Title: Ulk1(S555) inhibition alters nutrient stress response by prioritizing amino acid metabolism

Authors: Orion S. Willoughby[1], Anna S. Nichenko[1], Matthew H. Brisendine[1], Niloufar Amiri[1,2], Shelby N. Henry[1], Daniel S. Braxton[4], John R. Brown[1], Kalyn S. Specht[1], Adele K. Addington[1], Alexey V. Zaitsev[5], Steven T. Burrows[5], Ryan P. McMillan[1,3*], Haiyan Zhang[1,3], Spencer A. Tye[6], Charles P. Najt[1,3], Siobhan M. Craige[1,7], Timothy W. Rhoads[6], Junco S. Warren[1,5,7,8], Joshua C. Drake[1,3,7]

Affiliations:

[1] Department of Human Nutrition, Foods, and Exercise. Virginia Polytechnic Institute and State University. Blacksburg, VA. 24061, USA.

[2] Institute for Critical Technology and Applied Science. Virginia Polytechnic Institute and State University. Blacksburg, VA. 24061, USA.

[3] Metabolism Core. Virginia Polytechnic Institute and State University. Blacksburg, VA. 24061, USA.

[4] Department of Biological Sciences. Virginia Polytechnic Institute and State University. Blacksburg, VA. 24061, USA.

[5] Fralin Biomedical Research Institute at Virginia Tech Carilion, Virginia Tech, Roanoke, VA, USA.

[6] Department of Nutritional Sciences, University of Wisconsin Madison, Madison, WI, USA

[7] Translational Biology, Medicine, and Health Program, Virginia Tech, Roanoke, VA 24016, USA.

[8] Center for Exercise Medicine, Fralin Biomedical Research Institute, Virginia Tech, Roanoke, VA, USA

[*] Posthumous

Lead Contact

Joshua C. Drake, PhD

1981 Kraft Dr.

Blacksburg, VA

24061

Phone: 540-231-5119

Email: joshuacd@vt.edu

Summary

Metabolic flexibility, the capacity to adapt fuel utilization in response to nutrient availability, is essential for maintaining energy homeostasis and preventing metabolic disease. Here, we investigate the role of Ulk1 phosphorylation at serine 555 (S555), a site regulated by AMPK, in coordinating metabolic switching following short-term caloric restriction and fasting. Using Ulk1(S555A) global knock-in mice, we show loss of S555 phosphorylation impairs glucose oxidation in skeletal muscle and liver during short-term CR, despite improved glucose tolerance. Metabolomic, transcriptomic, and mitochondrial respiration analyses reveal a compensatory reliance on glucogenic amino acids, particularly alanine and serine, in Ulk1(S555A) mice, with sustained amino acid oxidation during fasting and blunted mitochondrial response to energetic stress. These findings establish Ulk1(S555) phosphorylation as a critical regulatory event linking nutrient stress to substrate switching and highlights an underappreciated role of Ulk1 in maintaining metabolic flexibility.

Introduction

Metabolic flexibility, the ability to respond and adapt to energetic demands (Goodpaster & Sparks, 2017), is necessary for survival. Regularly inducing a metabolic switch, such as switching from glucose to fatty acid oxidation during regular intervals of fasting, as occurs during intermittent fasting (IF) or caloric restriction (CR) has been shown to extend healthspan and lifespan in numerous organisms (Duregon et al., 2021; Lee et al., 2021). Consequently, metabolic inflexibility indicates metabolic dysfunction, contributing to many metabolic-related diseases (e.g., diabetes, sarcopenia, and obesity) and characteristic of aging (Riera & Dillin, 2015; Zhou et al., 2023). Skeletal muscle is the primary utilizer of glucose, accounting for 60-80% of glucose metabolism (Ng et al., 2012). CR and/or IF increase capacity to take up glucose (Pak et al., 2021; Teong et al., 2023), particularly in skeletal muscle (Sharma et al., 2014; Wang et al., 2017), as well as mitochondrial enzymatic processes (e.g., tricarboxylic acid cycle and oxidative phosphorylation) to utilize the pyruvate produced from the breakdown of glucose through glycolysis (Chen et al., 2015; Pak et al., 2021; Rhoads et al., 2020). Depletion of glucose with fasting initiates gluconeogenesis, with skeletal muscle providing amino acids via protein breakdown to be transaminated to pyruvate primarily in the liver via the Cahill cycle (Petersen et al., 2019) or within the muscle itself (Neinast et al., 2019) to meet skeletal muscle energy requirements. However, the utilization of amino acids as glucogenic precursors is inefficient, with 20-33% of the energy from amino acid-derived pyruvate being lost to the energetic cost of protein breakdown and urea synthesis (Hall, 2006; Veldhorst et al., 2009).

A primary axis for the molecular response to changes in cellular energy is coordination between 5'-AMP-activated kinase (AMPK) and mechanistic target of rapamycin 1 (mTORC1) (Green et al., 2022). AMPK responds to increased AMP/ADP by suppressing energetically costly processes, like protein synthesis, to conserve cellular energy, while mTORC1 promotes anabolism during energetic abundance. A shared substrate between AMPK and mTORC1 is Unc-51-like autophagy activating kinase (Ulk1), which is best characterized for its role in recycling cellular components through autophagy in response to energetic stress (Pareek & Kundu, 2024). Inducible loss of Ulk1 in skeletal muscle is sufficient to impair exercise training-mediated improvements in glucose tolerance (Drake et al., 2021; Laker et al., 2017), whereas silencing downstream autophagy genes (e.g., *ATG5, ATG7, BCN1*) *in vitro* does not affect glucose metabolism (Li et al., 2016), suggesting a direct role for Ulk1 in metabolic regulation. Indeed, *in vitro,* Ulk1 has been shown to directly phosphorylate glycolytic enzymes, hexokinase (HK), phosphofructokinase 1 (PFK1), and enolase 1 (ENO1), as well as the gluconeogenic enzyme

fructose-1,6-bisphosphatase (FBP1) in response to nutrient stress (Li et al., 2016). Ulk1-dependent glucose metabolism may function to modulate protein synthesis only when sufficient energy is available to support the energetic investment (Park et al., 2023; Yoon et al., 2020). Despite this evidence for Ulk1 having regulatory functions modulating metabolic responses to energetic stress, phosphorylation site-dependent roles for Ulk1 in modulating metabolism *in vivo* are not well defined.

AMPK phosphorylates Ulk1 at multiple sites (Park et al., 2023), including S467, S555, T574, and S637 (murine sites) (Egan et al., 2011). Of these, S555 is most readily phosphorylated in response to energetic stressors like exercise (Laker et al., 2017) and fasting (Bujak et al., 2015) *in vivo*. Based on evidence that deletion of Ulk1 or simultaneous impairment of S467, S555, T574, and S637 (4SA) disrupts the autophagy response to energetic stress (Egan et al., 2011; Laker et al., 2017), we and others have interpreted the phosphorylation of Ulk1 at S555 to initiate its autophagy function, as S555 is most responsive *in vivo* and precedes autolysosome formation (Bujak et al., 2015; Egan et al., 2011; Laker et al., 2017). However, recent evidence in cell culture models has challenged this paradigm by demonstrating that AMPK-dependent Ulk1(S555) phosphorylation, in coordination with other AMPK-dependent phosphorylation events, inhibits autophagy to conserve cellular energy when glucose is limited, depending on the availability of amino acids (Kazyken et al., 2024; Park et al., 2023). CRISPR-engineered Ulk1(S555A) mice maintain a higher RER during exercise, delayed crossover time (RER < 0.85), and have elevated circulating lactate following exercise in the absence of an effect on autophagy/mitophagy (Guan et al., 2024). Therefore, phosphorylation of Ulk1 at S555 may have an unappreciated role in mediating metabolic flexibility *in vivo*.

Here, we utilized Ulk1(S555A) global knock-in mice (Guan et al., 2024) to interrogate site-specific requirement(s) of Ulk1(S555) phosphorylation for metabolic flexibility under different durations of nutrient stress. We discovered inhibition of Ulk1(S555) altered the rate of body weight loss during 8 weeks of 40% caloric restriction (CR) and blunted CR-mediated increases in glucose oxidation capacity of skeletal muscle and liver via C^{14}-glucose tracing, despite improved glucose tolerance. GC-MS-based metabolomics of skeletal muscle and liver revealed that Ulk1(S555A) mice relied on glucogenic amino acids (e.g., alanine) as a primary energetic substrate during CR. We confirmed Ulk1(S555A) mice maintain circulating amino acid and pyruvate levels in plasma during prolonged fasting, coinciding with a delayed metabolic switch. Also, mitochondria isolated from

Ulk1(S555A) skeletal muscle utilize glucogenic amino acids to fuel basal and complex I-mediated respiration but are unable to further increase respiration during robust energetic stress.

Results

Attenuated glucose oxidative capacity following caloric restriction in the absence of Ulk1(S555) phosphorylation.

The effects of caloric restriction (CR) are associated with extended life span and amelioration of age-related pathologies, in part, through improved coordination between skeletal muscle and liver metabolism, leading to improved glucose tolerance and homeostasis, insulin sensitivity, and metabolic flexibility (Fok et al., 2013; Matyi et al., 2018). WT and Ulk1(S555A) mice were designated to either *ad libitum* (AL) or 8-week 40% CR (Fig. 1A). Food intake per cage for CR was determined by measuring three consecutive days of food consumption (Fig. 1A; S1A). *Ad libitum* fed Ulk1(S555A) mice had higher body mass over the course of the study compared to wild-type mice (Fig.1B). CR Ulk1(S555A) mice lost body mass at a slower rate than CR WT mice (Fig. 1B-C). WT mice maintained lean and fat mass as a percentage of body mass over the duration of CR, whereas Ulk1(S555A) mice maintained more fat mass with CR (Fig. S1B, C). Pre- and post-CR glucose tolerance tests (Fig. 1D) demonstrated improved glucose clearance in both WT and Ulk1(S555A) with CR (Fig. 1E-F), although Ulk1(S555A) were overall less glucose tolerant than WT (Fig. 1E). Contrary to deletion of Ulk1, which blunts exercise training-mediated improvements in glucose tolerance and insulin sensitivity (Drake et al., 2021; Laker et al., 2017), our findings suggest inhibition of S555 does not overtly affect improvement in glucose tolerance with CR, which is analogous to our recent findings following exercise training (Guan et al., 2024).

Skeletal muscle is the primary site for glucose metabolism during nutrient stress (Ng et al., 2012). To assess if increased glucose tolerance with CR was accompanied by an increased capacity to oxidize glucose, we utilized U-[^{14}C]-Glucose to uniformly assess oxidation of all carbons within glucose through glycolysis and the TCA cycle (Fig. 1G) (Specht et al., 2021). CR increased U-[^{14}C]-Glucose oxidation in both red (oxidative) and white (glycolytic) muscle fibers from quadricep, as well as liver of WT mice (Fig. 1H-J), suggesting an increased capacity to oxidize glucose following short-term CR. However, glucose oxidation capacity, as assessed by U-[^{14}C]-Glucose, did not change in skeletal muscle or liver from Ulk1(S555A) mice. Suggesting phosphorylation of Ulk1 at S555 (Fig. S1D-F) may have undefined roles in metabolic regulation to meet skeletal muscle and liver energetic needs during prolonged nutrient stress, with S555A causing an irregular shift away from glucose-derived substrates.

Caloric restriction leads to increased reliance on amino acids in the absence of Ulk1(S555) phosphorylation.

As Ulk1(S555A) mice did not increase capacity to oxidize glucose in skeletal muscle following 8 weeks of 40% CR, we performed GC-MS-based metabolomics to define metabolic profiles in skeletal muscle (quadricep). Metabolic pathway impact of skeletal muscle showed elevations in pathways associated with amino acid metabolism, including lysine degradation, ammonia recycling, and alanine metabolism (Fig. 2A; S2B). Analysis of individual amino acids showed skeletal muscle from Ulk1(S555A) mice had elevated levels of glucogenic and ketogenic amino acids that were either elevated in Ulk1(S555A) and unaffected by CR (e.g. glycine, methionine, and lysine, with alanine and threonine nearing significance) or further elevated in response to CR (e.g. serine) (Fig. S2A-C).

Metabolic flexibility during nutrient stress occurs, in part, through coordination between skeletal muscle as the primary utilizer of glucose and liver as the primary site for gluconeogenesis via amino acids (Sarabhai & Roden, 2019). Metabolic pathway impact of liver showed elevations in urea cycle, glucose-alanine cycle, and alanine metabolism in Ulk1(S555A) mice following CR (Fig. 2B; S3B). As in skeletal muscle, analysis of individual amino acids showed liver from Ulk1(S555A) mice had elevated levels of glucogenic and ketogenic amino acids that were either elevated in Ulk1(S555A) and unaffected by CR (e.g. glycine, methionine, serine, threonine, and lysine) or further elevated in response to CR (e.g. aspartic acid, glutamate, glutamine, and tyrosine) (Fig. S3C). Therefore, skeletal muscle and liver in Ulk1(S555A) mice may preferentially rely on amino acids to maintain skeletal muscle demand for pyruvate and/or acetyl-CoA to propel the tricarboxylic acid cycle (TCA) during nutrient stress (Fig. S2D; S3D).

Alanine in particular supports muscle metabolism during prolonged nutrient stress when glucose is limited (Okun et al., 2021; Petersen et al., 2019). Additionally, alanine is the primary amino acid that is released and synthesized from skeletal muscle and predominantly transaminated in the cytoplasm and mitochondria of the liver via the Cahill Cycle, and to a lesser extent within skeletal muscle (Felig, 1973; Hewton et al., 2021). Ulk1(S555A) mice tended to have more alanine in skeletal muscle, but CR increased α-ketoglutarate levels in skeletal muscle of Ulk1(S555A) mice (Fig. 2C), which receives the amino group from alanine during transamination, alluding to increased alanine transport to the liver. Compared to WT, liver from *ad libitum* fed Ulk1(S555A) mice had significantly higher alanine levels, suggesting increased transport and/or capacity for *de novo* synthesis of alanine as a glucogenic substrate in Ulk1(S555A) liver (Fig. 2D). Following short-term CR, Ulk1(S555A) liver alanine declined with a significant increase in α-ketoglutarate and glutamate (Fig. 2D),

indicating increased transamination of alanine. Subsequently, liver pyruvate concentration was elevated in both WT and Ulk1(S555A) following CR (Fig. 2D). We hypothesize Ulk1(S555A) lack of increased glucose oxidative capacity with CR (Fig. 1H-J) is due to a preferential reliance on glucogenic amino acid metabolism to provide pyruvate and propel the TCA cycle.

Caloric restriction impacts the expression of genes involved in glycolysis and gluconeogenesis in skeletal muscle (Lee et al., 1999) and liver (Dhahbi et al., 1999; Spindler, 2001). To understand how a potential reliance on glucogenic amino acids impacts gene expression in response to short-term CR, we performed bulk RNA sequencing of skeletal muscle (quadricep) and liver from AL and CR fed Ulk1(S555A) and WT mice and performed an integrated cross-correlation analysis focused on alanine. In both tissues, alanine was correlated with gene expression changes consistent with altered metabolic flexibility. In skeletal muscle, alanine positively correlated with Mrpl41 and Pip4pl, which are involved in mitochondrial adaptation (Rosivatz & Woscholski, 2011; Yoo et al., 2005) (Fig. 2E). In liver, alanine positively correlated with genes related to circadian rhythm (Per2 and Per3) and cholesterol biosynthesis (Srebp1, Srebp2, and Hmgcr) (Daniels et al., 2023; Mteyrek et al., 2016; Wang et al., 2020) (Fig.2F). Further, liver alanine negatively correlated with genes related to fatty acid metabolism (Cyp4a10 and Crot) (Lin et al., 2006; Sanford et al., 2023). Therefore, an increased reliance on alanine as an energetic substrate during CR in the Ulk1(S555A) may alter normal metabolic switching during prolonged periods of nutrient stress.

Altered metabolic switch during fasting in the absence of Ulk1(S555) phosphorylation.

Mice on CR, where the daily allocation of food is given all at once, as we performed, will eat all the provided food within a few hours (Acosta-Rodriguez et al., 2017; Duregon et al., 2023; Mitchell et al., 2019). Thus, self-imposing a daily, prolonged (20-22 hrs) fasted period. As Ulk1(S555A) mice did not increase their capacity to oxidize glucose within skeletal muscle and liver following CR (Fig. 1H-J), we hypothesized loss of Ulk1(S555) *in vivo* impaired metabolic switching during the daily self-imposed fasted window (Acosta-Rodriguez et al., 2017; Duregon et al., 2023; Mitchell et al., 2019). WT and Ulk1(S555A) mice were monitored in metabolic cages for 24 hours with *ad libitum* access to food, then fasted for 24 hours (Fig. 3A). There was no difference in respiratory exchange ratio (RER) during either light or dark phases when food access was unrestricted; however, Ulk1(S555A) mice were significantly less active despite energy expenditure being equivalent (Fig. 3A-B;

Fig.S4A). Once food was removed, RER of WT mice dropped below 0.75 within 6 hours of fasting (Fig. 3A), indicative of increased reliance on fat as a substrate. However, after an initial drop upon fasting, which may have been due to opening cages to remove food, Ulk1(S555A) mice RER did not drop below 0.75 until after ~18 hours of fasting (Fig.3A), indicating maintained reliance on mixed substrates. Despite the delayed metabolic switch in Ulk1(S555A) mice, Ulk1(S555A) mice remained less active while energy expenditure was equal between groups (Fig. 3B, Fig. S4F-D). These findings may support increased utilization of amino acids as inefficient glucogenic precursors in fasted Ulk1(S555A) mice (Fig. 2F-D), as protein breakdown and urea synthesis are energetically costly (Hall, 2006; Veldhorst et al., 2009), leading to the elevation of basal energy expenditure with a lack of increased physical activity.

To determine if glucose oxidation capacity was also impaired during acute fasting, we utilized U-^{14}C-Glucose and 6-^{14}C-Glucose to compare the fate of all carbons in glucose vs. those that are oxidized last in the TCA cycle, respectively (Fig. 3C). Capacity to oxidize glucose *ex vivo* via U-[^{14}C]-Glucose (Fig.3C) increased in red quadricep muscle fibers from WT in response to a 24-hour fast at 3 and 9 months of age but was unchanged by fasting in Ulk1(S555A) mice (Fig. 3D; Fig. S4B). A genotype effect was also observed in white quadricep muscle fibers following 24-hour fasting (Fig. 3D). We next utilized 6-^{14}C-Glucose to isolate the terminal end point of glucose oxidation in the TCA cycle (Fig. 3C) and found Ulk1(S555A) had lower capacity to oxidize 6-^{14}C-Glucose in red quadricep muscle fibers from Ulk1(S555A) (Fig. 3E). Put together, the lack of increased oxidative capacity of U-^{14}C-Glucose and 6-^{14}C-Glucose supports a preferential shift away from glucose during a prolonged fast to maintain energetic homeostasis when Ulk1 is unable to be phosphorylated at S555.

During fasting, skeletal muscle relies on the supply of nutrient substrates delivered through circulation (Sarabhai & Roden, 2019). To gain insight into preferred substrates in Ulk1(S555A) mice during fasting, we performed untargeted metabolomics of plasma following 24-hour fasting. Following fasting, circulating pyruvate significantly declined in WT mice (Fig. 3F), consistent with a shift towards fatty acids, which are broken down to acyl-CoA through β-oxidation in mitochondria, thus reflecting the observed RER (Fig.3A). However, in Ulk1(S555A) mice, potential elevation in circulating pyruvate following 24-hour fasting coincided with a significant increase in circulating urea (Fig. 3F-H), suggesting increased amino acid metabolism maintains circulating pyruvate. We found a mirrored effect of fasting on circulating amino acids within WT and Ulk1(S555A) mice, with Ulk1(S555A) fasted mice maintaining circulating glucogenic amino acids (Figs. 3F; S4D-J), particularly alanine

and serine (Fig. 3F, I-J), and the ketogenic amino acid lysine (Fig. S4E, I). In sum, these findings suggest inhibition of Ulk1(S555) impairs metabolic switching during prolonged fasting, resulting in a preference for glucogenic amino acids (e.g., alanine, serine) as substrates to maintain energetic homeostasis (Fig. 3K).

Loss of Ulk1(S555) phosphorylation increases mitochondrial oxidative capacity of glucogenic amino acids in skeletal muscle.

Alanine and serine can be metabolized either in the cytoplasm or imported and transaminated in mitochondria to pyruvate to supply the TCA cycle or into the reducing equivalent NADH (serine) for the electron transport chain (Fig.4A) (Lucas et al., 2018; Parker et al., 2020; Wu et al., 2023; Yang et al., 2020). To determine if sustained circulating levels of amino acids during prolonged fasting in Ulk1(S555A) mice reflected an increased reliance on amino acids as energetic substrates (Fig.3K), we assessed respiration of mitochondria isolated from quadricep skeletal muscle from overnight (~12 hours) fasted WT and Ulk1(S555A) mice in the presence of increasing concentrations (2mM, 5mM, 10mM) of either alanine or serine. Baseline oxygen consumption rate (OCR) was significantly elevated in the presence of alanine in a concentration-dependent manner in mitochondria isolated from Ulk1(S555A) skeletal muscle (Fig.4B-C). Baseline OCR was also elevated in Ulk1(S555A) mitochondria compared to WT mitochondria in the presence of serine at all concentrations (Fig.4F-G). In the presence of alanine or serine, OCR remained elevated in Ulk1(S555A) mitochondria with the addition of glutamate/malate (10mM/5mM) to stimulate Complex I-dependent respiration, though serine caused a concentration-dependent suppression of OCR (Fig.4D, H). However, with the addition of ADP, which mimics energetic stress conditions, only WT mitochondria were able to further elevate OCR in the presence of alanine or serine at any concentration (Fig.4E, I). In sum, these data suggest mitochondria from Ulk1(S555A) mice are adapted to better utilize glucogenic amino acids, but this preference limits energy production during periods of high or prolonged energetic stress, suggesting phosphorylation of Ulk1 at S555 is a critical metabolic checkpoint for metabolic switching *in vivo*.

Discussion

Inter-organ communication is critical for maintaining metabolic flexibility—the ability to adapt substrate source to changes in substrate availability (Goodpaster & Sparks, 2017). In healthy states, the body can efficiently transition between fuel sources depending on demand: insulin promotes glucose uptake and storage during feeding, while prolonged fasting stimulates lipolysis and glucose production to sustain energy. However, in metabolic disorders and aging, inter-organ communication breaks down, impairing fuel switching and promoting metabolic inflexibility (Castillo-Armengol et al., 2019; Goodpaster & Sparks, 2017). Skeletal muscle accounts for 60-80% of glucose utilization (Ng et al., 2012), accentuating its central role in regulating whole-body glucose homeostasis. During early nutrient stress, such as fasting, skeletal muscle relies on glucose oxidation. As circulating glucose levels fall, skeletal muscle transiently shifts to amino acid catabolism, primarily utilizing alanine as an energy source. Alanine is released and taken up by the liver, where it is converted to pyruvate and used in gluconeogenesis via the glucose-alanine cycle, thereby preserving plasma glucose levels for glucose-dependent tissues (Felig, 1973). As fasting progresses, both liver and muscle engage in coordinated metabolic reprogramming: the liver increases fatty acid oxidation and ketogenesis to produce alternative fuels, while skeletal muscle upregulates fatty acid uptake and β-oxidation, transitioning away from amino acid catabolism to conserve muscle protein. This adaptive inter-organ communication allows for efficient substrate switching—glucose to amino acids to lipids—ensuring that energy needs are met without depleting critical reserves. Our findings identify Ulk1(S555) phosphorylation as a key regulatory event that integrates nutrient-sensitive signals across metabolic tissues. In sum, the current study suggests phosphorylation of Ulk1 at S555 facilitates fuel switching during nutrient stress, highlighting its critical role in maintaining metabolic flexibility and potential target against metabolic dysfunction.

There are four known Ulk variants, with Ulk1 and Ulk2 being the most similar, with 55% homology (Chan et al., 2009; Hara et al., 2008). However, insulin-induced phosphorylation of Akt in skeletal muscle is impaired following exercise training in tamoxifen-inducible Ulk1 knockout mice, but not Ulk2 knockouts (Drake et al., 2021; Laker et al., 2017), suggesting Ulk1 is specifically needed for adaptation of glucose metabolism to energetic stress *in vivo*. In cell culture, Ulk1 has been shown to directly phosphorylate the glycolytic enzymes HK, thus promoting glucose uptake, and FBP1, continuing glycolysis over gluconeogenesis (Li et al., 2016). Further, Ulk1

has also been shown to phosphorylate PFK1 and ENO1, thus inhibiting their activity, causing the accumulation of glucose-6-phosphate, enhancing NADPH production, and maintaining redox homeostasis (Li et al., 2016). Our data extend these findings in vivo by demonstrating Ulk1 phosphorylation at S555 is the post-translational modification required for Ulk1-mediated regulation of glucose oxidation during nutrient stress in skeletal muscle and liver. Importantly, silencing of downstream autophagy genes, *ATG5, ATG7,* and *BCN1,* has been shown to not impair glucose metabolism (Li et al., 2016). We recently showed exercise-induced mitophagy is intact in Ulk1(S555A) mice (Guan et al., 2024), which echoes other recent work suggesting Ulk1(S555) does not induce autophagy (Kazyken et al., 2024; Park et al., 2023). Taken together, the inability of Ulk1(S555A) mice to enhance glucose oxidation in skeletal muscle and liver following different periods of nutrient stress suggests Ulk1 phosphorylation at S555 as a key event for metabolic flexibility that may be independent of Ulk1's more canonical role in autophagy.

Under normal conditions, the reliance on amino acids as an energetic substrate is either brief, as during the transition from glucose to fat, or as a last resort following prolonged fasting (Petersen et al., 2019). However, we show that in Ulk1(S555A) mice, reliance on amino acids as an energetic substrate is prolonged (~12 hours) during a 24-hour fast and a major energy source during short-term CR. Ulk1(S555) was recently demonstrated to be sensitive to amino acid availability in a variety of cell lines, with amino acid restriction increasing S555 phosphorylation and the addition of amino acids l-serine, l-hydroxyproline, and l-glutamine decreasing S555 phosphorylation (Kazyken et al.). In glucose-deprived HEK293 cells, protein synthesis is suppressed, and the essential amino acid leucine is made available as an energetic substrate through Ulk1-dependent phosphorylation of leucyl-tRNA synthetase 1 (LARS1) to maintain energetic homeostasis (Yoon et al., 2020). These findings suggest the absence of Ulk1(S555) creates a cellular environment where dramatic changes in glucose availability go unnoticed, prolonging the metabolic switch point, and therefore, amino acids become the default substrate.

Amino acids and glucose can suppress the utilization of each other to promote either anabolic or catabolic processes (Shao et al., 2018). Thus, reliance on amino acids as a substrate during nutrient stress in Ulk1(S555A) mice may also contribute to impaired capacity to oxidize glucose. For example, pharmacologically inhibiting

mitochondrial complex I-mediated respiration elevates serine catabolism, leading to an accumulation of NADH, suppressing other metabolic pathways known to produce NADH (glucose, glutamine, and fat oxidation) (Yang et al., 2020). Alternatively, serine-derived NADH may be an essential adaptive response to maintain mitochondrial energetics (Lucas et al., 2018). Alanine is predominantly released and synthesized in skeletal muscle (Felig, 1973), while the liver is the primary site for alanine transamination to pyruvate (Hewton et al., 2021). Notably, our metabolomics data show Ulk1(S555A) mice exhibit elevated alanine concentrations in both skeletal muscle and liver, coupled with increased hepatic alanine transamination and preferential utilization of alanine during fasting. In skeletal muscle mitochondria, Ulk1(S555A) mice had affinity to oxidize alanine and serine in skeletal muscle to support basal and complex I-mediated respiration, highlighting a shift in mitochondrial substrate preference that may reflect adaptive mechanisms when Ulk1 cannot be phosphorylated at S555. Alanine has been shown to outcompete glucose as a substrate to fuel mitochondrial respiration in pancreatic ductal adenocarcinoma (PDAC) (Sousa et al., 2016). While loss of Ulk1(S555) may directly impair glycolysis through direct interruption of glycolysis (Li et al., 2016), the resulting preference for gluconeogenic amino acids over glucose may further disrupt metabolic switching by outcompeting glucose-derived pyruvate and/or NADH to fuel mitochondrial metabolism. However, the inefficiency of amino acid oxidation may limit mitochondrial respiration during robust or prolonged nutrient stress (Hall, 2006; Veldhorst et al., 2009).

In the present study, the loss of Ulk1(S555) impairs metabolic flexibility, reduces glucose oxidative capacity, and promotes an adaptive reliance on gluconeogenic amino acids during short-term CR and acute fasting. This altered substrate preference reflects metabolic features of insulin resistance and may contribute to the development of obesity-related disorders and type 2 diabetes. Correlative analyses show that alanine levels in Ulk1(S555A) following CR are associated with transcripts involved in mitochondrial remodeling (Mrpl41, Pip4p1), circadian rhythm (Per2, Per3), and cholesterol biosynthesis (Srebp1, Srebp2, Hmgcr). This data complements prior findings in the knockdown of Ulk1, leading to decreased markers of fatty acid oxidation (Cyp4a10, Crot) and fatty acid uptake, suggesting further tissue-specific disruptions in metabolic adaptations (Ro et al., 2013). Furthermore, increased amino acid catabolism in the context of compromised glucose oxidation may predispose to sarcopenia and muscle loss, particularly since Ulk1(S555) may decline with age (Nichenko

et al., 2021). Future studies should investigate disease-dependent relevance of Ulk1(S555) as a therapeutic target to promote metabolic flexibility.

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Materials and Methods

Animals. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. *Ulk1(S555A)* knock-in mice were generated in C57BL/6J background were generated at the Genetically Engineered Murine Model (GEMM) core facility at the University of Virginia (Guan et al., 2024). Homozygotic knock-in mice were generated from homozygous breeding pairs in-house and compared to background controls (JAX). Mice were housed in temperature-controlled (21°C) quarters with a 12:12 h light-dark cycle and *ad libitum* access to water and chow (Purina) until designated for study.

Caloric Restriction. At 6 months of age (24 weeks of age), mice were designated to *ad libitum* fed or caloric restriction (CR). Mice designated for CR were maintained in cages of 4-5 mice to normalize competition for food between groups. To calculate daily food allotment, food consumption was monitored for three consecutive days and averaged. Pre-CR NMR and GTT were taken (described below). For CR, mice were given food once daily at 1600. At the onset, CR groups spent 1 week at 20% CR as a step down and then continued at 40% CR for the subsequent 8 weeks. Post-CR NMR and GTT were taken during weeks 6 and 7, respectively, of CR to avoid any confounding with tracer or metabolomic measures.

24-hour Fast. Mice were designated into fed or 24-hour fast groups. Fed groups had ad libitum access to food until sacrificed. 24-hour fast groups were placed into new cages without food access for 24 hours before sacrifice. All harvests began at the onset of the light cycle (7:00).

Glucose tolerance. Glucose tolerance was performed at approximately 0800. For AL mice, food was removed at approximately 1800 to normalize the fasting period across groups. Blood glucose from the tail vein was measured before and following intraperitoneal injection of sterilized d-glucose (2mg/g) at 30, 60, and 120 min as previously (Drake et al., 2021; Guan et al., 2024; Laker et al., 2017).

Nuclear Magnetic Resonance. NMR (Bruker Minispec) was utilized to assess body composition. Before the assessment, the mini spec performed a quality control check of internal voltages, temperature, magnets, and

NMR parameters using the manufacturer's standards. Mice were placed and immobilized in a plastic tube and placed into the sample chamber for ~2 minutes, or the duration of the scan.

Calorimetry. Mice were individually housed in Columbus Comprehensive Lab Animal Monitoring System (CLAMS) metabolic cages and acclimated before a 48-hour observation (24-hour ad libitum fed/24-hour fasted) to assess respiratory exchange ratio, energy expenditure, and activity.

Tissue Procurement. For the CR study, prior to tissue harvest, CR mice were fed at 1600, and then all mice had food removed at 1800 by placing them in clean cages without food. Beginning at approximately 0700, mice were anesthetized with 3% isofluorane. The quadriceps and liver were collected antemortem and divided, with one part for metabolomics, another part prepared for radio-labeled tracing, and the rest frozen in LN_2 for later analyses. The metabolomics-designated tissues were clamp-frozen immediately and placed in LN_2. The tracing-designated tissues were placed in phosphate-buffered saline (PBS) and immediately prepped for radio-labeled tracing. Additional tissues collected were done post-mortem.

Radio-labeled glucose tracing. The liver was surgically dissected (above), and the quadriceps were further dissected into red (oxidative) and white (glycolytic) regions. Approximately 15-50mg of tissue was placed in 200 µl of modified sucrose EDTA medium (SET buffer) containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, and 1 mM ATP at pH 7.4. Tissues were minced with scissors, then SET buffer was added to create an approximate 20-fold dilution (weight : volume). Samples were homogenized in a Potter-Elvehjem glass homogenizer for 10 passes over 30 s at 150 rpm with a Teflon pestle. and then incubated in U-^{14}C-glucose or 6-^{14}C-glucose (American Radiolabeled Chemicals, St. Louis, MO, USA). Production of $^{14}CO_2$ was assessed by scintillation counting (4500 Beckman Coulter) and interpreted for glucose oxidative capacity. Total protein content in tissue homogenates was measured via BCA (Thermo Fisher Scientific, Waltham, MA, USA) and used to normalize oxidation values.

Untargeted Metabolomics (GC-MS). Untargeted GC/MS-based metabolomics analysis was performed on skeletal muscle (quadriceps) and liver from Ulk1 (S555A) and WT mice. Metabolites were extracted with 90%

methanol, dried, and derivatized. Metabolomic profiling was performed in an electron-ion (EI) mode using a Shimadzu triple quadrupole GCMS-TQ8050 NX (GC-MS/MS). Data analysis relied upon a combination of MS-Dial and MetaboAnalyst to identify features that were most different between groups (PCA plot, volcano plot, and heat map).

RNA seq/Transcriptomics. Frozen liver and quadricep muscle were homogenized in LN_2 via mortar and pestle, then added to Trizol. Total RNA was isolated via Qiagen RNeasy Mini Kit (74106). For RNA sequencing (Novogene, Durham, NC, USA), mRNA was purified from total RNA using poly T oligo-attached magnetic beads. Following fragmentation, first-string cDNA was synthesized using random hexamer primers, and second-strand cDNA was synthesized using dTTP for a non-directional library and checked with Qubit and real-time PCR for quantification and a bioanalyzer for size distribution detection. Libraries were pooled and sequenced on the Illumina platform (NovaSeq X plus series) according to library concentration and data amount (9 G per sample). Reference genome was built using Hisat2 v2.0.05 and differential expression analysis was performed using DESeq2Rpackage (1.20.0).

MixOmics. To identify correlations between metabolomics and transcriptomics data, regularized Canonical Correlation Analysis (rCCA) was used (González et al., 2009). rCCA identifies pairs of linear combinations, canonical variates, that maximize the correlation between the two datasets under regularization constraints to prevent overfitting. Data was input as normalized fragments per kilobase of transcript per million mapped reads (FPKM) for transcriptomics data, while peak quantification data from MetaboAnalyst was used as-is for metabolomics data (see Untargeted Metabolomics (GC-MS) section, above). Transcripts with more than two zero or missing values in any single sample group were excluded. The rcc() function from the MixOmics R package was used to generate correlation structures, which were evaluated based on the strength of canonical correlations and the interpretability of component loadings (Rohart et al., 2017). Specific metabolites of interest were examined to identify the most strongly correlated transcripts, visualized via network plots.

Mitochondrial isolation. Quadricep skeletal muscle and liver tissues were dissected and immediately placed in ice-cold Isolation Buffer Mitochondria-1 (IBM-1). Tissues were minced for 2 minutes, incubated with 0.25%

trypsin in IBM-1 for 30 minutes on ice, and then centrifuged at 200 x g for 3 minutes at 4°C. The pellet was resuspended in IBM-1 and homogenized in a pre-chilled glass homogenizer (10 passes at 80 rpm). Homogenates were cleared by centrifugation at 700 x g for 10 minutes at 4°C. The resulting supernatant was filtered through a 100μm nylon mesh and transferred to chilled high-strength glass centrifuge tubes. Mitochondria were pelleted at 8,000 x g for 10 minutes at 4°C, resuspended in IBM-2, and re-pelleted under the same conditions. Final mitochondrial pellets were gently resuspended in 100μL IBM-2 and kept on ice. Protein concentrations were quantified using a BCA assay with 1:10 and 1:20 sample dilutions.

Mitochondrial respiration. Mitochondrial respiration was measured using the Seahorse XFe96 extracellular flux analyzer (Agilent). Liver and skeletal muscle mitochondrial fractions were isolated from n = 6 WT and Ulk1(S555A) mice. The six biological replicates were combined in pairs to create n = 3 biological duplicates and analyzed as two technical replicates per pairing and condition. Mitochondrial-enriched lysates were seeded into the cell plate at a 2 ug/ul concentration. Media was prepared at the following concentrations: pyruvate (10 mM)/malate (5 mM) 7.4pH, succinate (10 mM) / rotenone (2 uM) 7.4pH, alanine (2 mM, 5 mM, 10 mM)/malate (5 mM) 7.4pH, and serine (2mM, 5mM, 10mM)/malate (5mM) 7.4pH. Ports were prepared with A: glutamate (10 mM) / malate (5 mM) and B: ADP (4 uM). Oxygen consumption rate (OCR) was obtained and calculated as previously described (10, 14). Mitochondrial respiration measurement cycles consisted of 3 min mixing, 2 min wait, and 3 min measurement cycles.

Western Blotting. Total protein lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Briefly, tissues were electrically homogenized in ~1 mL fractionation (FRAC) buffer [20 mM HEPES, 250 mM Sucrose, 0.1 mM EDTA, plus protease (Roche Diagnostics) and phosphatase (Sigma) inhibitors]. Whole-tissue lysate was resuspended in 50uL of Laemmli sample buffer, boiled for 5 min at 97°C, then frozen at -80°C until further analysis. Protein concentration was determined via RCDC assay. Membranes were probed with the following primary antibodies at 1:1000 dilution, targeting pUlk1 (S555) (CST #5869), tUlk1 (Sigma #A-7481) , α-tubulin (Abcam #ab7291)

Statistics. Data are presented as the mean ± SEM and were analyzed and plotted via GraphPad Prism v10. Data were analyzed via Student's t-test when one variable was present, two-way ANOVA when two variables were present, or repeated measures two-way ANOVA when two variables were measured in the same animals over time. Post-hoc analyses were only performed when a significant interaction between a categorical and a quantitative variable was found, which is indicated in the figures where applicable. Statistical significance was established a priori as $P < 0.05$.

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Figure Legends

Figure 1. Attenuated glucose oxidative capacity following caloric restriction in the absence of Ulk1(S555). A) Caloric restriction study design. B) Weekly body mass (g) during CR. C) Weekly body mass% change from baseline during CR. D) glucose tolerance test. E) GTT area under the curve. F) GTT AUC delta. G) Uniformly labeled U-^{14}C-Glucose tracer metabolic pathway. H) U-^{14}C-Glucose oxidation in red fibers isolated from quadriceps. I) U-^{14}C-Glucose oxidation in white fibers isolated from quadriceps. J) U-14C-Glucose oxidation in liver. n=5 per group. Data presented as mean ± SEM and analyzed via repeated measures or two-way ANOVA with post-hoc analysis when a significant interaction effect was found (* = $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$).

Figure 2. Caloric restriction leads to increased reliance on amino acids in the absence of Ulk1(S555). A) Enriched metabolites in quadriceps. B) Enriched metabolites in the liver. C) Alanine transamination pathway in skeletal muscle. D) Alanine transamination pathway in the liver. E) Mixomics analysis of quadricep transcripts correlated with alanine. F) Mixomics analysis of liver transcripts correlated with alanine. n=5 per group. Data presented as mean ± SEM and analyzed via two-way ANOVA with post-hoc analysis when a significant interaction effect was found (* = $p < 0.05$, ** = $p < 0.01$).

Figure 3. Altered metabolic switch during fasting in the absence of Ulk1(S555). A) WT and Ulk1(S555A) 48-hour respiratory exchange ratio (RER). B) WT and Ulk1(S555A) 48-hour energy expenditure (EE). C) U-^{14}C-Glucose and 6-^{14}C-Glucose oxidation pathway. D) U-^{14}C-Glucose oxidation in red and white fibers isolated from quadricep skeletal muscle in fed and 24-hour fasted states. E) 6-^{14}C-Glucose oxidation in red fibers isolated from quadricep skeletal muscle in fed and 24-hour fasted states. F) Plasma metabolomics heatmap of amino acids in WT and Ulk1(S555A) mice in fed and 24-hour fasted states. G) Analysis of plasma pyruvate between groups. H) Analysis of plasma L-serine between groups. K) Illustration of gluconeogenic and ketogenic amino acid metabolism during fasting in the absence of Ulk1(S555). n=5 per group. Data presented as mean ± SEM and analyzed via repeated measures or two-way ANOVA with post-hoc analysis when a significant interaction effect was found (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Figure 4. Loss of Ulk1(S555) increases mitochondrial oxidative capacity of glucogenic amino acids in skeletal muscle. A) Illustration of the alanine and serine oxidation fates in mitochondria. B) Alanine incubated oxygen consumption rate (OCR) over time. C) Alanine incubated baseline OCR. D) Alanine incubated OCR with the addition of glutamate/malate, E) Alanine incubated OCR with the addition of 5 mM ADP. F) Serine incubated OCR over time. G) Serine incubated baseline OCR. H) Serine incubated OCR with the addition of glutamate/malate. I) Serine incubated OCR with the addition of 5 mM ADP. n=6 per group. Data presented as mean ± SEM and analyzed via two-way ANOVA with post-hoc analysis when a significant interaction effect was found (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Supplemental Figure Legends

Figure S1. A) Average food intake (g) over 3 days. B) NMR lean mass delta (%BM). C) NMR fat mass delta (%BM). n = 5 per group. Data presented as mean ± SEM and analyzed via two-way ANOVA with post-hoc analysis when a significant interaction effect was found (** = $p < 0.01$).

Figure S2. A) Skeletal muscle (gastrocnemius) phospho-Ulk1(S555), total Ulk1, and α-tubulin. B) Liver phospho-Ulk1(S555), total Ulk1, and α-tubulin. n=5 per group. Data presented as mean ± SEM and analyzed via two-way ANOVA with post-hoc analysis when a significant interaction effect was found (*** = $p < 0.001$).

Figure S3. A) PCA plot generated in Metaboanalyst. B) Pathway impact of metabolites. C) Amino acid (glucogenic, ketogenic, both) fold change (AU). D) Illustration of identified amino acids and their potential contribution to glycolysis and the TCA cycle in skeletal muscle. n=5. Data presented as mean ± SEM and analyzed via two-way ANOVA with post-hoc analysis when a significant interaction effect was found (** = $p < 0.01$).

Figure S4. A) PCA plot generated in Metaboanalyst. B) Pathway impact of metabolites. C) Detected amino acids concentrations in liver (glucogenic, ketogenic, both), D) Illustration of identified amino acids and their potential contribution to glycolysis, TCA cycle in liver. n=5. Data presented as mean ± SEM and analyzed via two-way ANOVA with post-hoc analysis when a significant interaction effect was found (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

Figure S5. A) Activity (m) during fed and 24-hour fasted periods. B) U-^{14}C-glucose oxidation pathway and oxidation in red fibers isolated from the quadriceps of 9-month-old animals. C) Plasma metabolomics PCA plot. D) Plasma metabolomics heatmap of amino acids in WT and Ulk1(S555A) mice in fed and 24-hour fasted states. E-I) Plasma concentration of detected amino acids. n = 4 - 5. Data presented as mean ± SEM and analyzed via two-way ANOVA with post-hoc analysis when a significant interaction effect was found (* = $p < 0.05$, ** = $p < 0.01$).

Figure 1

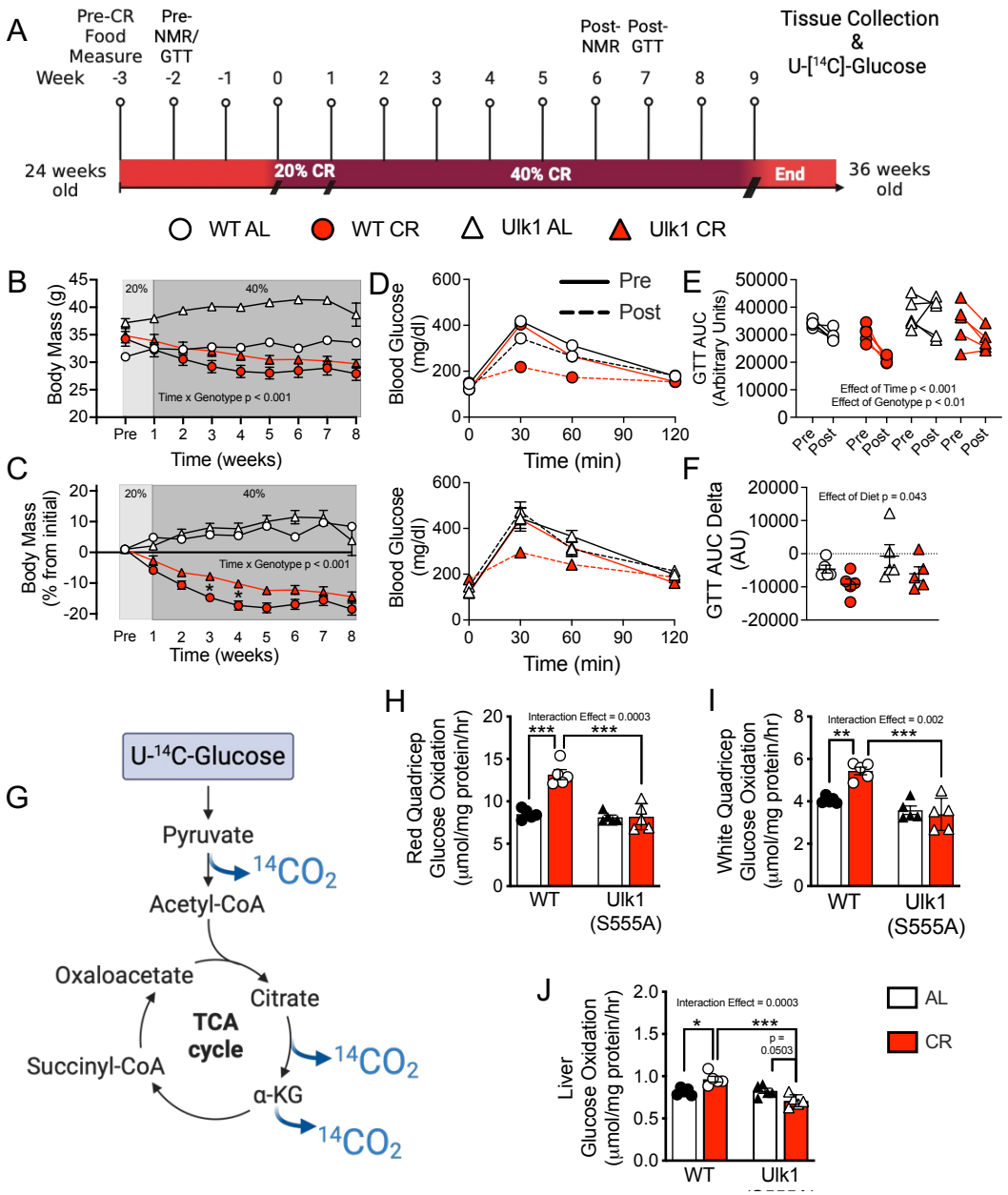

A
Pre-CR Food Measure
Pre-NMR/ GTT
Post-NMR
Post-GTT
Tissue Collection & U-[14C]-Glucose
Week -3 -2 -1 0 1 2 3 4 5 6 7 8 9
24 weeks old
20% CR
40% CR
End
36 weeks old
WT AL
WT CR
Ulk1 AL
Ulk1 CR
B
Body Mass (g)
20%
40%
Time x Genotype p < 0.001
Time (weeks)
C
Body Mass (% from initial)
Time x Genotype p < 0.001
Time (weeks)
D
Blood Glucose (mg/dl)
Pre
Post
Time (min)
E
GTT AUC (Arbitrary Units)
Effect of Time p < 0.001
Effect of Genotype p < 0.01
F
GTT AUC Delta (AU)
Effect of Diet p = 0.043
G
U-14C-Glucose
Pyruvate
14CO2
Acetyl-CoA
Oxaloacetate
Citrate
TCA cycle
Succinyl-CoA
α-KG
H
Red Quadricep Glucose Oxidation (µmol/mg protein/hr)
Interaction Effect = 0.0003
WT
Ulk1 (S555A)
I
White Quadricep Glucose Oxidation (µmol/mg protein/hr)
Interaction Effect = 0.002
J
Liver Glucose Oxidation (µmol/mg protein/hr)
Interaction Effect = 0.0003
p = 0.0503
AL
CR

Figure 2

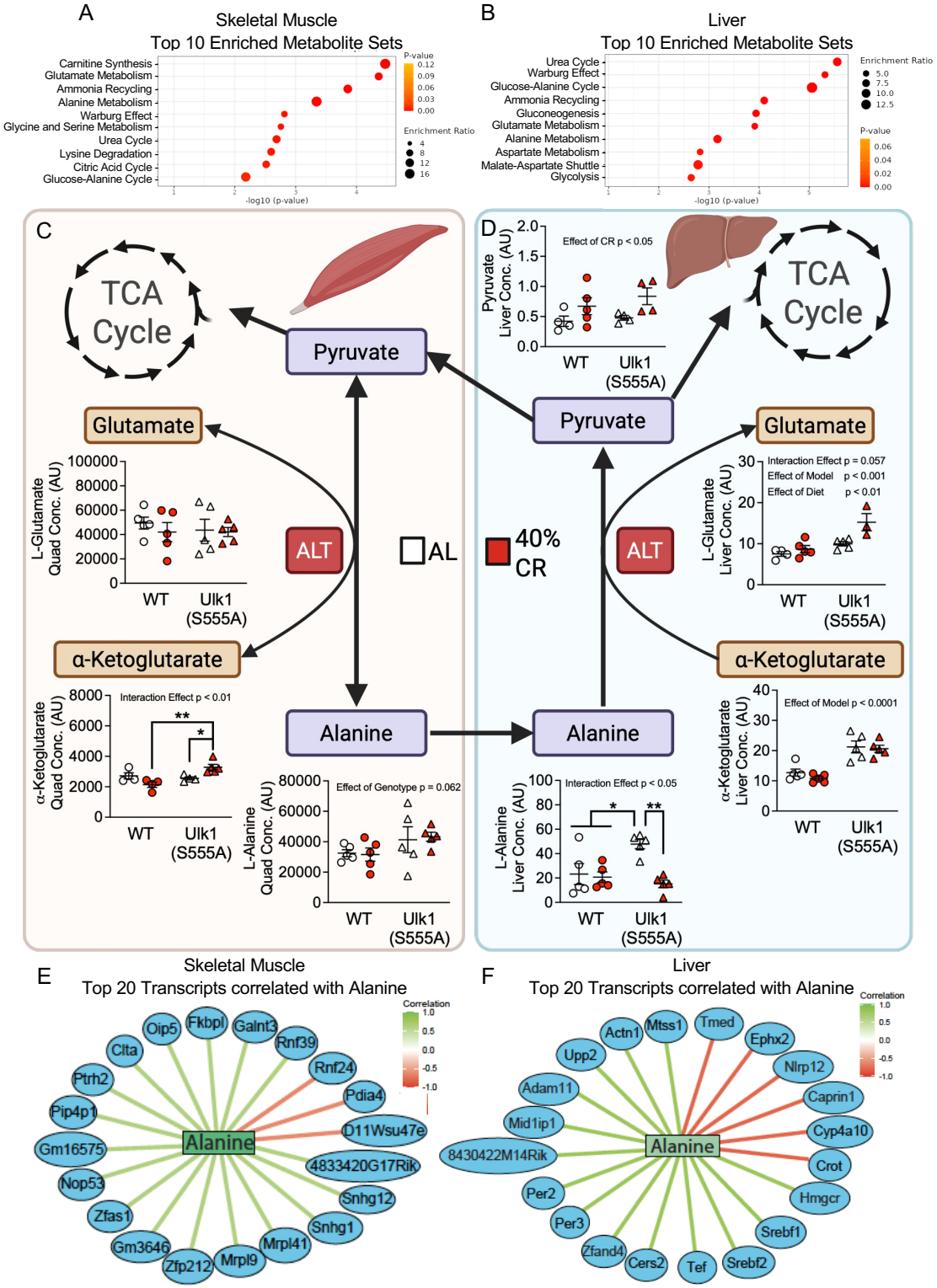
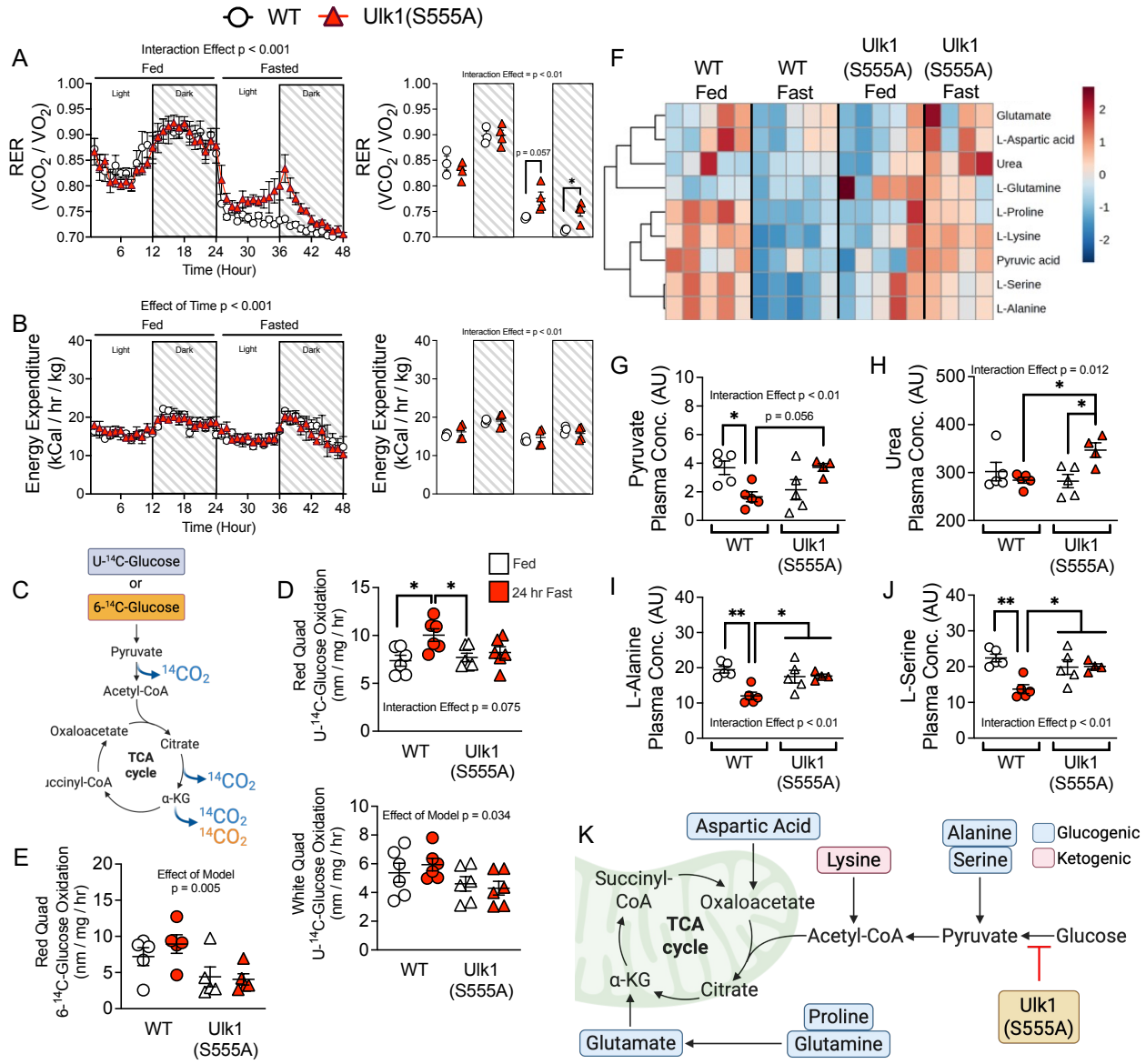
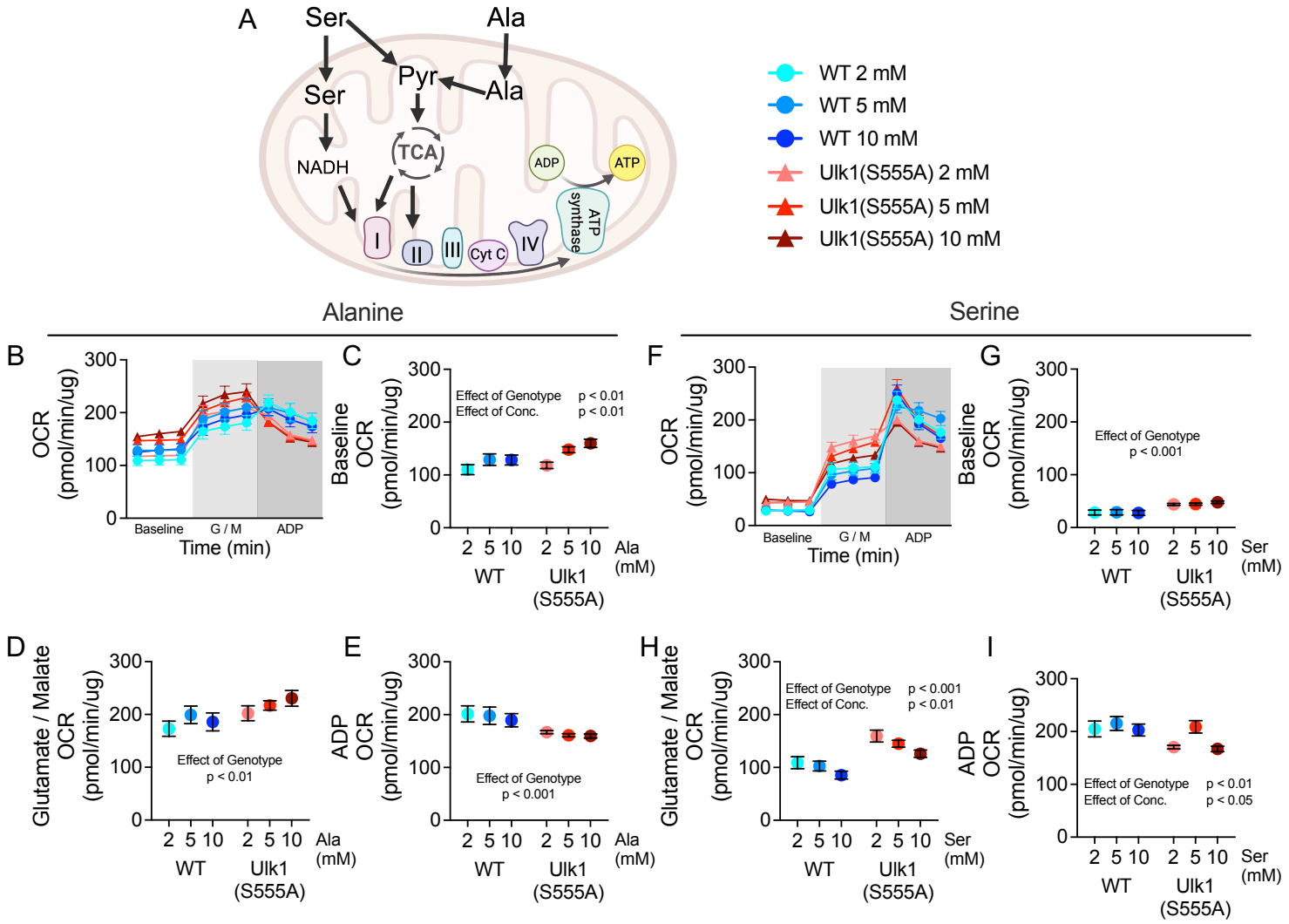

A
Skeletal Muscle
Top 10 Enriched Metabolite Sets
Carnitine Synthesis
Glutamate Metabolism
Ammonia Recycling
Alanine Metabolism
Warburg Effect
Glycine and Serine Metabolism
Urea Cycle
Lysine Degradation
Citric Acid Cycle
Glucose-Alanine Cycle
-log10 (p-value)
P-value
Enrichment Ratio
B
Liver
Top 10 Enriched Metabolite Sets
Urea Cycle
Warburg Effect
Glucose-Alanine Cycle
Ammonia Recycling
Gluconeogenesis
Glutamate Metabolism
Alanine Metabolism
Aspartate Metabolism
Malate-Aspartate Shuttle
Glycolysis
-log10 (p-value)
Enrichment Ratio
P-value
C
TCA Cycle
Pyruvate
Glutamate
L-Glutamate Quad Conc. (AU)
ALT
AL
α-Ketoglutarate
α-Ketoglutarate Quad Conc. (AU)
Interaction Effect p < 0.01
Alanine
L-Alanine Quad Conc. (AU)
Effect of Genotype p = 0.062
WT
Ulk1 (S555A)
D
Pyruvate Liver Conc. (AU)
Effect of CR p < 0.05
TCA Cycle
Pyruvate
Glutamate
L-Glutamate Liver Conc. (AU)
Interaction Effect p = 0.057
Effect of Model p < 0.001
Effect of Diet p < 0.01
40% CR
ALT
α-Ketoglutarate
α-Ketoglutarate Liver Conc. (AU)
Effect of Model p < 0.0001
Alanine
L-Alanine Liver Conc. (AU)
Interaction Effect p < 0.05
E
Skeletal Muscle
Top 20 Transcripts correlated with Alanine
Correlation
Alanine
Oip5
Fkbpl
Galnt3
Rnf39
Clta
Rnf24
Ptrh2
Pdia4
Pip4p1
D11Wsu47e
Gm16575
4833420G17Rik
Nop53
Snhg12
Zfas1
Snhg1
Gm3646
Mrpl41
Zfp212
Mrpl9
F
Liver
Top 20 Transcripts correlated with Alanine
Correlation
Alanine
Actn1
Mtss1
Tmed
Ephx2
Upp2
Nlrp12
Adam11
Caprin1
Mid1ip1
Cyp4a10
8430422M14Rik
Crot
Per2
Hmgcr
Per3
Srebf1
Zfand4
Cers2
Tef
Srebf2

Figure 3



Add 3-MA data to RER and metabolomics

Figure 4


A
Ser
Ala
Pyr
Ser
Ala
NADH
TCA
ADP
ATP
ATP synthase
I
II
III
Cyt C
IV
WT 2 mM
WT 5 mM
WT 10 mM
Ulk1(S555A) 2 mM
Ulk1(S555A) 5 mM
Ulk1(S555A) 10 mM
Alanine
Serine
B
OCR (pmol/min/ug)
Baseline
G / M
ADP
Time (min)
C
Baseline OCR (pmol/min/ug)
Effect of Genotype p < 0.01
Effect of Conc. p < 0.01
Ala (mM)
WT
Ulk1 (S555A)
D
Glutamate / Malate OCR (pmol/min/ug)
Effect of Genotype p < 0.01
E
ADP OCR (pmol/min/ug)
Effect of Genotype p < 0.001
F
G
Effect of Genotype p < 0.001
Ser (mM)
H
Glutamate / Malate OCR (pmol/min/ug)
Effect of Genotype p < 0.001
Effect of Conc. p < 0.01
I
ADP OCR (pmol/min/ug)
Effect of Genotype p < 0.01
Effect of Conc. p < 0.05