# 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 housed in the CLAMS for nine consecutive days. Mice received four days of vehicle treatment (1% Tween, 1% PEG-8000), followed by three days of either vehicle or rapamycin treatment (3 mg/kg/d). Mice were then switched to a high fat diet, 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 Tris buffer [200mM], pH7.4, containing nitro blue tetrazolium [245 µM] and NADH [1.13 mM]. 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 energy expenditure in single-housed mice during this dietary shift. As described in Figure 1A, animals were single housed and vehicle-injected daily for 3 days to acclimatize the animals. They were then placed in metabolic monitoring cages for 2 days, then were randomized into vehicle or rapamycin injected groups. After 2 days of these injections, 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 energy expenditure in the vehicle injected in the dark phase and a 6.8% increase in the light phase. For the animals injected with rapamycin, there was no increase in energy expenditure within the HFD phase, when compared to vehicle treated mice (p=1.24 x 10-5). These effects on energy expenditure 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 in response to HFD feeding.

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

To test whether muscle mTORC1 activation was sufficient to affect adiposity and energy expenditure, we performed indirect calorimetry studies on *Ckmm-Cre* driven *Tsc1* knockout mice. Consistent with prior reports [31] we observed a an elevation in energy expenditure in muscle specific *Tsc1* knockout mouse (Figure 1D, p<1 x 106). This increase in thermogenesis was more apparent during the active dark phase (17% increased in males, 7.5% increased in female knockout mice). There were no significant differences in physical activity during the monitoring period (Figure 1E­­­­). In terms of the respiratory exchange ratio (Figure 1F) while the male wild-type and knockout mice were very similar, female muscle *Tsc1* knockout mice had more lipid utilization at night, but more carbohydrate utilization during the day, potentially indicating sex-dependent increased metabolic flexibility. These data are consistent with a physiological role of mTORC1 in moderating organismal energy expenditure. 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

To understand the physiological significance of elevated baseline energy expenditure, we determined the body composition of male muscle *Tsc1* knockout animals over the course of 7 months on a normal chow diet. As animals aged the wild-type mice accreted more fat mass, but 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 cases lean mass was reduced as well [30,31], which was not observed in this model (Figure 2B, p=0.743 at endpoint). To determine any adipose depot-specific changes, we measured the weights of fat pads from wild-type and *Tsc1* knockout mice, and found that both subcutaneous and epididymal fat pads were reduced in size (79% and 76% respectively, each p<0.0001; Figure 2C).

To determine whether animals with TSC1 ablation in their muscle had different responses to a high fat diet, 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 majority of this difference was in fat mass which was reduced 60% in males and 58% in females by the end of the study (Figure 3A, p < 1.0 x10-6 for each). In terms of lean mass, there were no significant differences with the knockout (Figure 3B, p=0.941), though as expected female lean mass was lower than that of male mice. Upon sacrifice, fat pads were excised from these animals, and consistent with the body composition determinations, we observed a 75-80% reduction in perigonadal and inguinal fat pad mass for both males and females (all p<0.001; Figure 3C).

To test whether the reductions in fat mass were 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, both male and female knockout mice were more insulin sensitive after HFD treatment, consistent with the hypothesis that the lower adiposity results in improved insulin sensitivity. These data support the hypothesis that muscle *Tsc1I* knockout prevents adipose tissue expansion during a high fat diet, and is consistent with elevated energy expenditure.

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

In order to gain some insight into the changes that are occurring within the muscle tissue that may result in the resistance to diet-induced obesity in response to muscle *Tsc1* knockout, 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 some insights into the pathways and networks associated with these genes, we performed gene-set enrichment analyses on this dataset, finding 674 differentially regulated gene sets (see Supplementary Table 2). Among the significantly enriched gene sets were genes regulated by *Tsc2* deletion in MEFs and by treatment with rapamycin [46,53], indicating there are a core set of mTORC1 dependent genes in many tissue types. Consistent with this, we found that 58% of the differentially expressed genes in our muscles overlapped with previously published differentially expressed genes in *Tsc2* knockout MEFs [46]. We identified several gene sets that were upregulated including IGF1 targets in MCF-7 cells [54], those 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 next evaluated markers of muscle fiber types 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). Consistent with this and previous studies on *Tsc1* knockout muscles we observed substantially more NADH staining of cryopreserved muscle sections (Figure 4D). In terms of the molecular source of thermogenesis, we first evaluated the expression of the uncoupling proteins UCP1-3 and noted no significant elevations in these transcripts (not shown). However, we noted that there were dramatic increases in the ER Calcuim pump SERCA2 (encoded by *Atp2a2*, see Figure 4C), and its un-coupler Sarcolipin (encoded by *Sln*; Figure 4E). Sarcolipin has been reported to play a role in muscle-specific thermogenesis [55–58]. To evaluate Sarcolipin protein levels, we performed western blotting on these lysates and found a 4.1 fold increase in Sarcolipin protein (p=4.5 x 10-6; Figure 4F, pS6 is shown as a positive control). We therefore propose that the increased oxidative activity in these muscles may be related to increased futile cycling of ATP by uncoupled SERCA2 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). These changes are likely adaptive mechanisms to manage increased intracellular Ca2+ levels caused by SERCA2 uncoupling.

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

We next observed these animals without manipulation as they aged, asking whether the elevated metabolic rate had any effects on the lifespan of these mice. We observed increased signs of aging, including hunched and scruffy appearances at an earlier age in the knockout animals but not any of the control littermates. As shown in Figure 5, muscle-specific *Tsc1* knockout mice died of natural causes at a higher rate. 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).

A subset of mice were stored in formalin and sent for veterinary pathology, but no consistent cause of death was identified. In animals 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 (two out of four) but not knockout animals (none out of three). The lack of a specific diagnosis does not necessarily confirm the lack of lesions in examined animals; rather, 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.