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

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# Abstract

# 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 often due to 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 recent reports have implicated low-carbohydrate (or high protein) diets in enhancing energy expenditure in both overfeeding and weight loss paradigms [5,6]. The mechanism(s) by which these diets exert these effects have not yet been completely elucidated.

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 [7] for review). mTORC1 integrates these signals, and helps to co-ordinate anabolic processes such as lipogenesis [8–10], glycogenesis [11], cellular differentiation [12–14] while promoting insulin resistance [8,15]. These effects are often tissue-specific, reflecting the cell-type specific responses to elevated nutrient and energy status.

Although several studies have implicated mTORC1 inhibition as a mechanism of organismal lifespan extension in yeast, worms and mammals [16–18], the tissue or tissues that link mTORC1 activity to extended lifespan have not yet been identified. Skeletal muscle is an important potential target tissue for understanding aging, as functional differences in muscle strength predict lifespan in a longitudinal manner [19–24]. Furthermore, mTORC1 regulates several important, aging related processes in muscle; including oxidative stress, the unfolded protein response, autophagy and lipid metabolism [25–27].

Skeletal muscle is the major site of postprandial glucose disposal, and the primary determinant of resting energy expenditure [28,29]. Constitutive activation of mTORC1, via muscle-specific deletion of its negative regulator *Tsc1*, have identified age-related myoatrophy and a switch towards oxidative fiber types [26,30,31]. Consistent with this, cell culture models implicate mTORC1 as also a positive regulator of mitochondrial function and ATP production [32–34], whereas human skeletal muscle exhibits a fiber-type transformation towards more oxidative fibers during the aging process [Ciciliot S Int J Biochem Cell Biol 2013 45(10):2191-9], a change concomitant with increased mTORC1 activity [Sandri M 2013 Biogerontology 14(3):303-323; Markofski M 2015 Exp Genrontol 65:1-7]. Additionally, mTORC1 is strongly activated in skeletal muscle in response to high protein diets or supplementation with essential amino acids [35,36]. We therefore hypothesized that in oxidative tissues such as skeletal muscle, chronic mTORC1 activation promotes increases in energy expenditure, but may also play a role in reducing organismal lifespan.

# 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 lard (Research Diets D1492). This diet started when animals were approximately 10 weeks of age, animals were sacrificed 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 b, whereas body composition was determined using an EchoMRI 1100 at approximately ZT10. 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 [37] ~~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 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 just 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 were 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 would increase energy expenditure, we performed indirect calorimetry studies on *Ckmm-Cre* driven *Tsc1* knockout mice. Consistent with prior reports [31] we observed elevated energy expenditure in muscle specific *Tsc1* knockout mice (Figure 1D, p<1 x 106), and the magnitude of this increase 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 reduced 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 food intake in these animals on both NCD and HFD diets. As shown in Figure 1E, while the HFD animals ingested more calories, there was no significant difference in energy intake between wild-type and muscle *Tsc1* knockout mice.

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

Given the finding that mTORC1 activation in skeletal muscle caused elevated total 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% reduction in fat mass gains 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 reductions in fat mass [31,52], but in those reports there was a concomitant reduction of lean mass [30,31], a finding not replicated in this study (Figure 2B, p=0.743 at endpoint). To determine if the reduction in 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 reduced 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 reduced 60% in knockout males and 58% in knockout females by the end of the study (Figure 3A, p < 1.0 x10-6 for each). 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 data for total fat mass, we observed a 75-80% reduction in the weights of both the perigonadal and inguinal fat pads from both male and female mice (all p<0.001; Figure 3C).

To test whether the attenuation of fat mass gains in muscle *Tsc1* knockout mice was the result of aberrant fat accumulation in non-adipose tissue, or a form of lipodystrophy we performed insulin tolerance tests. As shown in Figure 3D, compared to wild-type mice, both male and female muscle *Tsc1* knockout mice were more insulin responsive after HFD treatment, consistent with the hypothesis that the adiposity is inversely related to insulin sensitivity. These data support the hypothesis that mice with muscle *Tsc1I* knockout are protected from adipose tissue expansion in response to a high fat diet.

## Ablation of Muscle *Tsc1* Results in Increased Oxidative 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 help protect *Tsc1* knockout mice from diet-induced obesity, we performed RNA sequencing studies. 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 gain insight into 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 [46,53], 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, including IGF1 targets in MCF-7 cells [54], 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 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. These findings are consistent with our observation that skeletal muscle from *Tsc1* ablated mice has substantially more NADH activity (Figure 4D), as well as findings from previous studies on *Tsc1* knockout muscles.

To identify the molecular mechanisms causing increased energy expenditure in skeletal muscle *Tsc1* knockout mice, we evaluated the expression of transcripts previously reported as being important for the determination of skeletal muscle thermogenesis. We observed dramatic increases in the ATP-dependent 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 [55–58]. 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). We noted no significant differences in the uncoupling proteins UCP1-3 (not shown). Thus, we propose that the increased oxidative activity in muscles from mice with *Tsc1* ablation may be caused by increased futile cycling of Ca2+ by uncoupled SERCA2 (therefore increasing ATP hydrolysis) at the ER. 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). 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

Skeletal muscle is an extremely important organ for both energy balance and aging, as humans with high baseline grip strength have 20-217% decreased risk of all-cause mortality, irrespective of gender or body mass index [19–24]. Candidate gene studies on aging have also implicated genes with important roles in muscle tissue, such as *IGF1R*, *AKT1* and *FOXO3A* [59,60]. For example, in humans, polymorphisms in *FOXO3A* have been associated with lengthened lifespan[60–66]. Both mouse and fruit fly models of *FOXO3A* loss of function result in stronger and longer living model organisms [67–69].

The rapamycin experiments presented here do not speak to tissue specificity they are consistent with previous reports demonstrating rapamycin sensitivity in cold-induced thermogenesis [70,71]. 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 muscle tissues may also 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 [27,72,73]. We propose that it is reasonable that nutrient overload may also promote ineffective catabolism to reduce nutrient stress.

One mechanism linking muscle mTORC1 activity to elevated energy dissipation may be an indirect pathway, associated with elevations in muscle-derived FGF21 [31,74,75] or other factors. Another possibility is that these effects are autonomous to the muscle tissue. The pathways underlying alterations in fiber type are unclear but since amino acids and fatty acids are both reliant on mitochondria to generate fuel, it is possible that an alternation in substrate flux could result more oxidative fibers, which then leads to more increased lipid oxidation and less adiposity. This hypothesis is consistent with anti-obesogenic effects of muscle specific *Cd36* overexpression [76] and with the observation that elevated free fatty acids can promote mitochondrial biogenesis [77]. Increased nutrient uptake, coupled with elevated mitochondria would be likely to produce more ATP in these cells. This in and of itself would not be sufficient to produce heat, so we also propose that increased uncoupling of SERCA2 by sarcolipin may consume ATP to generate heat [55,78]. This hypothesis would be consistent with upregulation of sarcolipin by HFD, exacerbated obesity when *Sln* is ablated [55–57] and prevention when it is overexpressed [58]. As such muscle *Tsc1* knockout animals could be plausibly thermogenic due to multiple muscle-autonomous adaptations including amino and fatty acid transport, mitochondrial activity and sarcolipin-mediated uncoupling.

These findings support the hypothesis that activation of mTORC1 and its downstream targets, specifically in muscle tissue may play a role in nutrient-dependent thermogenesis and therefore may be effective targets for weight loss interventions, but may come at a cost to organismal lifespan.

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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.