# Muscle mTORC1 Activation Causes Reduced Adiposity 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 known, 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 have 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 such as insulin, amino acids and energy abundance (see [7] for review). mTORC1 integrates these signals, and helps to co-ordinate such anabolic processes 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.

Several studies have implicated mTORC1 inhibition as a mechanism of organismal lifespan extension in yeast, worms and mammals [16–18]. While several pathways underlying extended lifespan have been proposed, the tissue or tissues that link mTORC1 activity to extended lifespan have not 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]. In this work we tested the hypothesis that activation of mTORC1, specifically within striated muscle tissue can affect organismal lifespan, in concert with impairments in muscle autophagy.

Muscle tissue is the major site of postprandial glucose disposal, and energy expenditure [28,29]. Muscle specific inhibition of mTORC1 (via ablation of *Rptor*), leads to weakened and short-lived mice [30,31]. 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 [26,32,33]. Consistent with this, studies in cell culture models implicate mTORC1 as also a positive regulator of mitochondrial function and ATP production [34–36]. mTORC1 is strongly activated in co-ordination with high protein diets or supplementation with essential amino acids [37,38]. We therefore hypothesized that in oxidative tissues such as heart and skeletal muscle, mTORC1 may promote increases in energy expenditure, but may also play a role in 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.

For muscle specific knockouts, FVB-Tg(Ckmm-cre)5Khn/J transgenic mice (stock 006405) were crossed 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. These parents 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 strains, 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 and cervical dislocation at approximately ZT3. The University of Michigan and UTHSC Institutional Animal Care and Use Committees approved all animal procedures.

## Body Composition and Calorimetry

Body composition was determined using an echoMRI 1100 at approximately ZT10. Body weights were determined using a standard scale. Tissue weights were determined for both the left and right hand side tissue and combined as total weights. Heart and muscle weights were determined by

For calorimetry studies, physical movement and calorimetry was determined using a comprehensive laboratory animal monitoring system from Columbus Instruments. These experiments were performed in light and temperature-controlled enclosures at 25C, in home-cage style cages with hanging feeders. The first 6h of measurements were discarded, after which animals were acclimatized to their new surroundings. Single-animal measurements were collected over the course of 3 days. Oxygen consumption was normalized to lean body mass (as determined by echoMRI, described above) and analyzed by mixed linear models with the considerations described in [39].

For rapamycin treatments, animals were injected with 3 mg/kg of rapamycin or a vehicle (1% Tween, 1% PEG-8000). For acute studies, animals were injected daily before switching diet to a high fat diet.

## 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. For PTG determination, glycogen binding proteins were enriched by amylose pull-down as previously described [11,40,41]. Proteins were separated by SDS-PAGE and blotted with antibodies described in the figure legends. Antibodies used in this study were raised against pS6K (pThr389, Cell Signaling cat #9206), S6K (Cell Signaling #2708), pAkt (pSer473, Cell Signaling #4060) and Akt (Cell Signaling #2920), TSC1 (Cell Signaling #4963), TSC2 (Cell Signaling #4308), pS6 (pSer236/236, Cell Signaling #2211), S6 (Cell Signaling #2317), SREBP1 (Santa Cruz #sc-366), pGS (pSer641, Cell Signaling #3891), GS (Cell Signaling #04-357) and PTG [11]. Blots were visualized on a LiCor Odyssey and quantified using near-IR secondary antibodies Image Studio software.

## Triglyceride and Glycogen Determination

Triglyceride and glycogen levels were determined from snap frozen tissues as previously described [11].

## Insulin and Glucuose Tolerance Tests and Glucose Clamp Studies

Insulin sensitivity was determined by injecting animals fasted for 6h with 0.7mU/g insulin (Humulin HR, Lily) and then collecting tail vein blood every 15 minutes via a glucometer (accucheck). For high fat diet studies, because fat mass was dramatically different between groups, we injected insulin at 0.7mU/g of fat free mass rather than body weight. For glucose tolerance tests 1 mg/kg glucose was injected into 6h fasted mice and glucose was analysed as per the insulin tolerance tests. To determine *in vivo* glucose-stimulated insulin secretion, glucose was injected before and 30 minutes after a retro-orbital bleed. Insulin was determined using an ultrasensitive mouse ELISA (CrystalChem).

## RNAseq Analysis

## NADH Staining of Muscle Sections

## Statistical Analyses

All statistical analyses were performed using the R, version 3.2.2 [42]. 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 [43]. Insulin tolerance tests were analyzed similarly with each time point treated as a repeated measurement. Statistical significance was determined via χ2 tests between models containing or missing the genotype or diet 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)[44,45]. 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 [46]. 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 obesegenic diets, we measured energy expenditure in single-housed mice during this dietary shift. As described in Figure 2A, 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 2B-C, this caused an increase in energy expenditure in the vehicle injected but not rapamycin treated animals. This was not associated with differences in physical activity as measured within these cages (Figures 2D-E). These data support the hypothesis that mTORC1 is required for the increase in energy expenditure in response to adaptation to HFD feeding. Together with the reduced adiposity and mTORC1-activating effects of high protein diets, we investigated a mouse model of mTORC1 activation, specifically in muscle tissues to test whether muscle mTORC1 activation was sufficient to affect adiposity and energy expenditure.

## Deletion of *Tsc1* in Muscle Results in Reduced Fat Mass and Increased Energy Expenditure

We followed the body weights and composition of the muscle *Tsc1* knockout animals over the course of XX months. While we did not observe any differences in lean mass, we did observe a striking lack of fat mass accumulation as these animals grew in size on a normal chow diet, becoming more apparent as the animals aged (Figures 4A-C). Previous studies using *ACTA1-Cre* mediated knockout of *Tsc1* also observed reductions in fat mass, but in those cases lean mass was reduced as well [32,33]. 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 epdidymal fat pads were reduced in size (Figures 4D-E).

To identify the cause of these reductions, we performed indirect calorimetry studies on these mice, prior to differences in adiposity (90 days of age).

## *Ckmm-Cre* driven knockout of *Tsc1* Impairs Glucose Disposal but Does Not Cause Insulin Resistance *in vivo*

mTORC1-dependent pathways have been implicated as causative of insulin resistance, and as a mediator of obesity associated insulin resistance [33,47,48]. To test whether these animals had altered glucose disposal, we performed glucose tolerance tests on the muscle *Tsc1* knockout animals. As shown in Figure XXA, these animals had a marked impairment in the ability to maintain glucose levels after a glucose challenge. Surprisingly, insulin tolerance tests revealed little to no insulin resistance in normal-chow fed knockout animals (Figure XXB). To further probe this effect, we evaluated insulin levels, both before and after a glucose injection. As shown in Figure XXC, we observed an inability of insulin levels to be induced by glucose *in vivo*. These data support an insulin secretion defect in muscle *Tsc1* knockout animals as the root cause of the glucose intolerance.

To test insulin sensitivity in a more controlled manner, we performed euglycemic hyperinsulinemic glucose clamp studies. Consistent with the insulin tolerance tests, we observed no differences in glucose disposal rates, or endogenous glucose production or their changes with insulin (Figure XXD-E). Using 2-deoxyglucose tracer studies, we also evaluated where the glucose was being disposed of at the end of these studies. As shown in Figure XXXF, there were no changes in glucose disposal into muscle tissues, but we did observe an increase in glucose disposal into adipose tissue depots (Figure XXXG). Together these data suggest that in contrast to *ACTA-Cre* induced *Tsc1* knockout animals, *Ckmm-Cre* induced *Tsc1* knockout animals have normal insulin sensitivity.

## Muscle *Tsc1* Deletion Elevates Fasting Glycogen and Triglyceride Levels

To test whether activation of mTORC1 in muscle caused the reduced fat mass observed in the high protein diet fed animals, we generated muscle specific *Tsc1* knockout mice using a floxed *Tsc1* allele [49] and the *Ckmm-Cre* transgene (muscle creatine kinase; expressed in both cardiac and skeletal muscle [50]). This driver differs from the *ACTA1-Cre* driven *Tsc1* knockout models previously described [26,32]. As shown in Figure 3A, we observed efficient knockout of TSC1 and TSC2 proteins, corresponding to an increase in mTORC1 activity in quadriceps lysates from these animals as determined by an increase in S6 phosphorylation (Figure 3B).

We next evaluated the cell autonomous changes in muscle tissue associated with *Tsc1* ablation. We found that both triglycerides and fasting glycogen levels were substantially elevated in *Tsc1* knockout muscles from fasted mice (Figures 3C-D). We repeated these studies in both fed and fasted muscle *Tsc1* knockout animals and found that this increase is only apparent in the fasted state (Supplementary Figure 2). The increase in fasting glycogen was correlated with increases in both processed SREPB1c (42% increase p=0.052), PTG protein levels and a decrease in the phosphorylation of GS (51% decrease, p=0.0012; Figure 3A-B). PTG has previously been shown to anchor protein phosphatase activity to the glycogen pellet [11,51], and the dramatic increase in muscle PTG protein is consistent with reduced GS phosphorylation and higher glycogen levels in these *Tsc1* knockout muscles. Mechanistically, our findings in muscle are consistent with our previous report of mTORC1-dependent, SREBP1c co-activation of the PTG promoter in the liver and *Tsc2* knockout embryonic fibroblasts [11].

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

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. As shown in Figure 6A, both male and female muscle-specific *Tsc1* knockout mice were highly resistant to weight gain on high fat diet. The majority of this difference was in fat mass (XXXX; Figure 6B) as there was only a modest increase in fat free mass in knockout males, and a modest decrease in knockout females (Figure 6C). In terms of percent fat mass, there was a XX% reduction (p < ; Figure 6D). Upon sacrifice, fat pads were excised from these animals, and consistent with the body composition determinations, we observed a XXX-YYY% reduction in perigonadal and inguinal fat pad mass for both males and females (all p<). In this study, we observed no changes in the mass of quadriceps, triceps surae or heart tissues (Supplementary Figure X). These data support the hypothesis that muscle *Tsc1I* knockout prevents adipose tissue expansion during a high fat diet, consistent with [33]. We tested whether insulin sensitivity was affected in these animals. As shown in Figure 6 E and F, we observed improved insulin sensitivity as determined by an insulin tolerance test, consistent with their resistance to the development of obesity.

## Ablation of Muscle *Tsc1* Results in 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. As shown in Figure 7A, we identified 4451 significantly differentially expressed genes in these animals, including 2481 upregulated genes and 1970 downregulated genes (also see Supplementary Table 1). To gain some insights into the pathways and networks associated with these genes, we performed GSEA analysis on this dataset. We identified enrichment of genes regulated by *Tsc2* deletion in MEFs and by treatment with rapamycin [52,53], indicating there are a core set of mTORC1 dependent genes in many tissue types. We found that 58% of the differentially expressed genes in our muscles overlapped with differentially expressed genes in *Tsc2* knockout MEFs [53] (Figure 7B). As shown in Table XX we identified several interesting 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 XXX), while both the fatty acid transporter *Cd36* and binding protein *Fabp3* were increased at the transcriptional level (Figure XXX). We confirmed the increase in CD36 protein levels by western blotting (Figure XXX)

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 7C-D). Consistent with this and previous studies on *Tsc1* knockout muscles we observed substantially more NADH staining of cryopreserved muscle sections (Figure 7E). We did not observe any significant changes in transcript levels of mitochondrial genes, or differences in mitochondrial copy number (Supplementary Figure XXA-B), nor significant elevations in UCP1-3 mRNA. This suggested that the increased metabolic rate in these muscles may be due to a different uncoupling mechanism.

In terms of the source of metabolic, we noted that there were dramatic increases in the ER Calcuim pump SERCA2 (encoded by *Atp2a2*), and its uncoupler Sarcolipin (encoded by *Sln*). Sarcolipin has been reported to play a role in muscle-specific thermogenesis [55–58]. To evaluate SERCA2/Sarcolipin protein levels, we performed western blotting of these lysates to evaluate expression of SERCA2 and Sarcolipin and observed that these proteins are also upregulated. 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* see Supplementary Figure XXX). We also observed reductions in *Ryr1* expression and reductions several plasma membrane Ca2+ transporters (see Supplementary Figure XXX). 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 1C, 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 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

We report here that activation of mTORC1 in muscle tissue results in the accumulation of glycogen and triglycerides, with a shift towards more oxidative muscle fiber types. This, coupled with no detectable decrease in *in vivo* insulin sensitivity is reminiscent of the “athlete’s paradox”, in which muscles of endurance trained athletes are insulin normosensitive, in spite of increased nutrient deposition [59,60]. These findings are concordant with a role of mTORC1 in this process, as this kinase is also activated during resistance exercise and muscle growth [61,62].

Skeletal muscle is an extremely important organ for 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* [63,64]. For example, in humans, polymorphisms in *FOXO3A* have been associated with lengthened lifespan[64–70]. Both mouse and fruit fly models of *FOXO3A* loss of function result in stronger and longer living model organisms [71–73].

Clinical studies have shown that energy expenditure can rapidly increase in response to overfeeding [3,74], consistent with the observation that obese individuals have higher energy expenditure than lean subjects [3,75–77]. Similarly, weight loss results in a rapid reduction in energy expenditure [2,3,6]. This compensation, when combined with increased feelings of hunger after weight loss [4], contributes to body weight set point maintenance and make successful, long term weight loss very difficult. Recent studies have shown that low carbohydrate (and high protein) diets are more successful in maintaining weight reductions [78], and that high protein diets also result in smaller reductions in energy expenditure [6]. A comparison of low-carbohydrate and low-fat calorie restricted diets (with equal protein intake) showed similar reductions of energy expenditure, consistent with a key role for protein in maintaining elevated energy expenditure during weight loss [79]. Similarly, overfeeding studies have shown that over-feeding induced increases in energy expenditure are even more pronounced when the diets are high in protein [5,80]. This is consistent with the elevated thermogenic effect of protein feeding [81], but also data correlating elevated protein intake with shortened lifespan [82,83].

The rapamycin experiments presented here do not speak to tissue specificity they are consistent with previous reports demonstrating rapamycin sensitivity in cold-induced thermogenesis [84,85]. While the focus of those studies have 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.

Our findings of reduced diet-induced obesity and increased energy expenditure are also generally congruent with the findings of Guridi *et al.* [33]*,* however our model has several important differences. We do not observe insulin resistance in our animals, nor do we observe substantial myoatrophy as measured either by reduced lean body mass or increased expression of atrogenes (data not shown). Whether muscle atrophy, insulin resistance and the expression of these genes are linked is unclear, but these differences may reflect different Cre drivers used, as *ACTA1-Cre* was used in those studies, while *Ckmm-Cre* was used in our studies.

One mechanism linking muscle mTORC1 activity to reduced fat mass may be an indirect pathway, associated with elevations in muscle-derived FGF21 [33,86,87] 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-obesegenic effects of muscle specific *Cd36* overexpression [88] and with the observation that elevated free fatty acids can promote mitochondrial biogenesis [89]. 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 [55,90]. 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: Effects of high protein diet on body composition and energy expenditure.** Body composition was determined weekly with body weight (A), lean mass (B) and fat mass (C). D) Weights of fat pad depots at the end of the 14 week feedint study. Statistical significance (p<0.05, n=12/group) was denoted via asterisks based on χ2 test (A-C) or Student’s *t-*test (D).

**Figure 2: Regulation of mTORC1 by amino acids and protein feeding.** A) XXXXXX C) Western blotting of quadriceps RIPA lysates from 16h fasted or non-fasted animals D) Quantification of the blots in B.

**Figure 3: Knockout of *Tsc1* in muscle leads to increased fasted glycogen and triglyceride levels in quadriceps.** A)Protein lysates from quadriceps dissected from animals fasted overnight were prepared in HNTG buffer and blotted as indicated. B) Quantification of processed SREBP1 and the phosphorylation ratio of glycogen synthase. Statistical significance (p<0.05, n=5/7) was determined via a Wilcoxon Rank-Sum test (C) after correcting for multiple hypotheses.

# Supplementary Figure Legends

**Supplementary Table 1: Gene expression differences in muscle *Tsc1* knockout quadriceps.**