christoffolete et al., 2015). For example, the fatty acid palmitate can be catabolized via β-oxidation to produce acetyl-CoA for TCA oxidation and can be used in preference to

carbohydrates when available (Baugh et al., 2020; Turcotte et al., 1994). When muscle cells lose the ability to switch between fuel sources, this initiates a state of metabolic disease (Stefanik et al., 2024). This state is evidenced by livestock between feeding periods where blood glucose declines and muscle cells must rely on other fuel sources (Ravelo et al., 2025).

The TCA cycle meets cellular energy demands by oxidizing substrates and generating reducing equivalents NADH and flavin adenine dinucleotide ($FADH_2$) as well as CO_2 and ATP. The loss of carbons as CO_2 is counterproductive for their utilization in biosynthetic processes. This is a concern in regard to metabolic efficiency in meat producing animals (Bogorad et al., 2013). The TCA cycle, being amphibolic, meets cellular energy demand through catabolism and supports biosynthesis through its intermediates, serving various needs of the cell and organism (Arnold & Finley, 2023).

In addition to TCA cycle intermediates playing a critical role in anabolic and catabolic processes, these intermediates can also be used in metabolic signaling pathways. For example, citrate can be exported from mitochondria by the mitochondrial citrate carrier to the cytosol where ATP citrate lyase (ACLY) converts cytosolic citrate into acetyl-CoA and OAA which are then used in many biological processes (Das et al., 2015; Zara et al., 2022). Citrate export is influenced by both insulin and nutritional state (Zara et al., 2022). Acetyl-CoA has roles in histone acetylation, a mechanism of epigenetic regulation, and in fatty acid synthesis (Das et al., 2015), while OAA is used in other anabolic pathways or recycled into the TCA cycle (Fink et al., 2025). Through these molecules, ACLY can impact cellular pathways beyond the TCA cycle including biosynthetic and transcriptional pathways.

Perhaps the largest role of the TCA cycle is to generate reducing equivalents for the electron transport chain (ETC), whose complexes are embedded within the inner mitochondrial

membrane (Arnold & Finley, 2023). The ETC consists of a series of complexes and generates the membrane potential between the inner membrane space and matrix of mitochondria. These 5 ETC complexes generate and sustain mitochondrial membrane potential by pumping protons across the inner membrane into the inner membrane space. Complex V then can harbor this electron gradient to phosphorylate ADP into ATP (Arnold & Finley, 2023). This process, known as oxidative phosphorylation, relies on oxygen as the final electron acceptor to generate water. This process is essential to cellular energy generation and relies upon reducing equivalents derived from the TCA cycle.

Unique profiles of metabolic enzymes exist between pigs characterized with high- compared to low-FE. Specifically, high-FE pigs down-regulated proteins in the TCA cycle and oxidative phosphorylation as compared to low-FE, suggesting these processes are associated with decreased efficiency (Dorji et al., 2021; Fu et al., 2017). As these processes are major producers of ATP, some suggest high-FE pigs produce lower levels of ATP with this alternate metabolism. High-FE pigs notably upregulated glycolytic proteins (Fu et al., 2017). Low-FE animals have been associated with greater mitochondrial uncoupling and lower substrate oxidation, exhibiting decreased efficiency of oxidative metabolism (Bottje et al., 2006; Toyomizu et al., 2011). This reduces efficiency of ATP synthesis on a cellular level and could contribute to their overall decreased efficiency (Bottje et al., 2006). These distinct metabolic traits indicate efficiency differences are correlated with a heavier reliance upon glycolytic metabolism as opposed to the TCA cycle.

Biosynthetic pathways

Throughout the series of enzymatic reactions of glycolysis, TCA, and oxidative phosphorylation are branching biosynthetic pathways that represent avenues of potential carbon

sparing for efficient lean accretion (Mantyselka et al., 2024). The PPP stems from G6P in glycolysis and serves several cellular functions, producing carbon backbones, the nucleotide precursor ribose-5-phosphate, and nicotinamide adenine dinucleotide phosphate (NADPH), a versatile molecule essential to many cellular processes including synthesis of amino and fatty acids and serving as an antioxidant (Chandel, 2021b; TeSlaa et al., 2023). This pathway is essential for skeletal muscle hypertrophy and regeneration. For example, genes associated with the PPP were upregulated to support induced hypertrophy (Valentino et al., 2021).

The Serine Biosynthesis Pathway (SBP) is essential to muscle hypertrophy (Baumert et al., 2024) and begins with 3-phosphoglycerate, a glycolytic intermediate. As its name suggests, the SBP is responsible for synthesizing the amino acid serine. In turn, serine is used to synthesize other amino acids including glycine and cysteine which are essential to hypertrophy. Inhibiting the SBP is demonstrated to impair skeletal muscle protein synthesis and upregulate glucose metabolism, although this is upregulation is not enough to rescue hypertrophy (Mantyselka et al., 2024). These findings demonstrate the SBP is essential to skeletal muscle metabolism and myogenesis.

The Hexosamine Biosynthesis Pathway (HBP) begins with glucose-derived F6P and produces substrates including UDP-N-acetylglucosamine (UDP-GlcNAc) for glycosylation and O-GlcNAcylation (Hussain, 1998). Glycosylation holds the potential to allow the translocation of GLUT4 to the cell membrane. The GLUT4 transporter is responsible for glucose transport into skeletal muscle where it can be used as an energy source in glycolysis. Glycosylation also initiates signaling cascades through protein kinase C activation (Chaveroux et al., 2016; Hussain, 1998). O-GlcNAcylation serves as a nutrient sensing pathway and can modulate metabolism in skeletal muscle by affecting glucose uptake and shifting nutrients towards the PPP (Hawkins et

al., 1997; Rao et al., 2015). The HBP can be activated in times of nutrient shortages and holds the potential to regulate glucose metabolism while connecting glucose and fatty acid metabolism to insulin (Chaveroux et al., 2016; Hussain, 1998).

Acetyl-CoA is a metabolite central to not only the TCA cycle but also fatty acid synthesis within the mitochondrial matrix and the cytosol, essential to cellular functionality (Wedan et al., 2024). Derived from the ACLY-mediated catalysis of citrate, acetyl-CoA molecules are converted to malonyl-CoA and condensed into fatty acids, which are components of lipids and cell membranes and are vital to cell signaling and storage (de Carvalho & Caramujo, 2018; Du et al., 2022). These fatty acids can also be used for ATP synthesis via β-oxidation, providing an energy source alternative to glucose and altering skeletal muscle metabolism in times of energy demand. Fatty acids can also be stored in skeletal muscle, saving nutrients for future use and altering glucose utilization (Miranda et al., 2011).

These biosynthetic pathways are critical to skeletal muscle function and growth. They hold the ability to regulate nutrient usage, alter metabolism, synthesize biomolecules, and produce signaling molecules (de Carvalho & Caramujo, 2018; Hussain, 1998; Miranda et al., 2011). Improper function of any of these pathways is detrimental to normal skeletal muscle functions, including hypertrophy (Mantyselka et al., 2024).

Metabolic regulation

Skeletal muscle is a complex, adaptable tissue reliant upon many mechanisms of regulation (Zumbaugh et al., 2022). Skeletal muscle metabolites are regulated by metabolic gene expression. For example, Fu et al. (2015) identified more efficient pigs differed in their metabolic transcriptome and proteome as compared to less efficient pigs, suggesting the metabolic profile of muscle differs in high- and low-FE animals. Metabolism can also be

regulated by the expression of metabolic proteins. For example, skeletal muscle enzymes such as ACLY can alter genome accessibility to transcription machinery, altering the expression of proteins (Das et al., 2017). The pathways and substrates within skeletal muscle metabolism are tightly controlled by many levels of regulation.

Altering levels of available metabolites has been associated with changes in other substrates not just downstream but across both catabolic and anabolic pathways and even throughout signaling cascades. For example, increasing pyruvate available to porcine muscle has been shown to alter the overall metabolic state by increasing TCA intermediate abundances, providing various substrates for biosynthetic pathways and phosphorylating metabolic enzymes (Ledee et al., 2015). The addition of α-ketoglutarate to the diet stimulates amino acid synthesis (Chen et al., 2018). While the mechanism needs further investigation, metabolites such as α-ketoglutarate and pyruvate have strong roles in regulating muscle and its hypertrophy.

ATP Citrate Lyase

Beyond its role in citrate catalysis, ACLY has been established as a regulator of mitochondrial function, stimulating production of cardiolipin and thus the formation of mitochondrial supercomplexes for ATP production (Das et al., 2015). ACLY-mediated citrate catalysis produces acetyl-CoA. These acetyl groups, when bound to histone tails, loosen DNA-histone interactions for transcription machinery access to DNA, promoting transcription (Reinke & Horz, 2003). Histone acetylation is a mechanism of regulation controlling gene expression including that of muscle specific genes. For example, myogenic regulatory factors, specifically the transcription factors that regulate muscle differentiation and regeneration including myogenic differentiation 1 (MYOD), are increased by acetylation (Sincennes et al., 2016). Through

acetylation of the MYOD locus, myogenesis is improved with ACLY upregulation. Conversely, inhibiting ACLY inhibits differentiation in skeletal muscle (Das et al., 2017).

In other cell types, through its contributions to histone acetylation, ACLY has been identified as a regulator of many cell functions including vascular remodeling, immune cell function, stem cell replication, and bone marrow regeneration, to name a few (Grobs et al., 2024; Hochrein et al., 2022; Umemoto et al., 2022). ACLY upregulation is characteristic of cancer cell physiology, and its inhibition is linked to inhibition of tumor growth specifically by inducing senescence and apoptosis. ACLY regulates cell fate in this instance by physically interacting with signaling molecule AMP-activated protein kinase (AMPK), not just through facilitating histone acetylation (Lee et al., 2015). In these ways, ACLY acts as a regulator of cell fates and functions, both through histone acetylation and enzymatic activity.

Conclusion

As global pressures for livestock production rise, innovative strategies to improve our production systems are now more crucial than ever. Understanding what drives efficient skeletal muscle hypertrophy holds promise for achieving this goal. Recent research has shown that both glycolysis and biosynthetic pathways have the potential to improve efficiency and justify further research. The manipulation of specific metabolites within these and other, less efficient pathways has helped to determine their downstream effects and potential points for improvement and intervention.

The intricacies of skeletal muscle metabolism make mechanistic research difficult, but strides are being made to characterize the effects of metabolites and their regulation. While associated with efficiency and facilitating intense hypertrophy, there is still a fundamental gap in knowledge of how ACLY regulates nutrient utilization and modulates gene expression in order

to support these alterations. We hypothesize ACLY is a master regulator of cellular metabolism, altering nutrient usage as a means to facilitate improved cellular efficiency. The purpose of this work is to investigate the role of ACLY in altering cellular nutrient preference and utilization in skeletal muscle cells.

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Chapter 2 - Investigation of ATP Citrate Lyase as a regulator of skeletal muscle nutrient metabolism

1. Introduction

Meeting the rising production challenges begins with understanding the cellular mechanisms regulating livestock growth efficiency. Knowing the metabolic characteristics of muscle can drive growth efficiency (Fu et al., 2017), these characteristics become of keen interest when evaluating potential avenues for efficiency improvement. Metabolic pathways such as glycolysis and the TCA cycle are essential to muscle functionality (Mantyselka et al., 2024). As the cellular utilization of glucose is correlated to efficiency (Faubert et al., 2013), investigating the regulatory mechanisms that dictate metabolic traits of skeletal muscle could lead to innovative strategies to improve livestock efficiency.

One such regulatory enzyme is ATP citrate lyase (ACLY). Once citrate is shuttled out of mitochondria via the mitochondrial citrate carrier, ACLY catalyzes the breakdown of cytosolic citrate to oxaloacetate (OAA) and acetyl-CoA (Das et al., 2015). OAA can be recycled in the TCA cycle or serve as a precursor for amino acid synthesis (Fink et al., 2025). Acetyl-CoA can be used for fatty acid synthesis or histone acetylation (Das et al., 2015). Acetyl groups from acetyl-CoA bind to histone tails, allowing transcription machinery to more readily access the genome and allowing for altered gene expression (Reinke & Horz, 2003).

Of great interest in regards to efficient nutrient utilization, ACLY has been identified as a regulator of metabolism in other cell types (Das et al., 2015; Li et al., 2023; Sohn et al., 2025; Zhao et al., 2016). In fact, overexpression of ACLY results in a bout of skeletal muscle hypertrophy *in vivo* (Das et al., 2017). However, the mechanism by which ACLY modulates metabolism and a hypertrophic response are yet to be identified. Upregulation of these pathways

holds potential for improved muscle growth. Whether ACLY is regulating cellular functions to improve efficiency in skeletal muscle and whether it can alter the utilization of nutrients to achieve this, remains unknown. Regardless, manipulating skeletal muscle metabolism has implications on animal growth.

The advantage of glycolysis, from a growth standpoint, lies in its carbon conservation (Bogorad et al., 2013). Throughout this metabolic pathway no carbons are lost and two 3-carbon pyruvate molecules emerge (Spriet et al., 2000). Alternatively, the TCA cycle loses carbons as waste products, which are counterproductive to anabolic processes essential for myogenesis and growth (Bogorad et al., 2013). To determine if ACLY modulates nutrient utilization between these two major metabolic pathways, ACLY was overexpressed in C2C12 myoblasts and pulsed with $^{13}C_6$-glucose or $^{13}C_{16}$-palmitate to evaluate patterns of ^{13}C labeling in glycolytic and TCA cycle intermediates.

2. Materials and Methods

2.1 Cell culture

C2C12 myoblasts (CRL 1772, ATCC) were cultured in growth medium (GM) containing Dulbecco's Modified Eagle's Medium (DMEM) (#25-500, GenClone, Genessee Scientific, El Cajon, CA, USA), 1% Penicillin/Streptomycin (#25-512, GenClone, Genessee Scientific, El Cajon, CA, USA), 10% Fetal Bovine Serum (#25-550H, GenClone, Genessee Scientific, El Cajon, CA, USA), 1:500 Normocin (NC9273499, Invivogen, ThermoFisher Scientific, Waltham, MA, USA), and 1:200 Gentamicin (G1272, Sigma-Aldrich, Saint Louis, MO, USA). Cells were incubated at 37ºC and 5% CO_2 and plated to seeding density of approximately 16,500 cells per cm^2 using a Countess 3 cell counter (ThermoFisher Scientific, Waltham, MA, USA). Cells were allowed to proliferate for 24 hrs then transfected with JetOPTIMUS transfection reagent (#55-

250, GenClone, Genessee Scientific, El Cajon, CA, USA). Each 10 cm plate was transfected with17.36 µg of plasmid DNA harboring either *M. musculus* ATP citrate lyase (ACLY) (#70765, Addgene, Watertown, MA, USA) or green fluorescent protein (GFP, control) (#60360, Addgene, Watertown, MA, USA) for 48 hrs. After 24 hrs, mammalian selection marker of either 10 µg/mL blasticidin (A1113903, Gibco, ThermoFisher Scientific, Waltham, MA, USA) for ACLY transfected cells or 500 µg/mL neomycin (N1142, ThermoFisher Scientific, Waltham, MA, USA) for GFP was used. Transfected plates were then used for subsequent analysis. Each experiement was conducted on 6 – 8 individually transfected plates (n = 6-8).

2.2 Western blot

After transfection, cells were collected using IntactProtein Cell-Tissue Lysis Kit (415S, GenuIN Biotech, Henan, China). Protein concentration was quantified using Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (23225, Pierce, ThermoFisher Scientific, Waltham, MA, USA). Samples were normalized to 1.9 µg/µL of protein in Laemmli Sample Buffer containing 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 0.005% bromophenol blue, and 5% β-mercaptoethanol. Lysates were loaded into a 4-14% Bis-Tris gel for SDS-PAGE and transferred onto PVDF membranes (IB34001, ThermoFisher Scientific, Waltham, MA, USA). Membranes were blocked with Oneblock fluorescent blocking buffer (20-314, Prometheus, Genessee Scientific, El Cajon, CA, USA) for 1 hr and incubated with 1:200 an anti-ACLY primary antibody (NBP1-90266, Novus Biologicals, Biotechne, Minneapolis, MN, USA) overnight. The next day, membranes were incubated with 1:5,000 Alexa Fluor 647 secondary antibody (A32733TR, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA) for 1 hr. Blots were imaged on an iBright imaging system (ThermoFisher Scientific, Waltham, MA, USA).

Total protein was quantified using No-Stain Protein Labeling Reagent (A44717, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA).

2.3 Metabolite concentration

Glucose concentrations of media after 24 hr glucose and palmitate pulses were quantified using glucose standards to calculate a standard curve. Samples and standards were diluted 1:2 and 1:1, respectively, in water. Buffer consisting of 6.25% triethanolamine, 12.5% 200 mM EDTA pH 7.4, 3.5% 1M $MgCl_2$, and 0.93 mg/mL NADP was added to diluted samples and standards. After a baseline reading at 340 nm, 35 µL of 1:10 glucose-6-phosphate dehydrogenase (10165875001, Sigma-Aldrich, Saint Louis, MO, USA) were added to samples and standards, incubated for 20 min at room temperature, and read again to determine glucose-6-phosphate concentration. Next, 21 µL of 1:10 hexokinase (11426362001, Sigma-Aldrich, Saint Louis, MO, USA) and 40 µL of 11 mg/mL Na-ATP (A2383, Sigma-Aldrich, Saint Louis, MO, USA) were added to samples and standards, incubated for 20 min at room temperature, and measured at 340nm using spectrophotometry to determine glucose concentrations using a standard curve. Glucose concentrations were normalized to total protein per lysate sample using the Pierce BCA Protein Assay Kit.

2.4 Metabolite tracing

After transfection, cells were pulsed with media containing no glucose DMEM (#11966025, Gibco, ThermoFisher Scientific, Waltham, MA, USA), 1% Penicillin/Streptomycin, 10% Fetal Bovine Serum, 1:500 Normocin, 1:200 Gentamicin, and 4.5g/L $^{13}C_6$-glucose (CLM-1396-0.5, Cambridge Isotopes Laboratories, Tewksbury, MA, USA) or 10mM $^{13}C_{16}$-palmitic acid (CLM-409-0.1, Cambridge Isotopes Laboratories, Tewksbury, MA, USA) for 24 hrs. After the

pulse, media was collected into a microcentrifuge tube and 2 M perchloric acid was added. Cells were washed with PBS and 2 M perchloric acid added to each well. Cells were scraped and collected into a mirocentrifuge tube. Cells and media were then incubated over ice for 15 min and centrifuged at 12,000 rpm for 5 min. Finally, the supernatants were transferred into 3M potassium hydroxide and frozen at -80ºC. Samples were then thawed and 5M hydroxylamine hydrochloride and 4M potassium hydroxide were added. Samples were then sonicated for 60 minutes at 65ºC and acidified with 6N hydrochloric acid. Saturating amounts of sodium chloride were then added and samples were extracted with ethyl acetate, shaken, centrifuged at 2,000 g for 5 min and the organic layer was separated and evaporated under nitrogen gas. The extraction process was then repeated with the samples and the organic layer was evaporated to dryness. 1:1 *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) and ethyl acetate were added to the evaporated vials which were then capped and heated for 1 hr at 65ºC. After heating, samples were transferred to inserts for gas chromatography-mass spectrometry (GC-MS) analysis (Agilent 6890N, Agilent Technologies, Santa Clara, CA, USA) as described by (Matarneh et al., 2021) with J&W HP-5ms GC column (30m, 0.25mm, 0.25 µm film, Agilent Technologies). Mass spectrophotometry (Agilent 5973N; Agilent, Santa Clara, CA, USA) was used to evaluate intermediate ions on mass-to-charge ratio (*m/z*). Pyruvate (274.10), lactate (261.10), succinate (289.10), fumarate (287.10), malate (419.20), oxaloacetate (432.10), α-ketoglutarate (446.20), and citrate (591.40) were among those investigated. The mole percent excess (MPE) was determined ((M + i/M + 0) × 100). M + i was defined as the molecule's isotopic enrichment.

2.5 Statistics

Data were analyzed as a completely randomized design comparing control to treatment using R (version 4.4.1, Indianapolis, IN, USA). Data were considered statistically significant when $p \leq 0.05$ and are presented as least square means +/- standard error. Means were separated using pairwise comparisons.

3. Results

3.1 Confirmation of ACLY Protein Overexpression

Plasmids overexressing GFP (control) or ACLY were used to transfect C2C12 myoblasts (Figure 1A). Confirmation of ACLY overexpression was confirmed using Western blotting. Protein abundance of ACLY increased 3-fold in ACLY compared to control myoblasts (Figure 1B, $p < 0.05$), confirming successful overexpression.

3.2 Glucose Pulse

To evaluate glucose uptake and utilization in transfected myoblasts, cultures were pulsed with $^{13}C_6$-glucose for 24 hrs and then collected for analysis. As ^{13}C are metabolized, their incorporation patterns show metabolite utilization which are detected using GC-MS (Figure 2). For example, Lactate M + 3 nomenclature denotes all 3 carbons in the lactate molecule are derived from the initial labeled substrate pulse of $^{13}C_6$-glucose. The isotopomer enrichment patterns provide insight into the proportions of Lactate M + 3 and other isotopomers.

Glucose uptake was measured following the 24 hr glucose pulse. Glucose uptake did not differ between ACLY overexpressing myoblasts and control myoblasts (Figure 3A, $p > 0.05$). Lactate M + 3 decreased in media of ACLY overexpressing myoblasts compared to controls (Figure 3B, $p < 0.05$).

Isotopic enrichment of lactate M + 3 decreased in ACLY overexpressing myoblasts compared to controls (Figure 4A, $p < 0.05$); however, pyruvate M + 3 did not differ between control and ACLY myoblasts (Figure 4B, $p < 0.05$). Further, citrate M + 1, M + 2, M + 3, M + 4, M + 5, and M + 6 decreased in ACLY overexpressing myoblasts compared to controls (Figure 4D, $p < 0.05$). Oxaloacetate M + 2, M + 3, and M + 4 labeling were lower in ACLY compared to control myoblasts (Figure 4C, $p < 0.05$). Citrate M + 1, M + 2, M + 3, M + 4, M + 5, and M + 6 labeling were lower in ACLY (Figure 4D, $p < 0.05$). Succinate M + 4 labeling was lower in ACLY (Figure 4E, $p < 0.05$). α-ketoglurarate labeling schemes M + 1, M + 2, M + 3, M + 4, and M + 5 were lower in ACLY (Figure 4F, $p < 0.05$). Fumarate M + 2, M + 3 and M + 4 were lower for ACLY cells (Figure 4G, $p < 0.05$).

3.3 Palmitate Pulse

Myoblasts pulsed with $^{13}C_6$-palmitate have ^{13}C incorporation patterns identifiable through GC-MS (Figure 5B). Isotopomer proportions, for example, Lactate M + 3, denotes all 3 carbons in the lactate molecule are derived from the $^{13}C_{16}$-palmitate substrate. When labeled palmitate was added as a cellular energy source (Figure 5A), ACLY overexpressing myoblast glucose uptake did not differ from control myoblasts at the time of collection when normalized to total cellular protein present (Figure 6A, $p > 0.05$). Palmitate-pulsed collected growth media lactate mole percent excess was lower for ACLY cells (Figure 6B, $p < 0.05$). ACLY was downregulated in Lactate M + 3 (Figure 7A, $p < 0.05$) and no differences were seen in lactate M + 1, M + 2, or pyruvate between cell types (Figure 7A and B, $p > 0.05$). Oxaloacetate isotopomers M + 2 and M + 3 were lower in ACLY cells (Figure 7C, $p < 0.05$). All labeling schemes of citrate and α-ketoglutarate (Figure 7D and F, $p < 0.05$) as well as succinate M + 4 and fumarate M + 1 and M

+ 2, the only fumarate isotopomers we detected, were lower in ACLY cells as compared to the control (Figure 7E and G, $p < 0.05$).

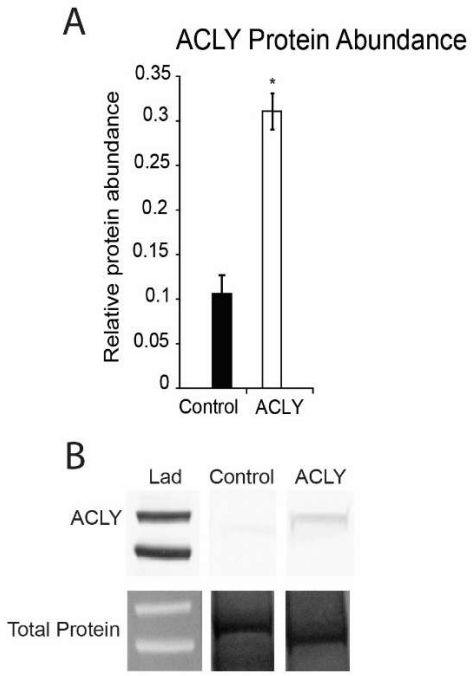

Figure 1. Relative abundance of ACLY protein normalized to total protein No-Stain in ACLY transfected and control myoblasts (A). Representative image of Western blot quantified in A (B). $p \leq 0.05$, statistical differences indicated by *.

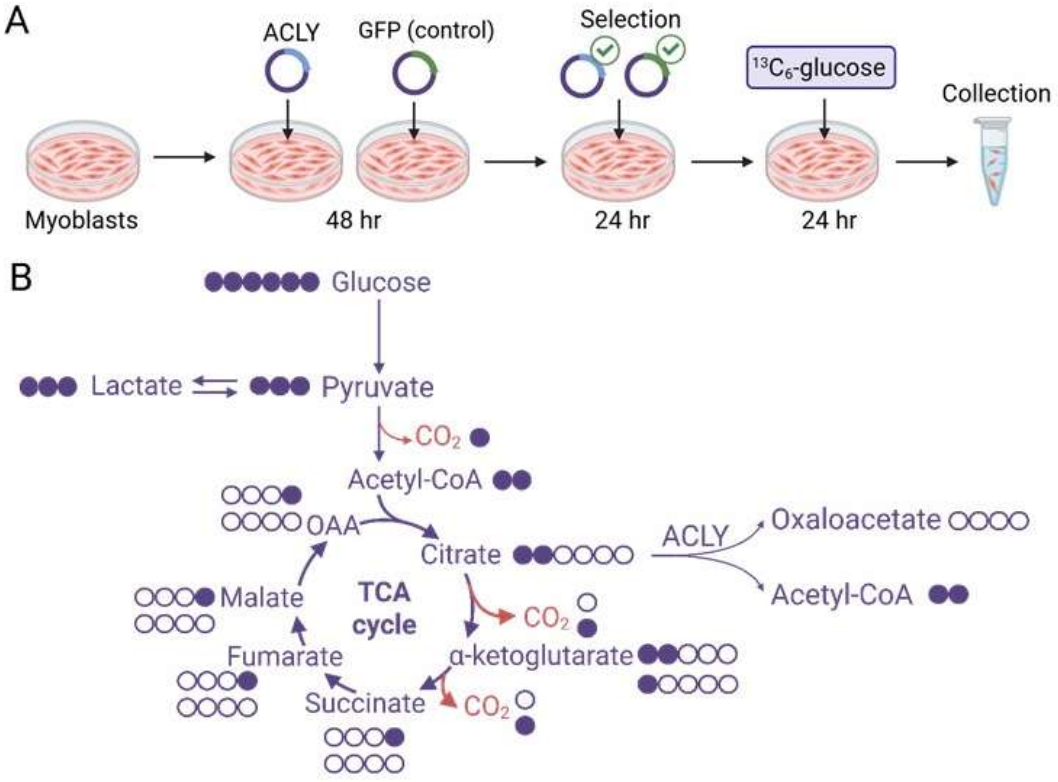

Figure 2. Depiction of $^{13}C_6$-glucose pulsed cell culture timeline including proliferation, transfection with ACLY or GFP plasmids, selection, and cell collection for analysis (A). Theoretical carbon labeling scheme derived from $^{13}C_6$-glucose. Purple circles depict labeled carbons derived from $^{13}C_6$-glucose and white depict unlabeled carbons (B).

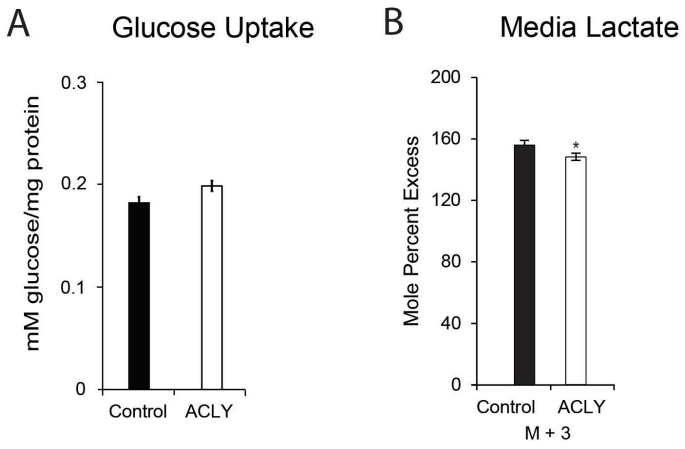

Figure 3. Quantification of glucose uptake after 24 hr glucose pulse from media. Glucose concentrations normalized to total protein of cells in each well (A). Mole percent excess of media lactate M + 3 isotopic enrichment after 24 hr $^{13}C_6$-glucose pulse (B). $p \leq 0.05$, statistical differences indicated by *.

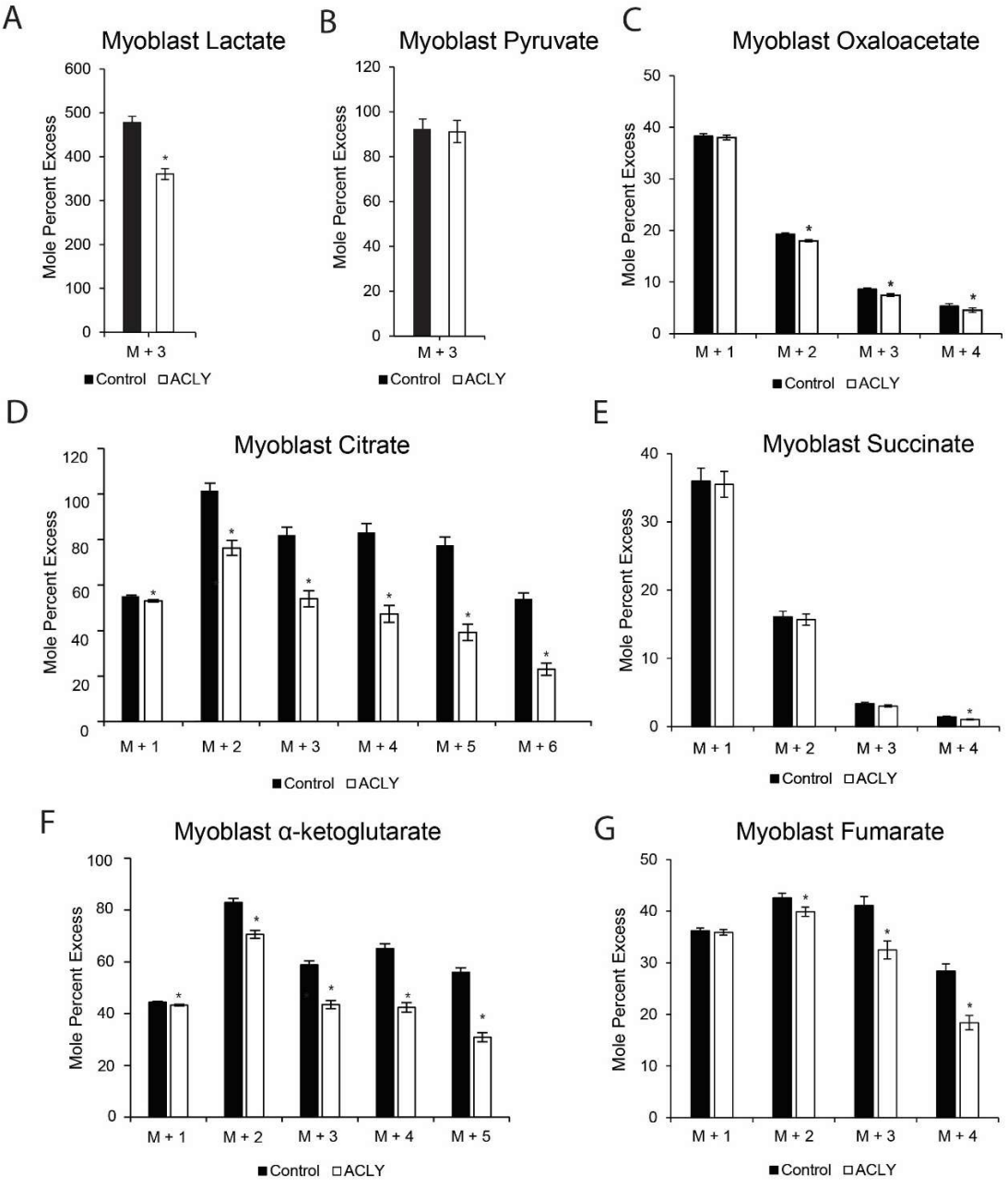

Figure 4. Mole percent excess of myoblasts after 24 hr $^{13}C_6$-glucose pulse including lactate M + 3 (A), pyruvate M + 3 (B), oxaloacetate M + 1 through M + 4 (C), citrate M + 1 through M + 6

(D), succinate M + 1 through M + 4 (E), α-ketoglutarate M + 1 through M + 5 (F), and fumarate M + 1 through M + 4 (G). $p \leq 0.05$, statistical differences indicated by *.

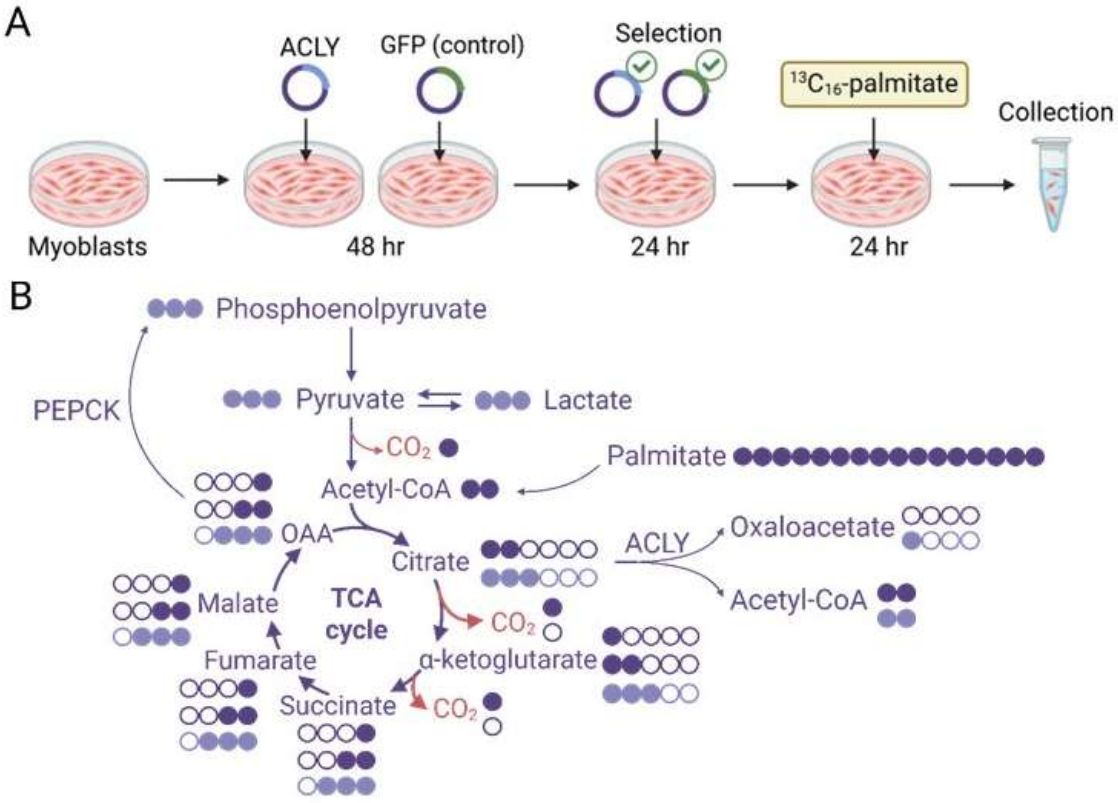

Figure 5. Depiction of $^{13}C_{16}$-palmitate pulsed cell culture timeline including proliferation, transfection with ACLY or GFP plasmids, selection, and cell collection for analysis (A). Theoretical carbon labeling scheme derived from $^{13}C_{16}$-palmitate. Purple circles depict labeled carbons derived from $^{13}C_{16}$-palmitate and white as unlabeled carbons with dark purple depicting one turn of the TCA cycle and light purple indicating a second turn of the TCA cycle (B).

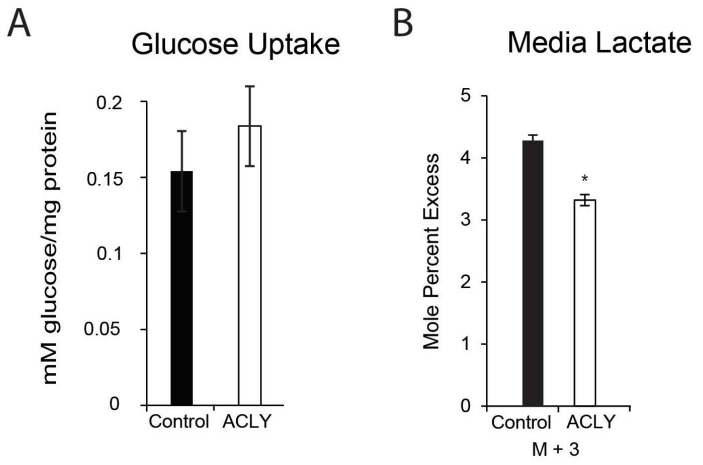

Figure 6. Quantification of glucose uptake after 24 hr palmitate pulse from media. Glucose concentrations normalized to total protein of cells in each well (A). Mole percent excess of media lactate M + 3 isotopic enrichment after 24 hr $^{13}C_{16}$-palmitate pulse (B). $p \leq 0.05$, statistical differences indicated by *.

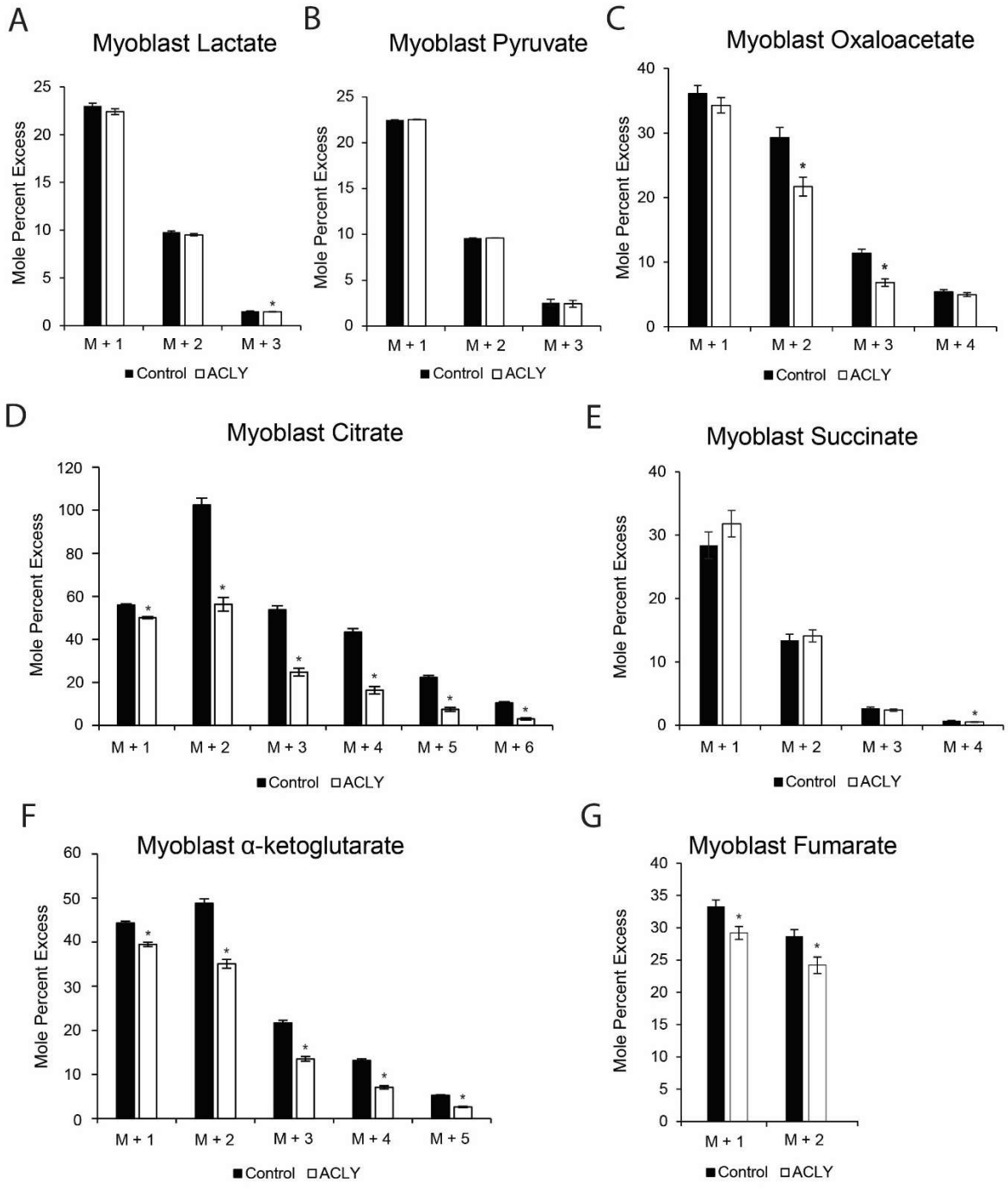

Figure 7. Mole percent excess of myoblasts after 24 hr $^{13}C_{16}$-palmitate pulse including lactate M + 1 through M + 3 (A), pyruvate M + 1 through M + 3 (B), oxaloacetate M + 1 through M + 4 (C), citrate M + 1 through M + 6 (D), succinate M + 1 through M + 4 (E), α-ketoglutarate M + 1

through M + 5 (F), and fumarate M + 1 through M + 4 (G). $p \leq 0.05$, statistical differences indicated by *.

4. Discussion

To determine if ACLY regulates nutrient metabolism and impacts cellular efficiency, we overexpressed ACLY to investigate its impact on nutrient flux through glycolysis and the TCA cycle. Overexpression of ACLY in skeletal muscle *in vivo* results in hypertrophy and seems to alter metabolism in the process, increasing the activity of ETC complexes, suggesting a remodeling to make metabolism more efficient (Das et al., 2015). Because the specific action of how ACLY alters nutrient usage has not yet been investigated, we overexpressed ACLY to determine how glucose and fatty acids are used in myoblasts.

ACLY-overexpressing myoblasts utilize similar amounts of glucose as controls ($p > 0.05$). In other cell types, ACLY has been established as a regulator of glycolytic nutrient utilization (Sohn et al., 2025). A possible mechanism for this regulation is histone acetylation by ACLY, although this remains vague (Wellen et al., 2009).

Lactate, a byproduct of glycolytic flux, can be excreted by the cell as waste and accumulate in the growth media (Rabinowitz & Enerback, 2020). A decrease in M + 3 lactate in media of ACLY myoblasts ($p \leq 0.05$) indicates nutrients from the $^{13}C_6$-glucose pulse are being directed elsewhere (Noe et al., 2021). This notion is supported by a decrease in myoblast lactate M + 3 in ACLY compared to control myoblasts ($p \leq 0.05$). Additionally, pyruvate conversion to lactate also regenerates NAD^+, which supports continued glycolytic flux (Chandel, 2021a). These data depict a shift in glycolytic flux in ACLY overexpressing myoblasts.

It is plausbile that pyruvate, the glycolytic end product, enters into mitochondria for utilization in the TCA cycle rather than being converted to lactate in the Cori cylce. However,

isotopomers of all TCA metabolites including citrate, α-ketoglutarate, oxaloacetate, succinate, and fumarate were lower in ACLY myoblasts as compared to the control ($p \leq 0.05$). This suggests a decrease in flux of $^{13}C_6$-glucose derived carbons through the TCA cycle in ACLY myoblasts. Although variations of heavy-carbon labeling schemes exist for individual TCA substrates, the corroboration of their collective downregulation is striking.

Interestingly, isotopic enrichment of pyruvate M + 3 did not differ between genotypes ($p > 0.05$). Given production of lactate and TCA cycle intermediates, its derivatives, are decreased in ACLY myoblasts, this questions if pyruvate handling is modified in these myoblasts. Others have demonstrated pyruvate is critical to energy generation via oxidative metabolism in mammalian cell types (Yako et al., 2021). Our observed accumulation of pyruvate could indicate ACLY myoblasts have a lower energy requirement as they are not utilizing pyruvate in the TCA cycle for energy production. Further investigation would be required to decipher the energy state of ACLY cells and activity status of pyruvate dehydrogenase (PDH), the complex that converts pyruvate to acetyl-CoA.

Others have demonstrated ACLY contributes to lipid synthesis and increases activity of mitochondrial complexes in myoblasts *in vitro,* which could contribute to the diminished glucose oxidation (Das et al., 2015) (Scheffler et al., 2014). This would explain the observed decrease in TCA cycle flux that is contradictory to expected energy demands for the bout of hypertrophy associated with ACLY overexpression in skeletal muscle in *vivo* (Cai et al., 2022) (Das et al., 2017). However, it is plausbile ACLY overexpression shifts nutrient preference from glucose to an alternative fuel source.

To assess if ACLY myoblasts shift to prefer a fatty acid source, cells were pulsed with $^{13}C_{16}$-palmitate and the results are remarkably similar to those of the $^{13}C_6$-glucose pulse. Given

media was supplemented with $^{13}C_{16}$-palmitate, media glucose levels were still measured to ensure addition of the fatty acid does not drastically alter overall glucose use. Again, glucose uptake did not differ between ACLY overexpressing myoblasts and controls ($p > 0.05$). As both glucose and palmitate are available as nutrient sources in this experiment, supplementing fatty acids does not appear to impact overall glucose uptake, which is not surprising as glucose is still the primary nutrient source for cultured cells (Mulukutla et al., 2010).

To evaluate if palmitate-derived carbon flux through the TCA cycle is altered, the same tracing strategy was implemented as described previously with glucose. Isotopic enrichment of lactate M + 3 in ACLY myoblasts and media decreased in ACLY myoblasts ($p \leq 0.05$). Palmitate is not directly converted to lactate in skeletal muscle (Figure 5). Palmitate enters the TCA cycle as acetyl-CoA after β-oxidation and as the cycle proceeds, these carbons are incorperated into TCA cycle intermeidates. After multiple "turns" of the TCA cycle, plamitate-derived carbons continue to incorporate allowing isotopic enrichment of oxaloacetate to be detected.

Oxaloacetate can be combined with acetyl-CoA to form citrate or exit the cycle through several catapleuric reactions. While oxaloacetate has many potential fates, it can be converted to the glycolytic intermediate, phosphoenolpyruvate, by phosphoenolpyruvate carboxykinase (PEPCK). This intermediate is converted to pyruvate, which can enter into the TCA cycle or the Cori cycle to generate lactate (Brown et al., 2016). Given the length of the incubation period, it is possible for several turns of the TCA cycle and thus this series of reactions to occur (Gibala et al., 1998). Interestingly, these data suggest that fatty acid-derived carbons are used to produce lactate, albiet still at a lower level in ACLY myoblasts compared to controls ($p \leq 0.05$). Given media in this experiment still contained glucose, it is plausble utilization of a fatty acid source in the absence of glucose may reveal different outcomes (Wellen et al., 2009). However, we

implemented this approach to evaluate if fatty acid utilzation increases in the presence of glucose because the ACLY signaling axis can increase histone acetylation or fatty acid synthesis.

Similar to incubations with glucose, pyruvate M + 3 did not differ between ACLY and control myoblasts ($p > 0.05$). The observed similarity amongst genotypes pulsed with palmitic acid could be explained by the fact that palmitate can undergo β-oxidation to be broken down into acetyl-CoA molecules which are able to directly enter the TCA cycle, thus skipping pyruvate entry altogether. In this case, it is possible there is no difference in pyruvate abundances as acetyl-CoA is being derived from palmitate instead. Hepatic cells have exhibited similar pyruvate consistency when mitochondrial citrate export was altered, a precursor step to ACLY activity (Rauckhorst et al., 2025). Further, isotopic enrichment of most citrate, α-ketoglutarate isotopomers, oxaloacetate, succinate, and fumarate decreased in ACLY myoblasts similarly to the $^{13}C_6$-glucose pulse ($p \leq 0.05$). These findings suggest ACLY cells are not simply shifting nutrient preference from glucose to palmitate once it becomes available, as oxidation of both decreased in the major metabolic pathways ($p \leq 0.05$). Given glucose uptake is not altered, it is plausible these carbons are branching into other pathways within the cell.

Altogether, this observed metabolic downregulation raises questions surrounding the overall cellular energy state in ACLY upregulated cells. Do cells overexpressing ACLY use energy sources more efficienctly, requiring less substrate oxidation while still maintaining the same cellular energy balance as cells that have greater oxidation of glucose? If ACLY-overexpressing cells are instead in a lower energy state, characterized by ATP and ADP abundances, what is the growth potential and functionality of these cells?

Other reports of ACLY-mediated muscle hypertrophy represents a potential avenue for improvement to effeciency on a cellular level. However, additional investigation is needed to

clarify ACLY-mediated nutrient utilization and the mechanism to promote hypertrophy in skeletal muscle.

5. Conclusion

Our findings elucidate ACLY as a potent regulator of nutrient metabolism in skeletal muscle. While the mechanism remains unclear, we present strong evidence ACLY upregulation results in cells oxidizing fewer nutrients. These cells produce fewer glycolytic and oxidative substrates, apparently downregulating these pathways. Nutrient source, whether carbohydrate or fatty acid, does not appear to affect these findings. These effects were seen on a cellular level and once applied to the animal, could have great implications for cost savings and sustainability improvement for the livestock industry.

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