# 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.

Muscle tissue is the major site of postprandial glucose disposal, and energy expenditure [16, 17]. Muscle specific inhibition of mTORC1 (via ablation of *Rptor*), leads to weakened and short-lived mice [18, 19]. 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 [20, 21]. Consistent with this, studies in cell culture models implicate mTORC1 as also a positive regulator of mitochondrial function and ATP production [22–24]. mTORC1 is strongly activation in co-ordination with high protein diets or supplementation with essential amino acids [25, 26]. We therefore hypothesized that in oxidative tissues such as heart and skeletal muscle, mTORC1 may promote increases in energy expenditure.

# Methods and Materials

## Animal Husbandry

All mice were purchased from The Jackson Laboratory. For high protein diet (HPD) feeding studies, a custom high protein diet and a matched control diet (CD) were purchased from Research Diets (see Table 1). Male C57BL/6J mice were purchased at 8 weeks of age, and allowed to acclimate to the animal facility prior to changing diets for two weeks. Animals then given *ad libitum* access to CD or HPD starting at 10 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. UTHSC Institutional Animal Care and Use Committee approved all animal procedures.

## Food Intake and Body Composition

Food intake was determined throughout the CD/HPD feeding studies by weighing the food in the cages (4 mice per cage) throughout the study and calculated based on the caloric content of the food. Food intake was therefore the average food eaten by a cage of mice divided by the number of mice in that cage. Body composition was determined using an echoMRI 1100. 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.

## Energy Expenditure Studies

For high protein diet 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 [27].

## Insulin/Glucose/Pyruvate Tolerance Tests and Euglycemic Hyperinsulinemic Clamp Studies

Insulin tolerance tests were performed as previously described [11, 28] by fasting mice for 6h, followed by an intraperitoneal injection of 1 mU/kg insulin (Humulin HR, Lily) with monitoring of blood glucose from the tail vein measured over time using a handheld glucometer (Accuchek). Glucose tolerance tests were performed by injecting 1 mg/g glucose into mice fasted for 16h, with blood glucose being identified as described above. For pyruvate tolerance tests, 2.5 mg/g pyruvate was injected intraperitoneally after a 6h fast. All of these tests were performed at approximately ZT8. To perform hyperinsulinemic euglycemic clamps, conscious 100 day old muscle *Tsc1* knockout mice were analyzed at the Vanderbilt Mouse Metabolic Phenotyping Center and according to previously published protocols [29]. Insulin was determined using an ELISA assay (CrystalChem Ultrasensitive Mouse Insulin ELISA) from retro-oribital blood drawn from isoflurane-anaesthetized animals.

## 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, 30, 31]. 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 via ECL or on a LiCor Odyssey and quantified using Image Studio Lite software.

## Triglyceride and Glycogen Determination

## Statistical Analyses

All statistical analyses were performed using the R, version 3.2.2 [32]. 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 [33]. Pyruvate and insulin tolerance tests were analyzed similarly with each time point treated as an independent 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. 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 [34]. All raw data and reproducible statistical analyses for this manuscript are available at <http://bridgeslab.github.io/TissueSpecificTscKnockouts>.

# Results

## High Protein Diet Feeding Results in Lean Mice With Increased Energy Expenditure

To test the effects of a high protein diet, we fed 10-week old C57B/6J mice a diet containing either 10% protein or 40% protein (Table 1). We observed a modest decrease in body weight in these animals (29% reduction, p=0.036, Figure 1A). This was not due to a decrease in lean mass (Figure 1B) but rather was due to reduced accumulation of fat mass (44% reduction, p=0.057, Figure 1C). This corresponded to reductions of both subcutaneous and epididymal fat pads at the end of the 14 weeks of diet. Skeletal and cardiac muscle mass were slightly increased at the end of the diet (Supplementary Figure 1A).

In order to identify changes in energy balance in these animals, we monitored food intake of HPD and CD-fed animals throughout the study. As shown in Figures 1E-F, there was no significant difference between caloric consumption either weekly or cumulatively between the diets. To test whether there was differential insulin sensitivity in HPD fed animals, we performed an insulin tolerance test. As shown in Supplementary Figure 1B-C, there was no change in the rate at which glucose decreased in response to insulin, but we did observe a more rapid increase back to euglycemia in the HPD fed animals. We interpret these data to mean that reduced systemic insulin sensitivity does underlie the reduced lipid storage in HPD fed animals. The reduced fasting glucose levels, and more rapid return to euglycemia may underlie more efficient gluconeogenesis, but reduced glycogen levels in HPD fed animals. To test this directly, we determined the rate at which injected pyruvate is converted into glucose (Supplementary Figure 1D-E). These pyruvate tolerance tests showed that HPD fed animals are more efficient at converting pyruvate into glucose, consistent with elevated gluconeogenesis.

## High Protein Diet Feeding Results in mTORC1 Activation

Since mTORC1 is a major regulator of metabolism and is activated by elevated amino acids, we next evaluated whether mTORC1 activity is increased in C2C12 myotubes. As shown in Figure 2A supplementation of cultured myotubes with protein results in increased phosphorylation of S6K, in a rapamycin sensitive manner. To test this *in vivo*, we evaluated quadriceps lysates from HPD fed animals (Figure 2C-D). To avoid the confounding effects of acute protein feeding, animals were starved for 16h prior to sacrifice. We blotted lysates from quadriceps for the mTORC1 target S6K and found a 42% increase in phosphorylation of S6K, indicating increased mTORC1 activity in muscle tissue. We also blotted these lysates for Akt activation and found that the pAkt/Akt ratio was unchanged (Figure 2C-D). These data support an Akt-independent role of mTORC1 activation by protein feeding. Based on reduced adiposity and mTORC1-activating effects of high protein diets, we investigated a mouse model of mTORC1 activation, specifically in muscle tissues.

## 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 [35] and the *Ckmm-Cre* transgene (muscle creatine kinase; expressed in both cardiac and skeletal muscle [36]). This driver differs from the *HSA-Cre* driven *Tsc1* knockout models previously described [20, 21]. 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, 37], and the dramatic increase in muscle PTG protein is consistent with reduced GS phosphorylation and higher glycogen levels in these *Tsc1* knockout muscles. Mechanistically, this is consistent with our previous report of mTORC1-dependent, SREBP1c co-activation of the PTG promoter [11].

## 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). A previous study using *HSA-Cre* mediated knockout of *Tsc1* also observed reductions in fat mass, but in their case lean mass was reduced as well [21]. 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 calorimetry studies on these mice, prior to differences in adiposity (90 days of age).

## Activation of mTORC1 in Muscle Does Not Result in Insulin Resistance

## Ablation of Muscle Tsc1 Results in Increased Expression of Oxidative Fiber Type and Fatty Acid Uptake Genes

# 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 [38, 39]. These findings are concordant with a role of mTORC1 in this process, as this kinase is also activated during resistance exercise and muscle growth [40, 41].

Clinical studies have shown that energy expenditure can rapidly increase in response to overfeeding [3, 42], consistent with the observation that obese individuals have higher energy expenditure than lean subjects [3, 43–45]. 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 [46], 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 [47]. 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, 48].

Our identification of skeletal muscle mTORC1 as both a target of high protein diets, and as an anti-obesegenic provides a potential mechanism by which over-feeding can cause increases in energy expenditure. Our findings are consistent with the hypothesis that nutrient excess generally, and high protein feeding specifically may function via mTORC1-dependent changes in muscle oxidation, but do not preclude the role of other tissues and molecular targets that may underlie the increases in energy expenditure that accompany high protein diets. While the direct mechanisms by which mTORC1 increases energy expenditure have not yet been identified, our data are congruent with previous reports showing that activation of mTORC1 in muscle results in elevated mitochondrial biogenesis in fibroblasts [49] and an increase in oxidative muscle fibers [20]. Furthermore, our observation of elevations in the fatty acid transporters CD36 and FABP3 are consistent with anti-obesegenic effects of muscle specific *Cd36* overexpression [50]. Together these findings support the hypothesis that activation of mTORC1 or its downstream targets in muscle tissue may be beneficial for weight loss interventions.

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# Tables

**Table 1: Composition of Control and High Protein Diets.** Percentages indicate percent of calories from the indicated nutrient.

|  |  |  |
| --- | --- | --- |
|  | Control Diet | High Protein Diet |
| Lipids (Lard/Soybean Oil) | 10% | 10% |
| Sucrose | 17% | 17% |
| Starch | 53% | 33% |
| Protein (Casein) | 20% | 40% |

# 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 dietary intervention. Statistical significance (p<0.05, n=6) was denoted if reached via asterisks based on χ2 test (A-C) or Welch’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 Figure 1: Supplementary data related to high protein diet feeding studies.** A) Muscle weights at the end of the 14 weeks of diet. B) Insulin tolerance test of CD and HPD fed animals after XX weeks of diet. C) Data from B, normalized to reflect changes relative to fasting glucose levels. D) Pyruvate tolerance test of CD and HPD fed animals after XX weeks of diet. E) Data from D, normalized to reflect changes relative to fasting glucose levels.

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