# Muscle mTORC1 Activation Causes Increased Energy Expenditure and Reduced Longevity in Mice

Erin J. Stephenson, JeAnna R. Redd, Detrick Snyder, Quynh T. Tran, Binbin Lu, Matthew J. Peloquin, Molly C. Mulcahy, Innocence Harvey, Kaleigh Fisher, Joan C. Han, Alan R. Saltiel and Dave Bridges

# Abstract

The mechanistic target of rapamycin (mTORC1) is a nutrient responsive protein kinase complex that helps co-ordinate anabolic processes across all tissues. There is evidence that signaling through mTORC1 in skeletal muscle may be a determinant of energy expenditure and therefore components downstream of mTORC1 signaling may be potential targets for treating obesity. Here, we have generated mice with *Ckm-Cre* driven ablation of *Tsc1* which confers constituent activation of mTORC1 in skeletal muscle. We have conducted unbiased transcriptional analyses to identify pathways and candidate genes that may explain how skeletal muscle mTORC1 activity regulates energy balance and aging. We show that increases in energy expenditure following a high fat diet- are mTORC1-dependent and that elevated energy expenditure caused by ablation of *Tsc1* coincides with the upregulation of skeletal muscle-specific thermogenic mechanisms that involve sarcolipin-driven futile cycling of Ca2+ through SERCA2. These findings support the hypothesis that activation of mTORC1 and its downstream targets, specifically in skeletal muscle, may play a role in nutrient-dependent thermogenesis. Future studies will identify whether targeting Ca2+ cycling through mTORC1 activation in skeletal muscle might be an effective target for weight loss interventions.

# Introduction

Obesity is a worldwide health problem, with comorbidities including diabetes, cardiovascular and liver disease [1]. Current modalities to prevent or reverse obesity are ineffective and short-lived either due to poor adherence to lifestyle interventions or reductions in energy expenditure and increases in hunger after weight loss [2–4]. The genetic and dietary modifiers of energy expenditure are not well understood, , but there is evidence that signaling through the mechanistic Target of Rapamycin Complex 1(mTORC1) pathway may play a role [5–8].

mTORC1 is a nutrient responsive protein kinase complex expressed in all known eukaryotic cells. This complex is activated by anabolic signals including insulin, amino acids and energy abundance (see [9] for review). mTORC1 integrates these signals, and helps to co-ordinate anabolic processes such as lipogenesis [10–12], glycogenesis [13], cellular differentiation [14–16] while promoting insulin resistance [10,17]. These effects are often tissue-specific, reflecting the cell-type specific responses to elevated nutrient and energy status.

Skeletal muscle is the major site of postprandial glucose disposal, and the primary determinant of resting energy expenditure in mammals [18,19]. Constitutive activation of mTORC1, via muscle-specific deletion of its negative regulator *Tsc1*, results in age-related myoatrophy, dysregulation of autophagy induction and increased expression of mitochondrial enzymes [6,20,21]. Consistent with the latter, cell culture models implicate mTORC1 as a positive regulator of mitochondrial function and ATP production [22–24], whereas in humans, during the aging process skeletal muscle exhibits a fiber-type transformation towards a more oxidative phenotype [25–27]. In line with this observation, several studies have implicated mTORC1 inhibition as a mechanism of organismal lifespan extension in yeast, worms and mammals [28–30]; however, the tissue or tissues that link mTORC1 activity to extended lifespan have not yet been identified.

Skeletal muscle is an important target tissue for understanding both aging and changes in energy metabolism, as functional differences in muscle strength predict lifespan in a longitudinal manner [31–36]. Furthermore, mTORC1 regulates several important metabolic processes in muscle; including oxidative stress, the unfolded protein response, autophagy and lipid metabolism [20,37,38]. Thus, we hypothesized that chronic mTORC1 activation in skeletal muscle would promote increases in energy expenditure, but would also play a role in reducing lifespan. Here, we have performed unbiased transcriptional analyses to identify pathways and candidate genes that may explain how skeletal muscle mTORC1 activity regulates energy balance and aging.

# Methods and Materials

## Animal Husbandry

All mice were purchased from The Jackson Laboratory. Unless otherwise stated, animals were fed a normal chow diet from Harlan Teklad. For high fat diet studies, animals were provided *ad libitum access* to a diet with 45% of calories from fat (Research Diets D1492). This diet started when animals were approximately 10 weeks of age, animals were sacrificed by cervical disolocation at 25 weeks of age.

Muscle-specific knockouts were generated by crossing FVB-Tg(Ckmm-cre)5Khn/J transgenic mice (stock 006405) with floxed *Tsc1tm1Djk*/J mice (stock 005680). To generate F1, mice that were heterozygous for the floxed allele, and either had or lacked the *Ckmm-Cre* transgene were intercrossed to generate knockout mice (*Tsc1fl*/fl, *Ckmm-Cre*Tg/+), wild type mice (*Tsc1+/+*, *Ckmm-Cre*+/+) and controls containing the transgene only (*Tsc1+/+*, *Ckmm-Cre*Tg/+)or the floxed allele only (*Tsc1fl*/fl, *Ckmm-Cre*+/+). All four genotypes were evaluated for all experiments. If there were no significant differences between the three control genotypes, these were combined and labeled as Controls. Animals were sacrificed in either the fed or fasted state as indicated in the figure legends after isoflurane-induced anaesthesia followed by cervical dislocation at approximately ZT3. The University of Michigan and UTHSC Institutional Animal Care and Use Committees approved all animal procedures.

## Insulin Tolerance Test

Insulin tolerance tests were performed in high fat fed mice at 24 weeks of age. The day prior to the test, body composition was determined via EchoMRI (Echo MRI1100, Houston, TX) and lean mass values were used to calculate insulin dose (1 U/kg of lean mass; Humulin R-100, Lilly, U.S.A). On the day of the test, fasting blood glucose concentrations were determined following a 6 hr fast, after which mice received an intraperitoneal injection of insulin. Blood glucose was monitored over a two hour period post-injection (One Touch Ultra2 hand-held glucometer, LifeScan Europe, Zug, Switzerland).

## Body Composition and Indirect Calorimetry

Total body weights were determined using a standard scale, whereas body composition was determined in conscious animals by magnetic resonance (EchoMRI 1100, EchoMRI, Houston, TX). Adipose tissue depot weights were determined post-mortem for both the left and right hand side and combined as total weights. For indirect calorimetry studies, physical activity, VO2 and VCO2 were determined using a home-cage style Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) with hanging feeders, under light and temperature-controlled conditions (12:12hr, 25ºC). The first 6h of measurements were discarded to accommodate acclimation, after which measurements continued over three consecutive days. Oxygen consumption was analyzed by mixed linear models with the considerations described in [39] ~~and normalized to lean body mass~~. Energy expenditure was calculated as heat, using the Lusk equation in Oxymax software (Columbus Instruments, Columbus, OH). For rapamycin treatments, animals were individually housed for 10 consecutive days (days 3-10 were in CLAMS cages). Mice received four days of vehicle treatment (1% Tween, 1% PEG-8000), followed by three days of treatment with either vehicle or the selective mTOR inhibitor rapamycin (3 mg/kg/d, via intraperitoneal injection). Mice were then switched to a high fat diet and were monitored for an additional three days, during which time mice continued to receive daily injections of either vehicle or rapamycin.

## Western Blotting

Protein lysates were generated in RIPA buffer (50 mM Tris pH 7.4, 0.25% sodium deoxycholate, 1% NP40, 150 mM sodium choride, 1 mM EDTA, 100 μM sodium vanadate, 5mM sodium fluoride, 10 mM sodium pyrophosphate and 1X protease inhibitors) or HTNG buffer (50 mM HEPES, pH 7.4, 150 mM sodium chloride, 10% glycerol, 10% triton X-100 and 1X protease inhibitors) by mechanical disruption in a Qialyser for 5 minutes at 30Hz. Lysates were clarified at 14 000 RPM for 15 minutes and quantified by Bradford assays. Proteins were separated by SDS-PAGE and blotted with antibodies described in the figure legends. Antibodies used in this study were raised against pS6 (pSer236/236, Cell Signaling #2211), S6 (Cell Signaling #2317), GAPDH and Sarcolipin. Blots were visualized on a LiCor Odyssey and quantified using near-IR secondary antibodies Image Studio software.

## RNA Sequencing Analysis and Bioinformatics

## Total RNA was purified using a Pure Link RNA mini kit from Life Technologies and then analyzed using an Agielent Bioanalyzer DNA High Sensitivity kit. All samples had a RNA Integrity numbers >7.9. The RNA (1 μg) was enriched for Poly A RNA using an Ambion Dynabeads mRNA Direct Micro kit and barcoded libraries for sequencing were prepared using the Life Technologies RNAseq V2 kit for Ion Torrent according to manufacturer’s standard protocol. The libraries were pooled based on the concentration of each sample between 200-350bp, purified on a Pippin Prep gel, quantified by the Agilent Bioanalyzer and sequenced on an Ion Torrent Proton sequencer. Alignments were made to the mouse genome GRCm38.75 using Tophat 2.0.10 [40] and Bowtie 1.0.0 [41] to incorporate colorspace data. Counts tables were generated using HTSeq version 0.5.4p5 [42]. Differential expression analyses were performed using DESeq2 version 1.20.0 [43]. All results are presented in Supplementary Table 1, and deposited into the Gene Expression Omnibus as XXXX. To compare our results to other gene-sets we performed Gene Set Enrichment Analyses (GSEA) comparing our rank-ordered gene lists to annotated gene sets from Gene Ontology, KEGG, Biocarta, Reactome, TRANSFAC and CGP provided as part of MSigDB v6.2 [44,45]. All pathways that met significance at an adjusted p-value of 0.25 are presented in Supplementary Table 2. For comparison of differentially expressed genes we re-analyzed the *Tsc2* knockout MEFs from GSE21755 [46] and compared with our differentially regulated gene sets.

## NADH Tetrazolium Reductase Staining

## Quadriceps muscles were frozen in isopentane, mounted in OTC and sectioned using a cryostat to 10 µm thickness. Frozen sections were incubated at 37°C for 30 min in pre-warmed 200 mM Tris buffer pH7.4, containing 245 µM nitro blue tetrazolium and 1.13 mM NADH. Sections were rinsed in water, dehydrated, and mounted under coverslips. Staining was visualized and photographed using an Evos XL Core transmitted-light inverted imaging system (Thermo Fisher Scientific, Waltham, MA).

## Statistical Analyses

All statistical analyses were performed using the R, version 3.2.2 [47]. For longitudinal measurements (body weights, fat mass and lean mass), the data were analyzed by mixed linear models using uncorrelated random slopes and intercepts using the lme4 package version 1.1-8 [48]. Statistical significance was determined via χ2 tests between models containing or missing the genotype term. Pairwise comparisons were tested first for normality via a Shapiro-Wilk test, then for equal variance via Levene’s test. For survival analyses and Cox proportional hazard tests, the survival package was used (version 2.38-3)[49,50]. We tested the assumptions of proportional hazards (with Shoenfeld residuals) and found no significant deviation from this assumption (p=0.875). Based on these, appropriate pairwise tests were performed as indicated in the figure legends. Corrections for testing of multiple hypotheses were done using the method of Benjamini and Hochberg [51]. Statistical significance was designated at p/q<0.05 for all assays, except GSEA analyses where q<0.25 was used. All raw data and statistical analyses for this manuscript are available at <http://bridgeslab.github.io/TissueSpecificTscKnockouts>.

# Results

## Rapamycin Treatment Reduces High Fat Diet Induced Increases in Energy Expenditure

Both short term overfeeding and chronic obesity result in increased energy expenditure. To test whether mTORC1 plays a role in the short-term responses to obesogenic diets, we measured the total energy expenditure of C57BL6/J mice during a dietary shift between low fat and high fat diets in the presence or absence of the specific mTOR inhibitor rapamycin. As depicted in Figure 1A, individually housed animals were vehicle-injected daily for four days, followed by three days of either vehicle or rapamycin injection. After three days of treatment, all animals were moved from normal chow diets to a high fat diet (HFD). As shown in Figure 1B, the switch to HFD caused a 7.8% increase in VO2 in the vehicle injected in the dark phase and a 6.8% increase in the light phase. Rapamycin injection suppressed the HFD-induced increase in VO2 compared to vehicle treated mice (p=1.24 x 10-5), and these effects were not associated with differences in physical activity (Figure 1C). These data support the hypothesis that mTORC1 is required for the increase in energy expenditure observed in response to HFD feeding.

## Activation of mTORC1 in Muscle is Sufficient for Increased Energy Expenditure

To test whether skeletal muscle mTORC1 activation resulted in elevated energy expenditure, we performed indirect calorimetry studies on *Ckmm-Cre* driven *Tsc1* knockout mice. Consistent with prior reports [6] we observed elevated energy expenditure in muscle specific *Tsc1* knockout mice (Figure 1D, p<1 x 106), and the magnitude of this difference was greater during the dark phase (⭡17% in males, ⭡7.5% in females). There were no significant differences in physical activity during the monitoring period (Figure 1E­­­­). Although there were no differences in the respiratory exchange ratio between knockout and wild-type male mice (Figure 1F), female muscle *Tsc1* knockout mice had lower respiratory exchange ratio’s during the dark period (indicating greater lipid utilization), and higher respiratory exchange ratio’s during the light period (indicative of more carbohydrate utilization) compared to their wild-type counterparts. This suggests there may be a sexually dimorphic component of mTORC1 signaling that determines metabolic flexibility. These data are consistent with a physiological role for mTORC1 in moderating organismal energy expenditure.

**Activation of mTORC1 in Muscle does not alter energy intake**

We next evaluated the effect of *Ckmm-Cre* driven *Tsc1* knockout in animals receiving either standard laboratory chow or a high fat diet. As shown in Figure 1E, while all mice receiving the high fat diet ingested more calories than those receiving chow, there was no significant difference in energy intake between wild-type and muscle *Tsc1* knockout mice within each diet grouping. This is in contrast to previous reports that show mice with *ACTA1-Cre* driven *Tsc1* knockout eat more food than control mice when provided a diet consisting of 60% calories from fat [Guridi 2016 Skeletal Muscle].

## Knockout of *Tsc1* in Muscle Causes Resistance to Age- and Diet-Induced Obesity

Given the finding that mTORC1 activation in skeletal muscle caused elevated energy expenditure in the absence of increased energy intake, we sought to understand the physiological significance of mTORC1 activation on body composition. The body composition of male muscle *Tsc1* knockout mice receiving a standard chow diet was determined weekly, over the course of 7 months. As animals aged the wild-type mice accreted more fat mass, whereas we observed a striking 84% lower fat mass gain in the knockout animals as they aged (Figures 2A, p=1.7 x 10-10). Previous work using *ACTA1-Cre* mediated knockout of *Tsc1* also observed lower fat mass [5,6], but in those reports there was a concomitant reduction in lean mass [6,21], a finding not replicated in this study (Figure 2B, p=0.743 at endpoint). To determine if the reducd fat mass was adipose depot-specific, we determined the weights of subcutaneous (dorso-lumbar inguinal) and visceral (epididymal) fat pads from wild-type and *Tsc1* knockout mice, and found that both fat depots were smaller in size (⭣79% and ⭣76% respectively, each p<0.0001; Figure 2C).

To determine whether a palatable, hypercaloric diet would induce changes in body composition in mice with skeletal muscle ablation of *Tsc1*, we placed male and female mice on a diet containing 45% of calories from fat. We found that both male and female muscle-specific *Tsc1* knockout mice were resistant to weight gain on high fat diet. The difference in body weight was primarily determined by differences in fat mass, which, compared to wild-type mice, was 60% lower in knockout males and 58% lower in knockout females by the end of the study (Figure 3A, p < 1.0 x10-6 for each). These data are consistent with previous reports in *ACTA1-Cre* mediated Tsc1 knockout mice fed a diet containing 60% calories from fat [REFS], however in our model lean masses were similar between wild-type and knockout mice on the high fat diet (Figure 3B, p=0.941)~~, though female lean mass was lower than that of male mice~~. Consistent with the magnetic resonance data for total body fat, we observed a 75-80% difference in the weights of both the perigonadal and inguinal fat pads from male and female knockout mice compared to their relative control groups (all p<0.001; Figure 3C).

To test whether the attenuation of fat mass gains in muscle *Tsc1* knockout mice was a form of lipodystrophy relating to insulin responsiveness, we performed insulin tolerance tests. As shown in Figure 3D, compared to wild-type mice, both male and female muscle *Tsc1* knockout mice that received a high fat diet were more insulin responsive (effect sizes, p-values). This is consistent with the hypothesis that the adiposity is inversely related to insulin sensitivity and that these mice are not lipodystrophic *per se*, rather that mice with muscle *Tsc1I* knockout are protected from adipose tissue expansion through triglyceride accumulation in response to a high fat diet.

## Ablation of Muscle *Tsc1* Results in Increased Oxidative Enzymes in Muscle Fibers and Upregulation of Fatty Acid/Amino Acid Uptake Genes

To gain further insight into the mTORC1 activity-driven mechanisms within skeletal muscle that increase energy expenditure and help protect *Tsc1* knockout mice from diet-induced obesity, we performed RNA sequencing studies in quadriceps muslce. We identified 4403 significantly differentially expressed genes in these animals, including 2464 upregulated genes and 1939 downregulated genes (see Supplementary Table 1 for complete list). To identify the pathways and networks associated with these differentially expressed genes, we performed gene-set enrichment analyses, finding 674 differentially regulated gene sets (see Supplementary Table 2). Among the significantly enriched gene sets were genes also regulated by *Tsc2* deletion in MEFs, and by treatment with rapamycin [46,52], indicating there are a core set of mTORC1 dependent genes that are similarly regulated across different tissues. Consistent with this observation, we found that 58% of the differentially expressed genes in our muscles overlapped with previously published differentially expressed genes in *Tsc2* knockout MEFs [46]. Additionally, we identified several other gene sets that were upregulated in muscle, including IGF1 targets in MCF-7 cells [53], genes involved in calcium trafficking, protein synthesis, and amino acid and fatty acid transport. Most amino acid transporters were increased at the mRNA level (Figure 4A), while both the fatty acid transporter *Cd36* and binding protein *Fabp3* were also increased at the transcriptional level (Figure 4B).

We also evaluated transcriptional markers of muscle fiber type and observed increases in Type I oxidative fiber markers, including *Myh7* and *Atp2a2*, along with a downregulation of glycolytic fiber type markers, including *Myh4* and *Atp2a1* (Figure 4C). These data suggest that skeletal muscle mTORC1 activation increases the oxidative profile of skeletal muscle at the transcriptional level. These findings are supported by our observation that skeletal muscle from *Tsc1* ablated mice has substantially more NADH activity (Figure 4D), and are consistent with findings from previous studies on *ACTA1-Tsc1* knockout muscles that report the accumulation of mitochondrial enzymes and changes in muscle fiber size [REFS].

To identify the molecular mechanisms causing increased energy expenditure in skeletal muscle *Tsc1* knockout mice, we evaluated the expression of transcripts known to be important contributors to skeletal muscle thermogenesis. We observed dramatic increases in the ATP-dependent SR/ER Calcuim pump SERCA2 (encoded by *Atp2a2*, see Figure 4C), and its un-coupler Sarcolipin (encoded by *Sln*; Figure 4E), proteins previously reported as playing an integral role in muscle-specific thermogenesis [54–57]. At the protein level, Sarcolipin was increased 4.1 fold (p=4.5 x 10-6; Figure 4F, pS6 is shown as a positive control). Unlike previous reports that show increased *Ucp2* mRNA in *ACTA1-Tsc1* knockout muscles, we noted no significant differences in the expression of transcripts for *Ucp1*, -*2* or -*3* (not shown). Thus, we propose that the increased oxidative activity in muscles from mice with *Tsc1* ablation via the *Ckm-Cre* promotor may be caused by increased futile cycling of Ca2+ by uncoupled SERCA2 (therefore increasing ATP hydrolysis) at the SR. Consistent with this hypothesis, we observe increases in the expression of *Stim1*, *Mfn1-2* and the subunits of the mitochondrial calcium importer (*Mcu, Micu1* and *Micu2*). We also observed reductions in *Ryr1* expression and reductions several plasma membrane Ca2+ transporters (see Supplementary Table 1), changes that are likely adaptive mechanisms to manage increased intracellular Ca2+ levels caused by SERCA2 uncoupling.

## Deletion of *Tsc1* in Muscle Tissues Reduces Lifespan.

To determine whether skeletal muscle mTORC1 activation-induced increases in energy expenditure affected lifespan, we monitored muscle *Tsc1* knockout animals without manipulation as they aged. Increased signs of aging, including hunched and scruffy appearances at an earlier age, were observed in knockout mice compared to their wild-type littermates (anecdotal/not shown). As shown in Figure 5, muscle-specific *Tsc1* knockout mice died of natural causes earlier than wild-type mice. Based on a Cox-proportional hazard model the hazard ratio was 4.17-fold higher compared to non-knockout littermates (p=2.0 x 10-5).

To determine how muscle *Tsc1* ablation reduces lifespan, a subset of mice were fixed in formalin upon death and sent for veterinary pathology. However, we were unable to identify a consistent cause of death in these mice. In mice with histologic evidence of lesions, the predominant process was neoplasia, and the specific etiology was lymphoma/lymphosarcoma affecting multiple organs, though this was only true for wild-type mice (two out of four) but not knockout animals (none out of three). It is important to note that lack of a specific diagnosis does not necessarily confirm the lack of lesions in examined animals; rather, that autolysis and the small number of animals evaluated may have resulted in loss of identifiable processes or tissues in which an etiology was present in-life.

# Discussion

Here, we show that high fat diet-induced increases in energy expenditure are mTORC1-dependent and that elevated energy expenditure caused by constitutive activation of skeletal muscle mTORC1 coincides with the upregulation of skeletal muscle-specific thermogenic mechanisms that involve the futile cycling of Ca2+ Consistent with prior work [6,20], we also show transcriptional evidence for an mTORC1-driven fiber type transition to a more oxidative phenotype, along with other markers of altered substrate oxidation and energy transformation in skeletal muscle.

Skeletal muscle is an important determinant of both energy balance and healthy aging. Humans with high baseline grip strength have 20-217% decreased risk of all-cause mortality, irrespective of sex or body mass index [31–36], whereas interventions that increase muscle mass and strength are associated with improved health outcomes in both young and older populations [58,59]. Given the important role of skeletal muscle in healthy aging and that skeletal muscle is the primary determinant of resting energy expenditure [60], understanding the molecular mechanisms that influence skeletal muscle health could have important ramifications for the way we treat diseases associated with both obesity and aging.

Our understanding of how mTORC1 regulates skeletal muscle physiology largely consists of the translation-initiation and post-translational role of mTORC1 and its response to growth factors, nutrients and mechanical loading [9]. Less is known regarding the role of mTORC1 in the regulation of skeletal muscle substrate oxidation and energy expenditure; however, it has been shown that gene silencing of mTORC1 alters ATP generation through disruption of PGC-1α driven mitochondrial signaling in vitro [22], whereas the increase in *Ppargc1a* expression that occurs in muscle during the acute phase post-exercise has been shown to be potentiated in mouse skeletal muscle when mTORC1 is inhibited by rapamycin [61].

Previous studies have shown that activating mTORC1 in mice through deletion of *Tsc1* in skeletal muscle results in smaller mice that have significantly lower body fat than wild-type mice and are resistant to both diet-induced obesity and age-associated gains in adiposity [5,6]. Our results are in agreement with these data, and we provide evidence that the lower body fat observed in these animals may be conferred, in part, by an increase in skeletal muscle thermogenesis via sarcolipin-driven uncoupling of SERCA2.

Skeletal muscle thermogenic pathways are important contributors to both shivering and non-shivering thermogenesis and while mitochondrial-generated thermogenic pathways have been well described as potential targets for leveraging skeletal muscle thermogenesis to combat obesity [van den Berg 2011 Curr Opin Clin Nutr Metab Care 14(3):243-9], other, lesser-known heat generating pathways may also be important. The Sarco/Endo-plasmic Reticulum Ca2+-ATPase (SERCA) transfers Ca2+ from the sarcoplasm into the lumen of the sarcoplasmic reticulum, hydrolyzing ATP in the process. Sarcolipin, a small helical peptide, can interact with SERCA and alter the kinetics of Ca2+ re-sequestration by allowing slippage of Ca2+ back into the sarcoplasm, thereby ‘decoupling’ Ca2+ uptake from SERCA-dependent ATP hydrolysis by creating a futile cycle of Ca2+ movement that generates heat [Smith 2002 Biochem J 361 (2)277-86]. In the present study, *Ckm-cre* driven *Tsc1* deletion resulted in an increase in whole-body energy expenditure [**Fig. 1**], the transcriptional upregulation of both *Serca2a2* and *Sln* [**Fig's. 4C and 4E**], and an increased protein abundance of sarcolipin [**Fig. 4F**]. Increased amounts of sarcolipin would likely i the, resulting in the futile cycling of Ca2+ and the generation ofin the muscles [54,62]. This hypothesis is supported by changes in transcripts for a number of other Ca2+ transporters and Ca2+ responsive mRNA’s in the muscles of these mice [**Table X**]. Increased muscle thermogenesis through the sarcolipin-driven uncoupling of SERCA would likely contribute to the increase in energy expenditure and subsequent lower body fat we observe in muscle-specific *Tsc1*-knockout mice, areports that obesity is [54–56]or ed*Sln* is [57]

Other mechanisms linking muscle mTORC1 activity to elevated energy dissipation may be indirect, such as increased energy expenditure associated with elevations in muscle-derived FGF21 [6,63,64] or other factors. Or they may be more obvious, such as altered skeletal muscle mitochondrial metabolism. We and others have observed that skeletal muscle-specific activation of mTORC1 via deletion of *Tsc1* results in an increase in the oxidative profile of the skeletal muscle [**Fig. 4D**; Bentzinger 2013]. Furthermore, non-*Tsc1*-driven models of muscle-specific mTORC1 activation, such as those involving knockout of individual components of the GATOR1 complex, result in increased expression of mitochondrial components, including TCA cycle intermediates [Dutchak 2018 Cell Rep 23:1907-14], and increased mitochondrial respiration [Graber 2019 J Biol Chem, E Pub doi: 10.1074/jbc.RA118.005970]. Conversely, abolishing skeletal muscle mTORC1 activity via Raptor knockout increases mitochondrial coupling efficiency but lowers mitochondrial respiration and reduces the abundance and activities of mitochondrial enzymes [Risson 2009 J Cell Biol 187(6):859-74]. Taken together, these reports suggest that mTORC1 influences mitochondrial metabolism both through determining mitochondrial enzyme content and the coupling of oxidative phosphorylation to ATP production, mechanisms that would influence energy expenditure at the whole-body level.

An equilibrium between anabolic and catabolic processes is essential to the healthy functioning of any cell. An important factor worth taking into consideration when interpreting the data presented here (and elsewhere) is that dysregulation of autophagy has been previously reported in response to skeletal muscle mTOCRC1 activation [Castets et al]. The presence of vacuolated mitochondria and intracellular inclusions that contain mitochondrial enzymes in *Tcs1*-null skeletal muscle would suggest that the increase in oxidative markers reported in other studies [REF] and our own might be the result of impaired mitophagy/autophagy and the accumulation of damaged and dysfunctional mitochondrial structures. This would explain the apparent oxidative fiber-type shift without a change in other markers of improved mitochondrial function. If mTORC1 active muscle is limited by its ability to dissipate excess energy via mitochondrial mechanisms, it is possible the increase in sarcolipin and our proposed mechanism of futile Ca2+ cycling is a compensatory response to this deficit, rather than an mTORC1-specific adaptation.

The pathways underlying how mTORC1 influences mitochondrial metabolism in skeletal muscle are unclear, but since amino acids and fatty acids both require mitochondria for their metabolism, it is possible that alternations in substrate flux could result in a transition toward more oxidative fibers within the muscle in a muscle-autonomous way, depending on the tissues needs. For example, it has been suggested that skeletal muscle *Nprl2* deletion increases aerobic glycolysis in order to provide more TCA cycle intermediates for non-essential amino acid synthesis [Dutchak 2018 Cell reports]. Similarly, increased fatty acid uptake into the muscle might influence rates of lipid oxidation and, consequently, alter adiposity. This hypothesis is consistent with anti-obesogenic effects of muscle specific *Cd36* overexpression [65] and with the observation that elevated free fatty acids can promote mitochondrial biogenesis [66]. Increased nutrient uptake, coupled with elevated mitochondria could potentially produce more ATP in these cells. As such muscle *Tsc1* knockout animals could be plausibly thermogenic due to the combined effects of multiple muscle-autonomous adaptations, including (but not limited to) amino acid and fatty acid transport, mitochondrial activity and sarcolipin-mediated uncoupling

Another consideration that must be made is that the rapamycin experiments presented here do not speak to tissue specificity, as they are consistent with previous reports demonstrating rapamycin sensitivity in cold-induced thermogenesis [7,8]. While the focus of those studies has been on the important roles of mTORC1 in BAT function, they may also speak to the role of mTORC1 in muscle or other tissues. Future studies, with temporal and tissue specific loss of function of mTORC1 at thermoneutrality will be key to understanding the relative importance of muscle and BAT in both diet and cold-induced thermogenesis. Furthermore, findings that mTORC1 is important for thermogenesis in both BAT and skeletal muscle may indicate a broader role of mTORC1 in nutrient homeostasis. One response to nutrient overload is to promote anabolism, consistent with mTORC1-dependent activation of protein synthesis, lipogenesis, and glycogenesis [38,67,68]. Thus, it is reasonable to propose that nutrient overload may promote ineffective catabolism as a way of reducing systemic nutrient stress.

Transcriptional profiling across species has identified downregulation of mitochondrial genes in skeletal muscle as a common aging signature [Zahn 2006 PLoS Genetics 2:e115; Phillips 2013, PLoS Genetics 9(3):e1003389], whereas loss of skeletal muscle mitochondrial function is associated with age-related sarcopenia in C. elegans [Gaffney, 2018, Aging 10(11):3382-96]. Candidate gene studies on aging have implicated genes with important roles in skeletal muscle metabolism, including *IGF1R*, *AKT1* and *FOXO3A* [69,70], genes that are also linked to mTORC1 signaling. In humans, polymorphisms in *FOXO3A* have been associated with lengthened lifespan[70–76], whereas both mouse and fruit fly models of *FOXO3A* loss of function result in stronger and longer living model organisms [77–79]. Skeletal muscle-specific activation of mTORC1 via deletion of *Tsc1* caused an increase in the oxidative profile of the skeletal muscle [**Fig. 4D**; Bentzinger 2013]; however, constitutively activating mTORC1 in the muscle also shortened lifespan [**Fig. 5**, REF]. Indeed, nonagenarians show downregulation of mTOR pathway genes [Passtoors 2013, Aging Cell 12(1):24-31], supporting a role for decreased mTOR signaling in human longevity. Interestingly, mTORC1 sensitive genes, including ribosomal RNA’s, are also downregulated following chronic resistance exercise training and this effect is greater in those who are high responders for skeletal muscle hypertrophy [Phillips et al. 2013]. Here, we show that despite an apparent increase in muscles oxidative phenotype, constituent activation of mTORC1 in skeletal muscle decreases lifespan in mice, a finding that is in consensus with other models of mTORC1 activation [REFS].

In conclusion, we have shown that increases in energy expenditure following a high fat diet- are mTORC1-dependent and that elevated energy expenditure caused by ablation of *Tsc1* and thus constituent activation of skeletal muscle mTORC1, coincides with the upregulation of skeletal muscle-specific thermogenic mechanisms that involve the sarcolipin-driven futile cycling of Ca2+ through SERCA2. These findings support the hypothesis that activation of mTORC1 and its downstream targets, specifically in skeletal muscle, may play a role in nutrient-dependent thermogenesis. Future studies will identify whether targeting Ca2+ cycling through mTORC1 activation in skeletal muscle might be an effective target for weight loss interventions.

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# Figure Legends

**Figure 1: mTORC1 regulates energy expenditure.** A) Schematic of rapamycin/high fat diet experiments, with effects on B) energy expenditure and C) ambulatory activity. Muscle *Tsc1* knockout at 70 days of age mice are evaluated by indirect calorimetry for heat production (D), ambulatory activity (E), respiratory exchange ratio (F). G) Food intake for mice on a NCD or HFD.

**Figure 2: Reduced age-associated weight gain in muscle *Tsc1* knockout mice.** A) Fat and B) Fat-Free mass of male animals from birth followed for 28 weeks. C) Inguinal and gonadal fat pad weights. Statistical significance (n=7 and 25) is denoted by asterisks which indicate p<0.05 based on a χ2 test (A) or Mann-Whitney test (C, due to lack of normality).

**Figure 3: Muscle *Tsc1* Knockout mice are resistant to diet-induced obesity and insulin resistance.** C) Fat pad weights at sacrifice. Statistical significance (p<0.05, n=5/7) was determined via a Welch’s *t* test (C, males), a Mann-Whitney (C, females, due to lack of normality).

**Figure 4: Transcriptional and structural changes in *Tsc1* knockout muscles.** A) Expression of A) Amino Acid Transporters B) Fatty Acid Transporters from RNAseq data. Markers of oxidative and non-oxidative muscles (C) and sections from quadriceps stained with NADH/NBT (D). Darker staining indicates oxidative fibers. E) mRNA and F) protein levels of Sarcolipin from quadriceps muscles. Asterisks indicates adjusted p value of <0.05.

**Figure 5: Survival curve of male muscle *Tsc1* knockout mice on a normal chow diet.** Dotted lines indicate age at which 50% of animals died.

# Supplementary Figure Legends

**Supplementary Table 1: Gene expression differences in muscle *Tsc1* knockout quadriceps.** Full results of differential expression analysis.

**Supplementary Table 2: Gene set enrichment analysis of *Tsc1* knockout quadriceps.** All pathways that met an adjusted p-value of 0.25 are shown. NES (net enrichment score) indicates pathway effect size with positive numbers indicating positive enrichment of this gene set in these data. Gene details are the genes which drove this positive or negative association. Both nominal (NOM) and FDR adjusted (FDR) p/q values are shown.