Global deletion of BCATm increases expression of skeletal muscle genes associated with protein turnover

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Proposed Journal: Physiological Genomics
Proposed Journal Section: Nutrient Gene Interaction

Keywords
branched chain amino acids, leucine, α-ketoisocaproate, protein synthesis, protein degradation, glycolysis, mTOR, TCA cycle, eukaryotic initiation factor-2, integrin-linked kinase, signaling, myopathy, dystrophy
Abstract

Consumption of a protein containing meal by a fasted animal promotes protein accretion in skeletal muscle, in part through leucine stimulation of protein synthesis and indirectly through repression of protein degradation mediated by its metabolite, α-ketoisocapreqate. Mice lacking the mitochondrial branched-chain aminotransferase ($BCATm/Bcat2$), that interconverts leucine and α-ketoisocapreqate, exhibit elevated protein turnover. Here, the transcriptomes of gastrocnemius muscle from BCATm knockout (KO) and wildtype mice were compared using Next Generation RNA-Sequencing (RNA-Seq) to identify potential adaptations associated with their persistently altered nutrient signaling. Statistically significant changes in the abundance of $1486/\sim 39,010$ genes were identified. Bioinformatics analysis of the RNA-Seq data indicated that pathways involved in protein synthesis (eIF2, mTOR, eIF4 and p70S6K pathways including 40S and 60S ribosomal proteins), protein breakdown (e.g., ubiquitin mediated), and muscle degeneration (apoptosis, atrophy, myopathy and cell death) were upregulated. Also in agreement with our previous observations, the abundance of mRNAs associated with reduced body size, glycemia, plasma insulin, and lipid signaling pathways were observed in BCATm KO mice. Consistently, genes encoding anaerobic and/or oxidative metabolism of carbohydrate, fatty acids and BCAAs were modestly but systematically reduced. Although there was no indication that muscle fiber type was different between KO and wildtype mice, a difference in the abundance of mRNAs associated with a muscular dystrophy phenotype was observed, consistent with the published exercise intolerance of these mice. The results suggest transcriptional adaptations occur in BCATm KO mice that along with altered nutrient signaling may contribute to their previously reported protein turnover, metabolic and exercise phenotypes.
Introduction

The mitochondrial branched chain aminotransferase (BCATm, encoded by BCAT2 gene) catalyzes the rapid and reversible transamination of branched chain amino acids (BCAAs, including Leu, Ile and Val) in many peripheral tissues (36, 53). BCAAs are essential amino acids that frequently represent the most abundant component of dietary protein and dietary supplementation with BCAA has been linked to positive health benefits such as satiety, lean body mass, muscle protein accretion and glucose homeostasis (for review see 49). Paradoxically, increased plasma BCAAs have been shown to be predictive of an increased risk of diabetes mellitus and insulin resistance or have associated with these disorders (1, 7, 10, 14, 21, 47, 58, 63, 73, 75, 84, 85, 87). While understanding the factors underlying this paradox is an active area of investigation, these positive and negative health associations have frequently been attributed, rightly or wrongly, to the ability of BCAAs to act as direct or indirect nutrient signals. For example, BCAAs appear to regulate the synthesis and/or release of hormones such as ghrelin, GLP-1, insulin and leptin that are able to impact eating behavior, glycemia, body composition and energy balance. As direct nutrient signals they can, for example, activate protein synthesis and exert a brake on protein breakdown in muscle and other tissues (52).

Plasma BCAA concentrations represent a balance between their rate of appearance (Ra) and rate of disappearance (Rd). The factors effecting BCAA Ra include dietary intake (not a factor in fasting plasma studies) and protein breakdown in tissues arising from protein degradation (for review see 52). The Rd is affected by protein synthesis, BCAA catabolic metabolism (e.g., transamination, oxidation) and excretion. BCAA oxidative metabolism is a multistep process that begins with transamination. For example, Leu is transaminated to α-ketoisocaproate (KIC) by BCATm in many peripheral tissues including heart, skeletal muscle, kidney and fat. This reaction is reversible, and thus, depending on substrate concentrations, BCATm can also convert KIC to Leu.

It has been unclear whether Leu, its metabolite, α-ketoisocaproate, or both participate in the activation of mTOR, increases in protein synthesis and the inhibition of protein degradation that occurs after Leu treatment. Indeed, some of the negative associations between BCAAs and metabolic health have been recently hypothesized to be related to the effects of KIC, rather than Leu signaling (for review see, 52). One approach that has been used to address this question was through inhibition of BCATm using transaminase inhibitors. While the specificity of those inhibitors is arguable, Tischler et al. (81) used them to determine whether Leu or its transaminated metabolite, KIC, were responsible for the
effects of Leu on muscle protein accretion. They concluded that whereas Leu, but not KIC, stimulated protein synthesis, KIC, but not Leu, provided the brake to protein breakdown in muscle. Subsequent research identified phosphorylation of ribosomal proteins, such as ribosomal protein S6, and translation initiation factors in the actions of amino acids on protein synthesis along with the mammalian Target of Rapamycin (mTOR) and later its complex 1 (mTORC1) as the potential mediator of the activation of protein synthesis by nutrients, in particular, Leu (5, 6, 9, 11, 12, 18, 23, 31, 34, 39-43, 51, 54, 59, 67, 77, 86). However, how KIC might bring about a decrease in protein degradation is still not clear.

A second approach that has been used to assess the role of Leu and its metabolites in downstream signaling events involves genetic ablation of the gene encoding BCATm (e.g., 89). BCATm KO mice fed standard rodent chow exhibit extraordinarily high plasma concentrations of BCAAs compared to wildtype mice. Remarkably, when given a choice of two diets (a normal diet and a diet deficient in BCAAs), the KO mice balanced their consumption such that plasma BCAA concentrations were reduced compared to KO mice fed the standard chow diet. However, even under this feeding regimen plasma BCAAs remained elevated in BCATm KO mice, and, as expected, KIC concentrations were far lower (74, 90). In contrast to the prevailing view that these mice might exhibit metabolic syndrome (62, 63), they instead exhibited decreased adiposity and body weight, and resistance to diet induced obesity, even though they ate more food their wildtype counterparts. In addition, BCATm KO mice exhibited 33% reductions in their plasma glucose levels, a ~50% reduction in glucose tolerance test (GTT) areas under the curve along with ~50% reductions in plasma insulin during the GTT, and improved insulin sensitivity, but not maximal insulin action (76, 79). However, these mice also had reduced exercise tolerance (76). The lower fasting glucose and exercise intolerance might be related in part to the important role of BCAA transamination in gluconeogenic amino acid production that is likely important during exercise.

She et al. (74) observed that an increase in energy expenditure in BCATm KO mice explained their ability to eat more food while maintaining lower body weight. However, factors usually associated with increased energy expenditure in other mouse models did not explain their increased energy expenditure and resistance to diet induced obesity. For example, neither brown fat uncoupling protein 1 (Ucp1) nor muscle sarco/endoplasmic reticulum calcium ATPase 1 (SERCA1, encoded by Atp2a1 gene) where increased. Instead, increased energy expenditure in these mice was postulated to arise
from an increased protein turnover (24, 74) arising from an increase in mTOR signaling brought
about by elevated Leu along with loss of the putative KIC signal to protein degradation postulated
originally by Tischler et al. (26, 81). Moreover, the increase in protein synthesis in these mice helped
protect them from endotoxin-induced decrease in muscle protein synthesis and improve their survival
during sepsis (48). While BCATm skeletal muscle exhibits increased protein synthesis and
degradation, other tissues such as heart, kidney, and spleen appear to be protected as might occur in
starvation (61). Finally, BCATm KO mice were exercise intolerant (76). This was largely postulated to
be associated with their low circulating substrates and energy reserves, interruption of the muscle
malate-aspartate cycle, along with the increased lactate-to-pyruvate ratio and ammonia in skeletal
muscle observed after exercise. However other changes in muscle could be having an influence on
this phenotype.

In the present study, we have used mRNA sequencing to examine the adaptations in gene expression
that might support and help explain the skeletal muscle phenotype of the BCATm KO mice. The data
provide evidence of adaptations in protein synthetic and degradative pathways consistent with the
previously observed protein turnover phenotype. Alterations in the expression of sarcomeric, Integrin-
linked kinase (ILK) signaling and nutrient metabolism genes provide further insight into the metabolic
improvements and exercise intolerance exhibited by these mice.
Methods

Ethics Statement.

All of the vertebrate animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Penn State University College of Medicine (Hershey, PA). The Animal Resource Program is accredited by AAALAC International. All animal living conditions are consistent with standards laid forth in the Guide for the Care and Use of Laboratory Animals (2011), 8th edition, published by the National Research Council.

Isolation of gastrocnemius muscle from BCATm KO mice.

BCATm KO (-/-) mice and their homozygous wildtype (+/+littermates on the C57BL/6 genetic background were genotyped at weaning and thereafter permitted free access to both normal rat chow and a purified amino acid diet lacking branched chain amino acids. BCATm KO (-/-) mice and their homozygous wildtype (+/+) littermates on the C57BL/6 genetic background were genotyped at weaning, and thereafter permitted free access to both normal rat chow and a purified amino acid diet lacking branched chain amino acids. The breeding strategy is intercrossing of +/- heterozygotes. The heterozygotes are maintained by occasional backcrosses at 1-2 time(s) per year to new C57BL/6 representatives of the parental strain ordered from Jackson Laboratories.

Male mice (9-11 weeks old; 4 WT and 4 KO genotypes) in the freely fed state were anesthetized under isoflurane anesthesia (carried with 100% O₂) between 3-4 PM. Under anesthesia, the gastrocnemius muscle was surgically removed and frozen between two aluminum blocks cooled to the temperature of liquid nitrogen and then stored at -80°C until RNA was isolated. While under continuous anesthesia, the animals were then euthanized by cutting the diaphragm and removing the heart.

Food deprivation and time of day are important considerations for sampling gene expression. It should be recognized that regulation of gene expression, especially for amino acid and other intermediary metabolism, is regulated in a circadian fashion and by nutrient sensors (28, 64, 78). For this reason, a narrow window of tissue sampling was used. We also used freely fed not food-deprived animals. BCATm KO mice have persistently elevated BCAAs. However, food-deprivation in this model leads to even further BCAA elevations from the increased rate of appearance from protein breakdown (a) which is part of the changes in this model and (b) that associated with fasting, and the loss of the rate of disappearance owing to the BCATm gene knock out. So in comparisons of this model to wildtype,
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we avoid extended periods of food-deprivation as much as possible and instead opt for either short periods of food deprivation in the afternoon when the animals are eating less or the freely fed state. Here we chose the freely fed state that the state the animals are normally in, and the 3-4 PM time period, a period when the amount of food intake is reduced compared to when the dark cycle begins.

RNA Sequencing.

Total RNA was extracted as previously described with slight modification (22). Briefly, frozen muscle tissue was pulverized in liquid nitrogen using mortar and pestle, followed by bead mill homogenization (Bullet Blender, Next Advance) using stainless steel beads (Next Advance, cat# SSB14B) and mirVana RNA isolation kit (Life Technologies). Optical density values of extracted RNA were measured using NanoDrop (Thermo Scientific) to confirm an A260:A280 ratio above 1.9. RIN was confirmed as greater 7 for each sample using Bioanalyzer RNA 6000 Nano Kit (Agilent Technologies). The cDNA libraries were prepared using the TruSeq RNA Sample Prep Kit v2 (Illumina) as per the manufacturer’s instructions. The final product was assessed for its size distribution using Bioanalyzer DNA High Sensitivity Kit (Agilent Technologies) and for its concentration using Kapa library quantification kit (Kapa Biosystems). Eight libraries were pooled per HiSeq lane, followed by on-board cluster generation on a Rapid Run single-end flow cell and subsequent 50 cycles sequencing (v3 sequencing kit) according to the manufacturer’s instructions (HiSeq 2500, Illumina). Demultiplexed and quality filtered mRNA-seq reads were then aligned to mouse reference assembly (mm10) using TopHat (v.2.0.9). The uniquely mapped reads were used to calculate the normalized expression level of genes, as fragments per kilobase of exon per million fragments mapped (FPKM), using Cufflinks (v.2.0.2).

Biostatistics and Bioinformatic Analysis.

Student’s t-test was used to compare body weights. We used a Mann-Whitney test to determine whether statistical differences existed in the number count of genes detected by RNA-Seq (FPKM values greater than or equal to 1) in the biological replicates in the control compared to the BCATm KO group. To determine significant differences in gene specific FPKM values between wildtype control and BCATm KO groups, the DEGexp function of the DEGSeq 1.18.0 R package was used with the Likelihood Ratio Test (LRT) and default parameters. A p<0.05 was used to determine significant differences. We manually inspected the list of the significantly different genes identified by DEGSeq in comparison to raw counts (Supplementary Tables 1-2). Based on this inspection and the flagging...
function provided with that program, it was decided to reduced the list used for bioinformatic analysis and those genes are found in Supplementary Table 3. In scenario 1, a number of genes were removed for bioinformatic analysis because they were flagged by DEGSeq with the code “#VALUE!” which indicates they lacked FPKM values in the WT or KO group. Many of these gene names had only one biological sample with a FPKM value (example – Cox20). Such genes were not added to Supplementary Table 3. An exception was made for a few genes that had multiple biological replicates with low biological variability in FPKM counts. In scenario 2, some genes designated as significantly different in Supplementary Table S2 were not further studied if the statistical significance appeared to be based on a single outlier FPKM value based on analysis of the values using ROUT routine in Graphpad Prism (example 5830428H23Rik). Finally a Cufflinks error in the analysis of one biological replicate for the gene, Mhy4, led to a reevaluation of the data set with an n=3 for one group as indicated in Supplementary tables. In the line showing that data, one biological replicate removed as indicated. Of the remaining 1543 genes (Supplementary Table S3), 57 had “unmapped identifications” (unknown functions or poorly annotated genes) according to IPA and were eliminated from further analysis, leaving 1486 statistically significant genes from DEGSeq. Those remaining with a normalized fold change of +/- 1.4 (838 genes) were subjected to bioinformatic analyses to narrow the number of pathways to focus upon.

Functional annotation clustering, pathway analysis and gene ontology along with associated confidences indicating the strength of evidence for pathway effects were obtained using a series of tools including Ingenuity Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com), Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/), Kyoto Encyclopedia of Genes and Genomes (KEGG, www.kegg.jp), GeneCards (www.genecards.org), Panther Classification System (www.Pantherdb.org) and the Rat Genome Database (RGD, http://rgd.mcw.edu/). In contrast to IPA, neither Pantherdb nor the DAVID functional annotation tools takes into consideration the direction of gene expression change or its positive or negative influence on a pathway. Therefore we primarily used Pantherdb for its gene overrepresentation analyses at Pantherdb.org (release 20141219, PANTHER version 9.0; The Bonferroni correction was used for multiple testing for those analyses). In David, we separated obtained insights from separately entering (1) the significantly different genes comparing BCATm KO to WT, (2) the genes up regulated by BCATm KO and (3) the downregulated genes. Recognizing the caveat that the products of some genes are inhibitory mediators, we integrated this information with the output from IPA analyses that
does take into consideration the direction of change and function of proteins in pathways or cell
functions. Some programs such as DAVID and Ingenuity provide a Z-score to assess the predicted
activation or inhibited state of upstream regulators or downstream effects of the altered gene
expression in known pathways. The magnitude of this number reflects the intensity/significance of the
predicted change whereas the – or + value indicates inhibition or stimulation respectively.

Data Sharing
The processed data (Supplementary Table S1), associated metadata, and the “raw” Illumina fastq file
have been deposited with the National Center for Biotechnology Information (NCBI) Gene Expression
Omnibus database (GEO Accession number: GSE68915).
Results

Four 9-11 wk old male BCATm KO (-/-) mice and four wildtype littermates were chosen for RNA-Seq. Their body weights are shown in Table 1. Consistent with previous studies (74, 76), there was a trend (p=0.06) for BCATm KO to be ~15-20% lighter than their wildtype (WT, +/-) littermates at this age (Table 1). The gastrocnemius muscle was removed from freely fed mice while under isoflurane anesthesia and mRNA was isolated and was used to make cDNA libraries for Next Generation RNA sequencing. The mouse genome assembly, mm10, was used as reference for alignment of the sequencing data (Supplementary Table S1).

Initial analysis of mRNA sequencing

We examined the data for systematic issues such as whether a similar number of genes was detected in each tissues and whether the FPKM values were consistent with the tissue being from skeletal muscle. The number of genes detected with FPKM values greater than or equal to 1 was 10599 ± 187 for control and 10485 ± 429 for the BCATm KO was not significantly different (p=0.8, Supplementary Table S1). Initial analysis of mRNA sequencing of gastrocnemius muscle from mice in the control group gave rise to FPKM values expected for striated skeletal muscle tissue. For example, of the 39,009 genes in the mouse genome build, the most highly abundant protein coding gene in gastrocnemius was Sepw1 (encoding selenoprotein W muscle 1), and the next 99 highest FPKM values were associated with genes coding proteins of the sarcomere, cation transporters, glycolysis, oxidative metabolism/phosphorylation and protein synthetic machinery (Supplementary Table S1). DEGSeq was used to identify statistically differentially expressed muscle genes between WT and BCATm KO mice (Output in Supplementary Table S2). For example, that analysis shows that the BCATm KO mice expressed very little BCAT2 as expected (Fig 1, Supplementary Tables S1-S3).

A subset of the genes in Supplementary Table S2 identified as significantly differentially expressed between wildtype and KO mice was selected for bioinformatic analysis using the approaches described in the methods. These genes are listed in Supplementary Table S3. Analyzing this subset of genes using PANTHERdb overrepresentation analyses indicated that biological processes and functions associated with protein synthesis, metabolism and the sarcomere were statistically overrepresented (Supplementary Table S4). The results from IPA analyses of the data from Supplementary Table S3 are summarized in Table 2.
**Evidence of elevated protein synthesis.**

The functional annotation tool DAVID does not take into consideration the genes direction of change in gene expression. Therefore, we first used it to analyze significantly upregulated genes. This analysis indicated that genes upregulated by BCATm KO associated in the “very high” category (kappa= 1.0 and 0.85, respectively) with two functionally related terms: protein biosynthesis and ribosome.

Canonical pathway and downstream pathway analyses using IPA, performed on the 838 up- and downregulated genes mentioned earlier, was also consistent with increased protein synthesis. For example, two pathways associated with elevated protein synthesis had strong positive Z-scores. The first pathway was “EIF2 Signaling” (p=5.7e-9, Z-score 3.5, Fig 2A). Contributing to that Z-score was the finding of decreased expression (blue color) of the negative regulator of EIF2, the heme-regulated eIF2α kinase (*Eif2ak1*, also called Heme-Regulated Inhibitor, Hri), whose expression was decreased in BCATm KO mice. Also contributing to this conclusion was the general up-regulation of twenty-two genes encoding protein components of the large and small ribosomal subunit proteins (Fig 2A, red color or font). Notably, an additional 29 ribosomal protein genes were statistically altered but were below the 1.4 fold cutoff we used for IPA analyses (Fig 2A, pink font). A second pathway, “Regulation of eIF4 and p70S6K Signaling”, was also identified as being up regulated (p=2.1e-6, Z-score 1), as was the related “mTOR Signaling” pathway (Fig 2B). For example, there was reduced expression of 4E-BP1, a negative regulator of eIF4E, and a phosphatase that dephosphorylates it (PP2A), enhancing its inhibitory function. However genes encoding upstream regulators of mTOR were either decreased or not affected, and members of the mTORC1 complex were not affected. Nevertheless, downstream targets of mTORC1 that are part of the translational machinery were frequently statistically elevated (Fig 2B). Other gene expression changes that IPA indicated as a prediction of increased translation of protein were the increased expression of poly(A)-binding protein (*Pabpc1*) and eIF3g (88), decreased expression of eIF3F (88), and increased expression of *Fxr1, Calr, Eprs, Cpeb1* and *Ybx2* (Supplementary Tables S1-S3). Moreover, *JunB* was elevated ~2 fold in BCATm KO mice (Supplementary Tables S1- S3). JunB has been shown to increase skeletal muscle hypertrophy and mass by promoting FoxO3 binding to *Fbxo32* and *MuRF1* promoters and thereby reduces protein breakdown (68). The cyclin-dependent kinase inhibitor, p21 (*Cdkn1a*), that plays an important role in skeletal muscle regeneration (20, 33) was also significantly elevated in BCATm KO mouse muscle (Supplementary Tables S1-3). Interestingly, eIF4G2 (also known as Death Associated Protein 5, DAP-5) expression was almost 2-fold greater in muscle of KO compared to wildtype mice (Figs 2A and 2B, Supplementary Tables S1-3). That isoform of eIF4G was recently shown to bind to eIF2S1 and eIF4A...
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to promote cap-independent mRNA translation (50), and thus both cap-dependent and cap-

Independent mRNA translation may be affected in KO mice.

Evidence of elevated protein degradation.

BCATm mice tend to have lower body weights when compared to sibling controls attributed to a futile
cycle consisting of increased rates of protein synthesis and degradation (Table 1 and refs. 74, 76).
Consistently, in the present study, gene ontology for size of the body also had a negative Z score
(Table 2). A number of gene expression changes also implied increased protein degradation in
BCATm mice. Pathways associated with protein degradation, such as cell death, apoptosis, necrosis
and muscle myopathy had positive Z scores with significant changes in hundreds of genes suggesting
that those pathways were activated (Table 2, Supplementary Table S5).

Gene ontology analyses also identified changes in the expression of a number of genes that normally
contribute to protein turnover in skeletal muscle. For example, DNA-damage-inducible transcript 4
(Ddit4, alias: Redd1) is a major inhibitor of mTOR during stress and a mediator of atrophy and target of
glucocorticoids (13). Ddit4 gene expression was elevated almost 3 fold in the gastrocnemius of
BCATm KO mice (Fig 2B, Supplementary Tables S1-3). Another glucocorticoid target,
CCAAT/enhancer binding protein delta (Cebpδ), the expression of which is associated with starvation
and muscle atrophy (3), was elevated more than four fold. The gene encoding atrogin-1 (Fbxo32, also
called Muscle Atrophy F-Box Protein and MAFbx), which is activated in catabolic disorders, was
elevated 1.7 fold in BCATm KO mice muscle (Supplementary Tables S1-S3). BCATm KO was also
associated with up-regulation of the Hint family genes in skeletal muscle (Hint1, Hint2, Supplementary
Tables S1-3). Hint1 appears to regulate proteasomal degradation of proteins by the SKP2-CUL1-F-box
protein-E3 ubiquitin-protein ligase complex, whereas the mitochondrial isoform, Hint2, has been
described as an apoptotic sensitizer (16, 57). Increased expression of two genes that are linked to
positive regulation of apoptosis, cytochrome C somatic (Cycs) and BCL2/adenovirus E1B 19 kDa
protein-interacting protein 3 (Bbip3, the most highly expressed member of this family in muscle) were
also increased (Supplementary Tables S1-2, notably no caspase genes were affected though). The
gene encoding the Muscle-Specific RING Finger Protein 1 (Trim63, alias: MuRF1) that regulates
proteosomal degradation of sarcomeric proteins was elevated 1.6 fold (Supplementary Tables 1-3,
refs. 8, 29, 38, 69, 70). BCATm KO also led to increased expression of 20S proteosome core particle
components (Pmsa7, Pmsa3, Psmb1, Psmb2, Psmb3, Psmb4, Psmb5, Psmb6, Supplementary Tables
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S1-3). Cullins provide a scaffold for E3 ubiquitin ligases involved in proteosomal protein degradation; two of the most highly expressed cullins in muscle, *Cul1* and *Cul3*, were significantly elevated in BCATm KO mice (Supplementary Tables S1-3). Furthermore expression of genes encoding two ubiquitin proteins, *Rps27A* (alias *Uba80*) and *Uba52* was elevated, whereas a number of ubiquitin-specific protease genes that might be involved in reversing these effects (*Usp2*, *Usp13*, *Usp15*, *Usp16*, *Usp19*, *Usp47*) were decreased (Supplementary Tables S1-3). *Nedd4*, the E3 ubiquitin ligase which is elevated in atrophy associated with unloading and denervation, was also increased (45). There was an approximately 2-fold increase in *Degradation In Endoplasmic Reticulum Protein 2* (*Derlin2*, *Derl2*), thought to be a component of the endoplasmic reticulum-associated channeling of unfolded luminal glycoproteins for proteosomal degradation (ERAD). However the gene encoding the Ubiquitin C-terminal hydrolase-L3, *Uchl3*, which reduces stress in skeletal muscle and indirectly improves insulin signaling was increased (72).

Finally, one of the top canonical pathways, Integrin-Linked Kinase (ILK) signaling was predicted to be repressed in BCATm KO compared to wildtype mice (Table 2). Deletion of this pathway in skeletal muscle has been associated with a muscular dystrophy syndrome with multiple regenerating and degenerating fibers (25). Decreased activation of this pathway is also observed during mechanical unloading (Unloading models typically display muscle atrophy) as typified in reduced gene expression of *Col1A1*, *Col1A2*, *Col3A1*, *Fn1* and *Bgn*, (for review see, KJÆR, 44). The expression of these genes was decreased ~2 fold or more in BCATm KO muscle (Supplementary Tables 1-3), consistent with the exercise intolerance (76) and skeletal muscle atrophy pathways we observed to be activated in these mice (Table 2).

Metabolic Genes

Pathway analyses implicated changes in several metabolic pathways in BCATm KO mice. This includes moderately reduced expression of genes in glucose and fatty oxidation pathways that become very significant when examined by pathway analysis (Table 2, Fig 3 and Supplementary Table S5). Most genes in glycolysis were decreased as was the insulin responsive glucose transporter Glut4 (*Slc2a4*, Fig 3A). An exception was hexokinase 2 (*Hk2*) whose expression was increased (Fig 3A). A number of genes in the pentose phosphate pathway were also down regulated (Supplementary Tables S1-3). Genes encoding proteins facilitating non-oxidative and oxidative pyruvate metabolism were also affected. Thus LDH gene isoforms and the *Gpt* gene encoding alanine amino transferase (Fig 3A and
Supplementary Tables S1-3) were downregulated as were pyruvate dehydrogenase (PDH) component genes, \textit{Pdha}, \textit{Pdhx}, \textit{Dlat} and the inhibitory PDH kinase gene (\textit{Pdk2}) in BCATm KO mice. Most genes coding for oxidative glucose metabolism in the TCA cycle were also modestly decreased (Fig 3B) and there was a similar reduction in genes encoding the mitochondrial NADH shuttles (Fig 3C). However, no consistent pattern of change was observed in the KEGG Oxidative Phosphorylation Pathway for the electron transport chain. For example, genes encoding elements of complexes 1-4 were unaffected, upregulated or downregulated (Supplementary Tables S1-3).

Most genes involved in fatty acid oxidation were reduced by the knock out of BCATm. Genes in the BCAA metabolic pathway downstream from BCATm (gene name: \textit{Bcat2}) also displayed a generalized modest decrease in expression (Fig 1, Supplementary Tables S1-3).

\textbf{Muscle Fiber Type and Function.}

Muscle fiber phenotyping schemes can be devised with various levels of complexity and subdivisions of fiber type. A simple approach is to divide the fiber types into two (e.g., slow and fast) or three categories, slow (a.k.a., oxidative, type I fibers), fast oxidative-glycolytic (FOG; a.k.a. intermediate, type IIA and IIX), and fast-glycolytic (fastest in rodent, a.k.a., type IIb). This phenotyping may rely solely on evaluation of myosin heavy chain (Mhc family) isoform content. Alternatively more extensive muscle fiber phenotyping may consider other muscle fiber type specific genes or proteins/isoforms coding for cation, oxygen handling and sarcomeric components found in different muscle fiber types.

We observed a number of significant changes in gene expression implying transitions occurring in the types of fibers present in gastrocnemius due to BCATm KO (Table 4). While we noted no change in the expression of the myosin heavy chain I gene (\textit{Myh7}, found in slow oxidative/type I fibers,) \textit{Myh1} (found in FOG fibers, IIA) and \textit{Myh2} (a fast glycolytic fiber, IIX) showed decreased expression. FPKM values for myosin heavy chain 4 (\textit{Mhy4}), which is the most abundant myosin type in rodent gastrocnemius associated with the fastest most glycolytic fibers (IIb), was also decreased.\textsuperscript{1} Looking at other sarcomeric components, BCATm KOs expressed higher amounts of genes encoding slower fiber (I) types of troponin C and myosin binding protein C (\textit{Tnnc1}, \textit{Mybpc1}), whereas the faster twitch fiber types (\textit{Tnnc2}, \textit{Mybpc2}) were decreased (Table 4). The \textit{Mb} gene encoding the O\textsubscript{2} carrying protein, myoglobin, associated with more oxidative fiber types, was elevated (Table 4). Consistently, the gene encoding the myosin light chain associated with slower (\textit{Myl3}) fiber types was elevated. However those encoding the faster (\textit{Myl1}, \textit{Mylpf}) isoforms were also significantly increased.
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Other changes in gene expression also imply changes in muscle performance/function may be occurring based on calcium handling gene expression (4, 82). Thus the most highly expressed sarco/endoplasmic reticulum calcium ATPase in mouse gastrocnemius, SERCA1, was decreased. Furthermore two SERCA inhibitory peptides, myoregulin (2310015B20Rik, Min, Mrln) and sarcophilin (Sln) were increased (Supplementary Tables 1-3, ref. 4).

**Upstream Pathway Analysis.**

Upstream pathway analysis suggested a number of pathways in skeletal muscle appear to be affected by BCATm KO including a number of transcription factors and signaling pathways (Supplementary Table S6). This analysis does not require that expression of the upstream regulatory element of the pathway change. For example, our analysis predicted that the Ste20-like mitogen-activated protein 4 kinase 4 (Map4k4) pathway was activated (Z-score +4.4, p=8.8e-12), based on the altered expression of 23 genes. However Map4k4 expression was not affected (Supplementary Tables S1, S2 and S6). In C2C12 cells this pathway is a negative regulator of hypertrophy (83).

Activation was also predicted for other upstream regulators, some sharing the same pathway genes (Supplementary Table S6). For example IPA analysis predicted activation of α-Catenin (Ctnna1, 19 target genes affected, Z-score +2.9, p=3.3e-08), the Fas Cell Surface Death Receptor pathways (Fas, 31 target genes, Z-score +2.5, p=5.2e-06) and the small chromatin-binding protein p8 (Nupr1, 31 target genes, Z-score +2.5, p=5.2e-06) pathways. Ctnna1 may play a role in muscle cell differentiation, Fas is a physiological regulator of programmed cell death, whereas Nupr1 promotes myogenic regeneration (71). All of these observations are consistent with various elements of our findings and the phenotype of these mice.

IPA analysis predicted inhibition of other pathways (Supplementary Table S6). For example, most of the various peroxisome proliferator-activated receptors (PPARs) were predicted to be downregulated, including Ppara (38 target genes affected, Z-score -3.3, p=5.7e-08), Ppard (24 target genes affected, Z-score -2.2, p=1.3e-08), Pparg (52 target genes affected, Z-score -4.3, p=7.1e-16) and Ppargc1a (26 target genes affected, Z-score -2.0, p=1.7e-08). Decreases in Ppargc1a activity in muscle haven been linked to a generalized decrease in BCAA metabolism genes, as we observed in BCATm KO mice (Fig 1, and ref 32). Pathway analysis also predicted down regulation of a master regulator of antioxidant
defense (2), Nfe2l2 (35 target genes affected, Z-score -3.9, p=1.7e-07), and Klf15 (14 target genes affected, Z-score -3.6, p=1.6e-10), a master regulator of metabolic gene expression (78).

The insulin receptor (Insr, 44 target genes affected, Z-score -3.9, p=1.7e-07, Supplementary Table S6) pathway was predicted to be downregulated, along with two other physiologically redundant “conditions” hypoglycemia and glucose metabolism disorder (Table 2). These findings are consistent with substantially lower plasma glucose and insulin found in BCATm KO mice (74, 76).

A number of upstream regulators previously associated with muscle degeneration and regeneration were affected. For example, a negative regulator of muscle protein synthesis and mTOR in skeletal muscle, Smad3, was decreased (Supplementary Tables S1-3). However two genes that Smad3 is known to regulate, atrogin-1 (Fbxo32 which increased by Smad3) and irisin (Fndc5, which is negatively regulated by Smad3), did not change in a direction that might be expected based on previous studies of Smad3 expression (27, 80). Muscle atrophy is associated with increased TGFb3 signaling affecting a network of muscle genes including Gnb2l1 (alias RACK1), Cdkn1 and JunB (15).

Each of these genes was elevated in BCATm KO mice (Supplementary Tables S1-3). Another link to these genes and TGF-beta is decreased expression of Sparc (osteonectin) gene expression in BCATm KO mice (Supplementary Tables S1-3), which is associated with myofibrillar atrophy (60). A gene encoding another protein osteopontin (Spp1) is induced following muscle injury and is thought to be an important factor in the degenerative and regenerative events associated with muscle injury (for review see, 65). Spp1 was elevated more than 3 fold in BCATm KO gastrocnemius (Supplementary Tables S1-3 and S6)
Conclusions

Genetic ablation of BCATm leads to an overabundance of circulating branched chain amino acids and a decrease in their metabolites including the branched chain keto acids which appear to provide independent nutrient signals in BCATm KO. In this study we used a systems biology approach, Next Generation mRNA-Seq, to determine the effect of global BCATm deletion on skeletal muscle gene expression in order to (a) provide insights into the potential adaptations arising from the altered nutrient signaling and (b) to provide new research avenues for understanding the mechanisms of the increased protein turnover, metabolic phenotype and exercise intolerance of these mice. Gene ontology analysis of the observed changes in gene expression imply that altered gene expression may contribute to the phenotype of these mice, as genes associated with both protein synthesis and regeneration, along with protein degradation and muscle degeneration, were upregulated.

Consistent with the previously observed increase in protein synthesis in these mice, we observed changes in gene expression in pathways involved in protein synthesis such as EIF2, MTOR, eIF4 and p70S6K1. In the eIF4 and p70S6K1 pathway, components of the mTORC1 complex were not affected, and there was a modest decrease in the upstream regulator Irs1. Surprisingly, expression of the mTORC1 repressor, Ddit4, was also increased. Although increased Ddit4 expression is typically associated with muscle wasting conditions, we note that Ddit4 expression is also increased in other models where mTOR signaling is persistently elevated such as TSC2 KO, where its increased expression can be inhibited by rapamycin (66). In such scenarios, increased Ddit4 expression may serve as a feedback mechanism to limit the magnitude of mTORC1 activation. Contributing to the impression of increased protein synthetic machinery was a generalized increase in the expression of the genes encoding the ribosomal protein (RP) genes that help form the 40S and 60S ribosomal subunits. Eukaryotic RP genes and ribosome biogenesis is frequently coordinately regulated at the transcriptional and translational level from yeast to mammals (17, 30, 35, 56). However the mechanism underlying the transcriptional regulation does not appear to be evolutionarily conserved. In yeast, transcriptional regulation of RP genes can involve mTOR and the transcription factor FHL-1, both of which are expressed in mammals. In contradistinction to yeast, human and murine RP genes are scattered throughout their genomes, and while there is evidence of coordinate regulation of these genes in response to various stimuli (e.g., 35, 55), how this transcriptional regulation is coordinated in mammals has yet to be determined (35), but is thought to be mTOR independent (37). For example this set of genes is not affected in a microarray dataset when TSC-/- cells (which have a persistently
activated mTOR complex 1 that BCAAs normally activate) are treated with rapamycin that would inhibit mTORC1 (19).

BCATm KO mice have smaller, leaner bodies than WT mice along with increased global muscle protein turnover (74, 76). Our results identified a number of genes and pathways where increased protein degradation is implied, with proteosomal and apoptotic as opposed to autophagy pathways was predicted to be affected. We previously postulated that a relatively stable muscle protein pool was undergoing an increase rate of turnover in BCATm KO mice. While the results of the gene ontology analysis are consistent with that conclusion, they were also consistent with a different scenario in which fibers may be undergoing an increase in both degenerative and regenerative processes. Such processes can be activated when muscle is injured or dystrophic. In agreement with this concept, many of the affected genes or pathways were associated with terms such as apoptosis, injury, degeneration, necrosis, myopathy, atrophy and dystrophy. None of these findings contradict the hypothesis that these differences in BCATm KO mice arise from a mixed messaging from the excess of one nutrient signal (Leu) and the loss of another, its metabolite KIC, thought to be signaling separate processes of protein synthesis and protein degradation (24, 81). Further studies are needed to address the role of persistently lower KIC and persistently elevated Leu in these changes.

BCATm KO mice exhibit increased energy expenditure (74, 76). It was surprising, therefore, that modest but significant and systematic declines in gene expression associated with anaerobic and oxidative metabolism of carbohydrate, fat and amino acids occurred in these mice. It is unclear whether this is due to the fact that, while their energy expenditure increases, the circulating concentrations of metabolic fuels are decreased in BCATm KOs. While BCAA levels are elevated in BCATm KOs, they cannot be metabolized, so a reduction in the expression of enzymes that mediate steps beyond BCATm is not unexpected. Thus all of the observed changes, along with the pathway analysis indicating loss of fatty acid (PPAR) signaling and decreased insulin receptor signaling, may be related therefore to lower circulating metabolic substrate and consequent reduction in plasma insulin in this model (74, 76). Further studies at the protein level are needed to determine whether changes in these enzymes and transporters are in fact occurring in muscle of these mice. Glucose and insulin challenge along with clamp studies demonstrated improved not worsened disposal and insulin sensitivity in the muscle of these mice, with no change in capacity for disposal at very high insulin concentrations during a hyperinsulinemic, euglycemic clamp (74, 76).
Many changes occurred in sarcomeric, ion handling, O$_2$ handling and metabolic genes (oxidative and glycolytic) in BCATm KO mice consistent with muscles having slow, FOG or fast twitch types. Our findings support our previous hypothesis that BCATm disruption may be affecting the malate-aspartate shuttle (76), the glycerol phosphate shuttle genes were impacted as well. While a clear picture of muscle fiber type transition can be gleaned from our previous study in which the same RNA-Seq approach was employed (55), a seemingly uncoordinated pattern of changed emerged from BCATm gene deletion. Such lack of a coordinated change in muscle fiber specific genes and their fiber specific isoforms that are meant to work together, if translated into protein, might lead to a dystrophic muscle situation predicted by the pathway analysis. Further studies are needed to evaluate this possibility along with the mechanisms underlying these transcriptional adaptations including the involvement of the upstream pathways implicated by the gene ontology analyses.
Acknowledgements

The authors wish to thank Charles Lang for helpful discussions. The study was funded by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grant DK084428.

Footnote

1 During this part of our bioinformatics evaluation, it was noticed that *Myh4*, normally a major gene and protein component in muscle, had 0 FPKM values for several animals. Notably, *Myh4* has multiple Ensembl entries and a complicated exon structure for these entries (39 and 38 exons). Using a new version of the Cufflinks software (v2.2.1) provided very similar values for the other genes, but resolved this situation for *Myh4*, however wildtype animal 2 was still flagged with an error. These new FPKM values for *Myh4* are reported here and DEGSeq was rerun using these values without the ones for wildtype animal 2 in order to provide data for this gene. The flagged value is shown in Supplementary Table S1 and the other values from the wildtype n=3 analysis appear in Supplementary tables S2-S3 and are noted in those tables.
Table 1. Characteristics of BCATm mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body Weight</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcatm +/+ , Wt</td>
<td>24 ± 0.70</td>
<td>4</td>
</tr>
<tr>
<td>Bcatm -/- , KO</td>
<td>19.3 ± 1.95*</td>
<td>4</td>
</tr>
</tbody>
</table>

*P=0.06 when compared to control mice
Table 2. IPA analysis of RNA-Seq comparison of BCATm KO and WT mice gastrocnemius

<table>
<thead>
<tr>
<th>Top Canonical Pathways</th>
<th>Name</th>
<th>Z-score</th>
<th># of molecules(a)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>eIF2 Signaling</td>
<td>+3.5</td>
<td>26</td>
<td>5.8e-09</td>
</tr>
<tr>
<td></td>
<td>ILK Signaling</td>
<td>-1.5</td>
<td>25</td>
<td>2.8e-08</td>
</tr>
<tr>
<td></td>
<td>mTOR Signaling</td>
<td>+0.3</td>
<td>25</td>
<td>3.5e-08</td>
</tr>
<tr>
<td></td>
<td>TCA Cycle(b)</td>
<td>NAN</td>
<td>9</td>
<td>6.7e-08</td>
</tr>
<tr>
<td></td>
<td>Glycolysis</td>
<td>NAN</td>
<td>9</td>
<td>1.6e-07</td>
</tr>
<tr>
<td></td>
<td>Regulation of eIF4 &amp; p70S6K</td>
<td>+1.0</td>
<td>19</td>
<td>2.1e-06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Top Diseases and Bio Functions</th>
<th>Category</th>
<th>Name</th>
<th># of molecules(a)</th>
<th>p-value range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diseases and Disorders</td>
<td>Skeletal and Muscle Disorders</td>
<td>185</td>
<td>1.2e-03 - 2.4e-22</td>
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<tr>
<td></td>
<td>Molecular and Cellular Functions</td>
<td>Cell Death and Survival</td>
<td>288</td>
<td>1.4e-03 - 2.3e-17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cellular Growth and Proliferation</td>
<td>296</td>
<td>1.4e-03 - 9.4e-15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cellular Assembly and Proliferation</td>
<td>188</td>
<td>1.4e-03 - 1.7e-14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell Morphology</td>
<td>232</td>
<td>1.4e-03 - 2.2e-13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein Synthesis</td>
<td>135</td>
<td>1.1e-03 - 5.9e-12</td>
</tr>
<tr>
<td></td>
<td>Physiological System Development and Function</td>
<td>Skeletal and Muscle System Development and Function</td>
<td>167</td>
<td>1.4e-0.3 - 1.4e-15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diseases and Functions</th>
<th>Name</th>
<th>Z-score</th>
<th># of molecules(a)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell Death</td>
<td>+3.8</td>
<td>273</td>
<td>2.3e-17</td>
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<tr>
<td></td>
<td>Glucose Metabolism Disorder</td>
<td>+3.4</td>
<td>102</td>
<td>8.8e-07</td>
</tr>
<tr>
<td></td>
<td>Necrosis</td>
<td>+3.2</td>
<td>217</td>
<td>2.7e-15</td>
</tr>
<tr>
<td></td>
<td>Apoptosis</td>
<td>+3.2</td>
<td>221</td>
<td>2.8e-08</td>
</tr>
<tr>
<td></td>
<td>Insulin Sensitivity</td>
<td>+2.7</td>
<td>15</td>
<td>3.64e-04</td>
</tr>
<tr>
<td></td>
<td>Myopathy</td>
<td>+3.0</td>
<td>91</td>
<td>2.4e-22</td>
</tr>
<tr>
<td></td>
<td>Atrophy of Muscle</td>
<td>+2.5</td>
<td>18</td>
<td>2.5E-05</td>
</tr>
<tr>
<td></td>
<td>Hypoglycemia</td>
<td>+2.1</td>
<td>14</td>
<td>2.0e-04</td>
</tr>
<tr>
<td></td>
<td>Contractility of Muscle</td>
<td>-2.8</td>
<td>27</td>
<td>3.4e-8</td>
</tr>
<tr>
<td></td>
<td>Oxidation of Fatty Acid</td>
<td>-2.5</td>
<td>30</td>
<td>1.8e-11</td>
</tr>
<tr>
<td></td>
<td>Size of body</td>
<td>-6.32</td>
<td>67</td>
<td>1.3e-04</td>
</tr>
</tbody>
</table>

The 838 genes from Supplementary Table S3 with a normalized fold change of +/- 1.4 were analyzed by IPA. The summarization related to general tissue functions and either muscle or myocytes function including heart muscle are shown (Version 23814503). Footnotes: \(a\) refers to number of molecules with normalized fold changes of ±1.4 or greater; there may be more molecules with lower fold but statistically significant changes. \(b\) NAN indicates “not a number”. All of the TCA cycle IDs had lower FPKM values in BCATm KOs. Causal analysis approaches used to describe generation of p-values have been described (46).
Table 3. Significant Changes in Fatty Acid Oxidation Gene Expression in Gastrocnemius of BCATm KO mice compared to WT sibling controls.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Control WT, FPKM</th>
<th>BCATm KO, FPKM</th>
<th>Normalized Fold Change</th>
<th>Molecular Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acaa2</td>
<td>92±9</td>
<td>69±2</td>
<td>-1.4</td>
<td>Acetyl-CoA acyltransferase 2</td>
</tr>
<tr>
<td>Acad9</td>
<td>29±2</td>
<td>19±2</td>
<td>-1.7</td>
<td>Acyl-CoA dehydrogenase family, member 9</td>
</tr>
<tr>
<td>Acadl</td>
<td>113±14</td>
<td>76±5</td>
<td>-1.6</td>
<td>Acyl-CoA dehydrogenase, long chain</td>
</tr>
<tr>
<td>Acadm</td>
<td>133±11</td>
<td>109±6</td>
<td>-1.3</td>
<td>Acyl-CoA dehydrogenase, C-4 to C-12 straight chain</td>
</tr>
<tr>
<td>Acads</td>
<td>38±4</td>
<td>27±2</td>
<td>-1.5</td>
<td>Acyl-CoA dehydrogenase, C-2 to C-3 short chain</td>
</tr>
<tr>
<td>Acat1</td>
<td>65±3</td>
<td>55±2</td>
<td>-1.3</td>
<td>Acetyl-CoA acetyltransferase, mitochondrial also known as acetooacetyl-CoA thiolase</td>
</tr>
<tr>
<td>Acadvl</td>
<td>147±10</td>
<td>117±2</td>
<td>-1.3</td>
<td>Acyl-CoA dehydrogenase, very long chain; Obesity/T2D susceptibility</td>
</tr>
<tr>
<td>Acsl1</td>
<td>60±10</td>
<td>45±6</td>
<td>-1.5</td>
<td>Ayl-CoA synthetase long-chain family member 1</td>
</tr>
<tr>
<td>Cd36</td>
<td>115±36</td>
<td>91±18</td>
<td>-1.4</td>
<td>Fatty acid translocase (transmembrane)</td>
</tr>
<tr>
<td>Cpt1b</td>
<td>88±8</td>
<td>73±6</td>
<td>-1.3</td>
<td>Carnitine palmitoyltransferase 1b, muscle</td>
</tr>
<tr>
<td>Cpt2</td>
<td>33±7</td>
<td>24±1</td>
<td>-1.5</td>
<td>Carnitine palmitoyltransferase 2</td>
</tr>
<tr>
<td>Eci1</td>
<td>107±12</td>
<td>132±9</td>
<td>+1.2</td>
<td>Enoyl-CoA delta isomerase 1</td>
</tr>
<tr>
<td>Fabp3</td>
<td>339±62</td>
<td>246±26</td>
<td>-1.5</td>
<td>Fatty acid binding protein 3, muscle</td>
</tr>
<tr>
<td>Hadh</td>
<td>127±18</td>
<td>110±5</td>
<td>-1.2</td>
<td>Medium and short-chain L-3-hydroxyacyl-coenzyme A dehydrogenase, mitochondrial</td>
</tr>
<tr>
<td>Hadha</td>
<td>96±10</td>
<td>62±5</td>
<td>-1.7</td>
<td>Trifunctional protein, alpha subunit</td>
</tr>
<tr>
<td>Hadhb</td>
<td>141±20</td>
<td>91±25</td>
<td>-1.7</td>
<td>Trifunctional protein, beta subunit</td>
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<tr>
<td>Lpl</td>
<td>197±79</td>
<td>119±22</td>
<td>-1.8</td>
<td>Lipoprotein Lipase</td>
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<tr>
<td>Slc25a20</td>
<td>32±2</td>
<td>26±1</td>
<td>-1.3</td>
<td>Carnitine/acylcarnitine translocase (cytosol mitochondrial)</td>
</tr>
<tr>
<td>UCP3</td>
<td>23±4</td>
<td>18±1</td>
<td>-1.4</td>
<td>Uncoupling protein 3, mitochondrial</td>
</tr>
</tbody>
</table>

Data are mean means plus or minus (±) SE of the FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped). Normalized fold change values are shown, a positive value indicates a statistically significant increase whereas a negative value indicates a decrease when BCATm KO mice are compared to wildtype (WT) control mice.
Table 4. Genes Associated Skeletal Muscle Fiber Type.

<table>
<thead>
<tr>
<th>Fast Twitch or type IIb gene or homologs</th>
<th>WT Control, FPKM</th>
<th>BCATm KO, FPKM</th>
<th>Normalized Fold Change</th>
<th>Slow Twitch or non-IIb type genes or homologs</th>
<th>WT Control, FPKM</th>
<th>BCATm KO, FPKM</th>
<th>Normalized Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Atp1b2</strong> *</td>
<td>80±12</td>
<td>48±5</td>
<td>-1.8</td>
<td><strong>Atp1b1</strong></td>
<td>42±5</td>
<td>33±4</td>
<td>-1.3</td>
</tr>
<tr>
<td><strong>Atp2a1</strong></td>
<td>4918±657</td>
<td>4344±979</td>
<td>-1.2</td>
<td><strong>Atp2a2</strong> *</td>
<td>38±12</td>
<td>32±5</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Casq1</strong></td>
<td>1226±128</td>
<td>830±54</td>
<td>-1.6</td>
<td><strong>Casq2</strong></td>
<td>13±1</td>
<td>18±3</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Fhl3</strong></td>
<td>89±9</td>
<td>69±2</td>
<td>-1.4</td>
<td><strong>Fhl1</strong></td>
<td>300±26</td>
<td>274±44</td>
<td>-1.2</td>
</tr>
<tr>
<td><strong>Atp1b2</strong></td>
<td>80±12</td>
<td>48±5</td>
<td>-1.8</td>
<td><strong>Atp1b1</strong></td>
<td>42±5</td>
<td>33±4</td>
<td>-1.3</td>
</tr>
<tr>
<td><strong>Atp2a1</strong></td>
<td>4918±657</td>
<td>4344±979</td>
<td>-1.2</td>
<td><strong>Atp2a2</strong> *</td>
<td>38±12</td>
<td>32±5</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Casq1</strong></td>
<td>1226±128</td>
<td>830±54</td>
<td>-1.6</td>
<td><strong>Casq2</strong></td>
<td>13±1</td>
<td>18±3</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Fhl3</strong></td>
<td>89±9</td>
<td>69±2</td>
<td>-1.4</td>
<td><strong>Fhl1</strong></td>
<td>300±26</td>
<td>274±44</td>
<td>-1.2</td>
</tr>
<tr>
<td><strong>Atp1b2</strong></td>
<td>80±12</td>
<td>48±5</td>
<td>-1.8</td>
<td><strong>Atp1b1</strong></td>
<td>42±5</td>
<td>33±4</td>
<td>-1.3</td>
</tr>
<tr>
<td><strong>Atp2a1</strong></td>
<td>4918±657</td>
<td>4344±979</td>
<td>-1.2</td>
<td><strong>Atp2a2</strong> *</td>
<td>38±12</td>
<td>32±5</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Casq1</strong></td>
<td>1226±128</td>
<td>830±54</td>
<td>-1.6</td>
<td><strong>Casq2</strong></td>
<td>13±1</td>
<td>18±3</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>Fhl3</strong></td>
<td>89±9</td>
<td>69±2</td>
<td>-1.4</td>
<td><strong>Fhl1</strong></td>
<td>300±26</td>
<td>274±44</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

Gene expression of isoforms of fast and slow twitch muscle fiber associated genes from WT and BCATm KO mice are shown. Data are mean means plus or minus (±) SE of the FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped). Normalized fold change values are shown, a positive value indicates a statistically significant increase whereas a negative value indicates a decrease when BCATm KO mice are compared to wildtype (WT) control mice. N.S. indicates no significant difference.
**Figure Legends**

**Fig 1. Statistically different BCAA metabolism pathway genes expressed in BCATm KO and WT mice.** Genes in the mouse BCAA metabolism KEGG pathway that were statistically different based on DEGSeq analysis (Supplementary Tables S2-3) are plotted. The bars are mean FPKM plus or minus SE. Black bars, WT skeletal muscle; gray bars, BCATm KO.

**Fig 2. Effects of BCATm KO on protein synthesis pathways.** IPA analysis results are shown for genes in the EIF2 pathway (A) and eIF4 & p70S6K pathway (B). The beige border represents the sarcolemma. Arrows indicate a pathway direction and a blunted arrow indicates an inhibitory factor or process. Blue color indicates that the expression of the gene was decreased. Red color indicates expression of a gene or a number of RP genes composing 40S and 60S subunits were generally increased. Lighter shades of blue and red and regular rather than bold font indicates that the normalized fold change was significant but less than the ±1.4 normalized fold change used as an arbitrary cut off for IPA analysis (Supplementary Tables S1-3). Gray font and equal sign (=) indicates no significant change.

**Fig 3. Changes in genes encoding components of the glycolytic, TCA cycle and mitochondrial NADH shuttle pathways in gastrocnemius muscle in response to BCATm KO.** Effects of BCATm KO on muscle gene expression are shown for the glycolysis pathway (A), citric acid (TCA) cycle (B) and two mitochondrial NADH shuttles (C). Metabolites and glycolytic intermediates are shown in black font; gene names are italicized. The beige border represents the sarcolemma (A) or mitochondrial membrane (C). Arrows indicate a pathway direction. Blue color indicates that the expression of the gene was decreased (emphasized with a down direction arrow). Red color indicates expression of a gene was increased (emphasized with an up direction arrow). Lighter shades of blue along with regular rather than bold font indicates that the normalized fold change was significant, but less than the -1.4 normalized fold change used as an arbitrary cut off for IPA analysis (Supplementary Tables S1-3). Gray font and equal sign (emphasized with an = sign) indicates no significant change. Where the abundance of two isoforms is different, the font size is smaller for the isoform where the FPKM has a lower value (Supplementary Tables S1-3).
List of Supplementary Tables

Supplementary Table S1. Cufflinks output from RNA Sequencing data. FPKM values for each wildtype (WT1-WT4) and BCAT2 KO (KO5-KO8) mouse is shown along with the mean and SE for each genotype by gene and whether the differences were significant at the p<0.05 or p<0.001 level based on analysis in Supplementary Table S2.

Supplementary Table S2. Statistical analysis of Supplementary Table S1 using DEGSeq. DEGSeq output from analysis of Supplementary Table S1 is provided.

Supplementary Table S3. Subset of genes used for bioinformatic analysis. These genes were chosen for bioinformatic analysis. While all of these were provided for IPA software a cutoff of +/-1.4 was used for that analysis and those with a normalized fold change of 1.4 or more were used.

Supplementary Table S4. Over-represented biological processes and protein functions in BCATm KO mouse muscle. A PANTHER overrepresentation test of the statistically different genes in BCATm KO skeletal muscle using gene set in Supplementary Table 3 that had at least a positive or negative normalized fold changed of 1.4 or greater. Mouse reference indicates the number of genes with the indicated protein function or biological process in the *Mus musculus* reference genome. Obs indicates the number observed or entered into the test field that matched to each row category. Exp is the number of genes with the gene ontology tag in each row with the *Mus musculus* reference genome used by Pantherdb.org.

Supplementary Table S5. Diseases and functions pathways affected by BCATm KO. Output from the IPA “Disease and Functions” analysis was edited to include generalized functions or disorders that were not tissue specific, unless skeletal muscle was indicated. Disease or function is in column A, column B shows the number of molecules significantly affected (greater than +/- 1.4 normalized fold change), column C is p-value (46), column D is the predicted activation state based on Z score greater than +/- 2., column, column F are the category of the disease or function from column A, and column G contains a list of the affected genes interspaced by a comma.
**Supplementary Table S6. Upstream Regulators affected by BCATm KO Output.** IPA analysis of potential upstream regulators based on target molecules that the regulators have been known to regulate. The potential regulator is described in column A. If regulator is a gene whose expression changes significantly in our data set, the fold change is shown in column B (+ indicates elevated, and – indicates decrease in BCATm KO, respectively). A prediction of the potential activation state of the regulators activity is shown in column C (“Inhibited” indicates that the regulator appears to have its activity blocked while “Activated” indicates that the regulator may be active based on the genes affected and their direction of change.). Column D indicates a Z-score reflecting the relative magnitude of activation (+) or inhibition (-). E indicates the overlap P-value measuring enrichment of network-regulated genes in the dataset (46). Column F is the type of regulators, and Column G is the list of target genes in the dataset.
References

19. Düvel K, Yeaces JL, Menon S, Raman P, Lipovsky AI, Souza AL, Triantafellou E, Ma Q, Gorski R, Cleaver S, Vander Heiden MG, MacKeigan JP, Finan PM, Clish CB, Murphy LO, and...


Muscle mRNA-Seq after BCATm deletion


Muscle mRNA-Seq after BCATm deletion


C

Gly6P + NAD\(^+\) \rightleftharpoons 1,3-bPGly + NADH + H\(^+\)

NADH + H\(^+\) + DHAP \rightleftharpoons Glycerol-3P + NAD\(^+\)

Cytosol

Slc25a12 ↓

FAD \rightleftharpoons FADH2

Asp

Got1 ↓

OAA + NADH + H\(^+\) \rightleftharpoons Mdh1 ↓

Malate + NAD\(^+\) \rightleftharpoons Slc25a11 ↓

Got2 ↓

OAA + NADH + H\(^+\) \rightleftharpoons Mdh2 ↓

Asp

Mitochondria

Malate-Aspartate shuttle

Glycerol phosphate shuttle