# 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] in a tissue specific manner. Studies in fibroblasts also implicate mTORC1 as also a positive regulator of mitochondrial function and ATP production [16–18]. mTORC1 is strongly activation in co-ordination with high protein diets or supplementation with essential amino acids [19, 20]. 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. Animals were placed on CD or HPD at 10 weeks of age.

For High 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 kept on a 12h light/dark cycle. The 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 (echoMRI , Houston, TX). 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 [21].

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

Insulin tolerance tests were performed as previously described [11, 22] 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. 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 [23]. Insulin was determined using an ELISA assay (CrystalChem Ultrasensitive Mouse Insulin ELISA) from retro-oribital blood drawn from isoflurane-anaesthetized animals.

## Statistical Analyses

All statistical analyses were performed using the R package, version 3.2.2 [24]. 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 [25]. 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 [26]. 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 reduced mass 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.

## 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 2B). 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 increased phosphorylation of S6K, indicating increased mTORC1 activity in muscle tissue. We also blotted these lysates for Akt signaling and found that the pAkt/Akt ratio was unchanged. 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 [27] and the *Ckmm-Cre* transgene (muscle creatine kinase; expressed in both cardiac and skeletal muscle [28]). 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.

We next evaluated the cell autonomous changes in muscle tissue associated with *Tsc1* ablation. We found that both fasting triglycerides and fasting glycogen levels were substantially elevated in *Tsc1* knockout muscles (Figures 3B-C). The increase in fasting glycogen was correlated with increases in both processed SREPB1c protein and PTG protein (Figure 3A). This is consistent with our previous report of a mTORC1 -> SREBP1c -> PTG pathway that regulates fasting glycogen in the liver [11]. To determine whether this increased muscle glycogen content correlates with improved muscle function in these animals we evaluated both grip strength (Figure 3D) and amount of cycles spent on a running wheel (Figure 3E). This is consistent with previous reports of reduced muscle strength in *Tsc1* knockout muscles [29]. Our findings support the hypothesis that while short-term contractile function is reduced, overall muscle fitness is improved, potentially related to increased nutrient storage in these muscles.

## 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 reductions in lean mass, we did observe a striking lack of fat mass accumulation as these animals grew in size (Figures 4A-C). A previous study using a *HSA-Cre* mediated knockout of *Tsc1* observed reductions in both lean mass and fat mass [29].

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 [30, 31]. These findings are concordant with a role of mTORC1 in this process, as this kinase is also activated during resistance exercise and muscle growth [32, 33].

Clinical studies have shown that energy expenditure can rapidly increase in response to overfeeding [3, 34], consistent with the observation that obese individuals have higher energy expenditure than lean subjects [3, 35–37]. Similarly, weight loss results in a rapid reduction in energy expenditure [3, 2, 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 (or high protein) diets are more successful in maintaining weight reductions [38], and that these diets also result in smaller reductions in energy expenditure [6]. Similarly, overfeeding studies have shown that energy expenditure is even more increased when the diets are high in protein, but the mechanisms underlying these diet composition have not been clearly defined [5, 39].

Our identification of skeletal muscle mTORC1 as both a target of high protein diets, but as an anti-obesegenic provides a potential mechanism by which over-feeding can cause increases in energy expenditure. 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 [40] and an increase in oxidative muscle fibers [41]. Furthermore, our observation of elevations in the fatty acid transporters CD36 and FABP3 are consistent with anti-obesegenic effects of muscle specific *Cd36* overexpression [42]. 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.**

**Figure 3: Knockout of *Tsc1* in muscle leads to increased fasted glycogen and triglyceride levels in quadriceps.**

# 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.

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

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