## Supplemental Experimental Procedures

### Targeting vector

To delete the *BCATm (BCAT2)* gene in mice, we used a 13.0-kb DNA sequence containing exons 4-11 of the mouse gene. The mouse genomic BAC clone was obtained from Incyte Genomics 129/SvJ mouse BAC library. A 6.0 kb genomic fragment, a 2.2 kb genomic fragment, and a 4.8 kb genomic fragment from the mouse BAC clone were subcloned for the 5' homology arm, conditional knockout region, and 3' homology arm, respectively (Figure S1A). The conditional knockout region, which was floxed with 3 loxP sites, contained the active site Lys in exon 6 that binds the pyridoxal phosphate cofactor and other key active site residues are in these exons (Bledsoe et al., 1997; Davoodi et al., 1998; Yennawar et al., 2002). A neo cassette flanked by two *loxP* sites was inserted in the intron preceding exon 4 and was used as a positive selection marker; and a DTA expression cassette was included at end of the 3' arm for negative selection. In this way, *loxP* sites flanked exons 4-6.

### Gene targeting and production of chimeras

XSV1 mouse embryonic stem (ES) cells were cultured in standard conditions on a feeder layer of mitotic inactivated G418-resistant primary embryonic fibroblasts. The targeting vector was prepared with Qiagen MaxiPrep kit and was linearized using the single NotI site and electroporated into 1.0 X$10^7$ XSV1 ES cells at 500 mF and 250 V. The cells were than cultured in the presence of G418 and 147 ES clones were selected for primary Southern blot analysis using a 0.5 kb 5' external probe and SphI digestion of genomic ES DNA (Figure. S1B). Positive clones were identified by the presence of hybridization bands of both 17.5 kb and 11 kb. The hybridization band of ~11 kb, corresponding to the homologous recombinant allele, was visualized in clone #B6, B7, B9, B10, L1, L3, L11, and M12. Additional Southern blot analysis using a 0.2 kb 3' external probe and an internal Neo probe confirmed the correct targeting events (data not shown). With the 3' probe, positive clones were identified by the presence of hybridization bands of both 12.5 kb and 8.9 kb of EcoRV digested genomic DNA and a 10.5 kb hybridization band with SacI digested genomic DNA for the Neo probe (data not shown). The presence of the 3rd loxP site in intron 6 was confirmed by PCR using the following sense 5'-GAGATGGGTGGGTTAATGGG-3'and antisense 5'-CCTTGTTCCGACATGCCAGT-3'primers. Two correctly-targeted ES clones (L3 and M12) were used for microinjection.

### Generation of BCATm conditional and null alleles

C57BL/6N Tac female mice were used as blastocyst donor for microinjection. The highest percentage male chimeras were bred with C57BL/6N Tac female mice to produce germline-transmitted heterozygote mice. Genomic DNA prepared from tail biopsies was used for genotyping by PCR. Forward and reverse primers for the PCR were 5'-TCCCTGGTG CCTGACACTAAA-3' (primer 3 in Figure S1A and S1C) and 5'-AGAAGC CAC AGG GGAAATGT-3' (primer 4 in Figure S1A and S1C), respectively. These primers are complementary to the region of intron 6 that contains the 3' end of the loxP-flanked transgene. PCR with this primer pair generates a 534 bp product for the wild-type allele and a 600 bp product for the original floxed allele (data not shown).

The F1 mice carrying the targeted allele were bred with a cytomegalovirus promoter-induced *cre*-transgenic mouse (CMV-Cre) originally obtained from http://jaxmice.jax.org/strain/003465.html and backcrossed 4 generations to C57BL/6J. From this

mating we obtained mice with BCATm alleles that had lost the $Neo^R$ cassette and retained two loxP sites flanking exons 4-6 (loxB) as well as mice with BCATm alleles that had lost both the $Neo^R$ cassette and exons 4-6 and retained a single loxP site (*BCATm*$^-$). $BCATm^{+/-}$ progeny were interbred to generate homozygous null mutants (*BCATm*$^{-/-}$). Knockout mice were identified by PCR genotyping of genomic DNA using primers 1, 3 and 4 for wild-type and $BCATm^-$ alleles as shown in Figure S1D. Primer pair 1 and 2 shown in Figure S1 was used for screening wild-type allele and conditional allele, LoxB. Hetero- and homozygous mutant mice were obtained in Mendelian proportions suggesting no obvious effect on viability.

**Supplemental Experimental References**

Bledsoe, R.K., Dawson, P.A., and Hutson, S.M. (1997). Cloning of the rat and human mitochondrial branched chain aminotransferases (BCATm). Biochim Biophys Acta. 1339, 9-13.
Davoodi, J., Drown, P.M., Bledsoe, R.K., Wallin, R., Reinhart, G.D., and Hutson, S.M. (1998). Overexpression and characterization of the human mitochondrial and cytosolic branched-chain aminotransferases. J Biol Chem. 273, 4982-4989.
Hall, T.R., Wallin, R., Reinhart, G.D., and Hutson, S.M. (1993). Branched chain aminotransferase isoenzymes. Purification and characterization of the rat brain isoenzyme. J Biol Chem. 268, 3092-3098.
Yennawar, N.H., Conway, M.E., Yennawar, H.P., Farber, G.K., and Hutson, S.M. (2002). Crystal structures of human mitochondrial branched chain aminotransferase reaction intermediates: ketimine and pyridoxamine phosphate forms. Biochemistry. 41, 11592-11601.

**Supplemental Figure Legend**

**Figure S1. Generation of BCATm null mice.**
**A)** Gene targeting strategy using Cre-loxP system. Top, partial map of the wild-type *BCATm* allele. Exons are indicated as solid rectangles. Middle, map of the *BCATm* gene targeting vector. The vector also contains a *neo* cassette, a *DTA* cassette, and three *loxP* sites (triangles). Two of the *loxP* sites flank *neo*, and the third is located in intron 6 so that exons 4, 5 and 6 are flanked by *loxP sites* in the *BCAT2* gene. The targeted allele was generated by homologous recombination (HR) in ES cells. Bottom, the *BCATm* conditional and null alleles derived from the original targeted allele by Cre-mediated recombination. The original targeted mice with lox+neo allele were bred with CMV-Cre mice (Jackson laboratory) to excise the *neo* cassette and/or exons 4, 5 and 6 to generate conditional and null alleles.
**B)** Southern blot analysis in targeted ES cells. A 0.5 kb 5' external probe and SphI digestion of genomic ES DNA were used for Southern blotting. The recombination alleles (~11.0 kb band) and wild-type alleles (~17.5 kb band) in various clones were shown.
**C)** PCR primers used for genotyping various alleles generated by gene targeting and Cre-mediated recombination. The primer number corresponds to that shown in Figure S1A
**D)** PCR genotype analysis. For the wild-type and null alleles, tail or ear DNA was amplified by mix of primers 1, 3, and 4 as shown in Figure S1C to give wild-type and/or null (*BCATm*$^-$) bands.

**Figure S2. Relationship of absolute energy expenditure and fat free body mass (FFM).**
Energy expenditure data from the experiment in Figure 4B, in which mice were fed with choices of BCAA-containing and BCAA-free purified amino acid diets for three weeks was reanalyzed.

The data was re-expressed in absolute terms per mouse and plotted against the estimated FFM for each mouse because differing slopes are thought to provide evidence for an effect of the KO on EE when the lean body mass is different. Analysis of the slopes using Graphpad Prism software indicates that they are significantly different ($p=0.014$).

**Figure S3.** Western blot analysis of UCP1, UCP2 and UCP3 in various tissues.
Proteins (50 or 100μg) from tissue lysates (brown fat, epidydimal white fat or gastrocnemius) or particulate fractions containing mitochondria [kidney and liver, prepared as previously described (Hall et al., 1993)] were separated by SDS-PAGE and transferred to PVDF. PVDF blots were probed with peptide antibodies to UCP1 and UCP3 from Santa Cruz as well as UCP1, UCP2 and UCP3 antibodies generously provided from Dr Thomas Gettys at Pennington Biomedical Research Center. Whereas short ECL exposures (minutes) were possible for UCP1 in brown fat, much longer (~1h) exposures were needed for other antibodies and tissues. After exposure, blots were stripped and reprobed for loading controls [tublin, β-actin or cytochrome oxidase1 (Cox1)]. Recombinant UCP proteins (also from Dr Getty) and a muscle particulate/mitochondrial fraction were used as positive controls or to help identify the UCP proteins (not shown). Representative blots are shown. Blots were quantified as a ratio to a loading control and statistically analyzed as follows. Brown fat UCP1/tublin: +/+ 15920 ± 3290 vs. -/- 11530 ± 1520, n=4, not significant (NS); Epidydimal fat UCP1: not detected after 1 h; Epidydimal fat UCP2/β-actin: +/+ 6320 ± 728 vs. -/- 5360 ± 1030 n=5, NS; Gastrocnemius UCP1: not detected after 1 h; Gastrocnemius UCP2/tublin: +/+, 0.55 ± 0.09 n=8 vs. -/- 0.46 ± 0.05 n=7; Gastrocnemius UCP3/tublin: +/+, 0.44 ± 0.17 n=5 vs. -/- 0.41 ± 0.04 n=3, NS; Kidney UCP2/Cox 1: +/+, 2242 ± 436 n=4 vs. -/- 1377 ± 155.1 n=4, NS; Liver UCP2/Cox1: +/+ 533 ± 145 n=4 vs. -/- 425 ± 71 n=4, NS.

Table S1 Fed plasma amino acid concentrations (μM) in BCATm null and wild-type mice[a]

| | Male | | |
|---|---|---|---|
| | +/+ | -/- | Δ% |
| LEU | 115.5 ± 13.4 | 423 ± 200 | ns |
| ILE | 77.6 ± 11.5 | 587 ± 300 | ns |
| VAL | 190.7 ± 35.8 | 1974 ± 717 | 935% |
| CIT | 51.8 ± 2.9 | 67.0 ± 6.1 | 29% |
| B-ALA | 3.6 ± 0.2 | 2.9 ± 0.2 | -20% |

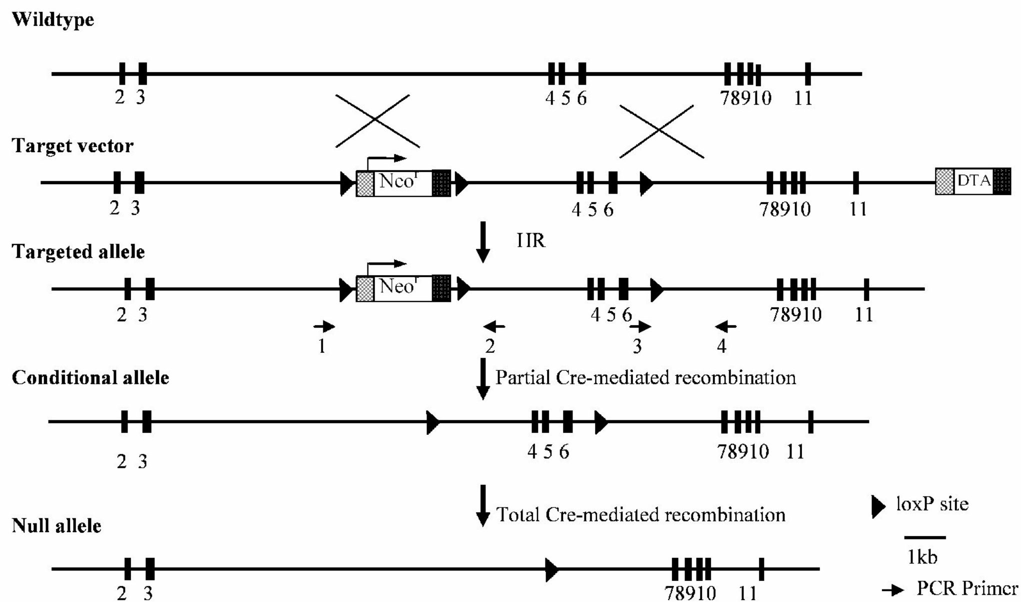
[a]Mice fed a mix of purified amino acid BCAA free and BCAA-containing diets. n=8, ns, not significant. Data for all other amino acids were altered and not shown.

Table S2. Metabolic parameters in urine measured in a metabolic cage[a]

| | +/+ | -/- | Δ% |
|---|---|---|---|
| Food intake (g/d) | 3.7 ± 0.2 | 4.4 ± 0.2* | 16% |
| 24-h Urine volume (ml) | 1.91 ± 0.0.17 | 2.53 ± 0.24* | 33% |
| Creatinine concentration (mg/l) | 209 ± 14 | 144 ± 12** | -31% |
| 24-h Creatinine amount (μg) | 373 ± 23 | 340 ± 25 | ns |
| 3-Methyhistidine concentration (mg/l) | 7.2 ± 2.5 | 8.2 ± 1.1 | ns |
| 24-h 3-Methyhistidine amount (μg) | 13.3 ± 1.3 | 20.2 ± 2.0** | 52% |

[a]Individual mouse was placed in an metabolic cage and fed with mixed powered food with 76% -BCAA/24% NC for the BCATm$^{-/-}$ mice and 45%-BCAA/55%NC for the wild-type for 2 days. $P < 0.05$, ** $P < 0.01$, n=16

A

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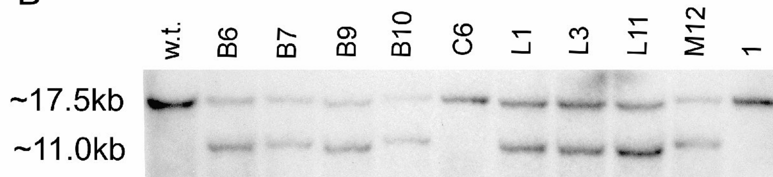


C

| Primer sequence | Primer pairs and PCR products |
|---|---|
| 1: 5'-GTTCTCAAGGTGGTGGGTGT-3' | Primers 1,3,&4: 534bp wildtype |
| 2: 5'-AACTGCTTGATTTCGGTGCT-3' | 770bp BCATm$^-$ |
| 3: 5'-TCCCTGGTGCCTGACACTAAA-3' | Primers 1&2: 530 wildtype |
| 4: 5'-AGAAGCCACAGGGGAAATGT-3' | 600 loxB |
| Cre1: 5'-TGATGGACATGTTCAGGGATC-3' | Primers Cre1 & Cre2: 865bp Cre |
| Cre2: 5'-CAGCCACCAGCTTGCATGA-3' | |

D

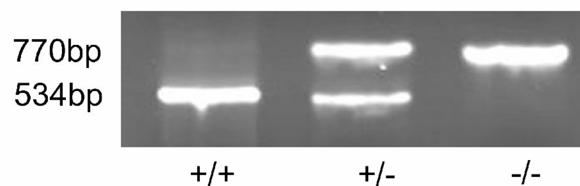


**Fig S1**

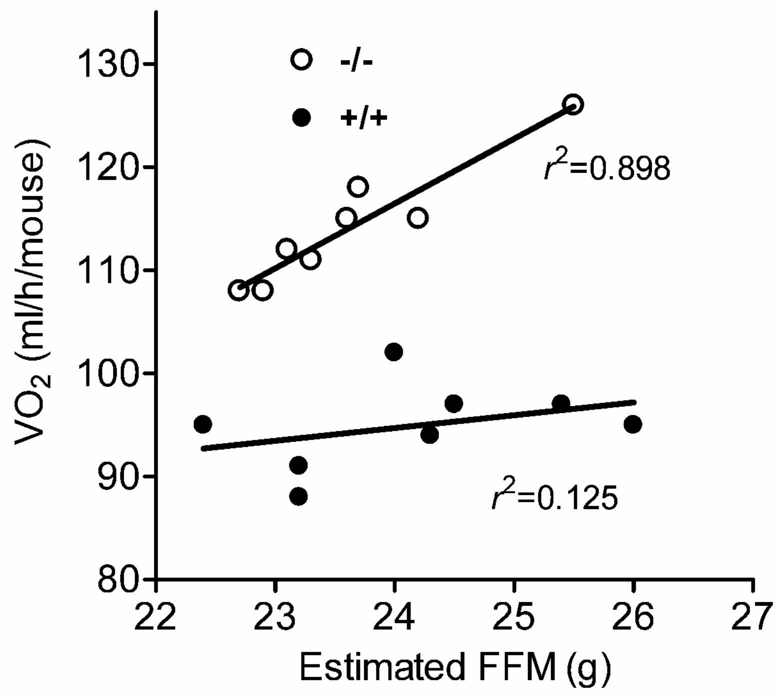


**Fig S2**

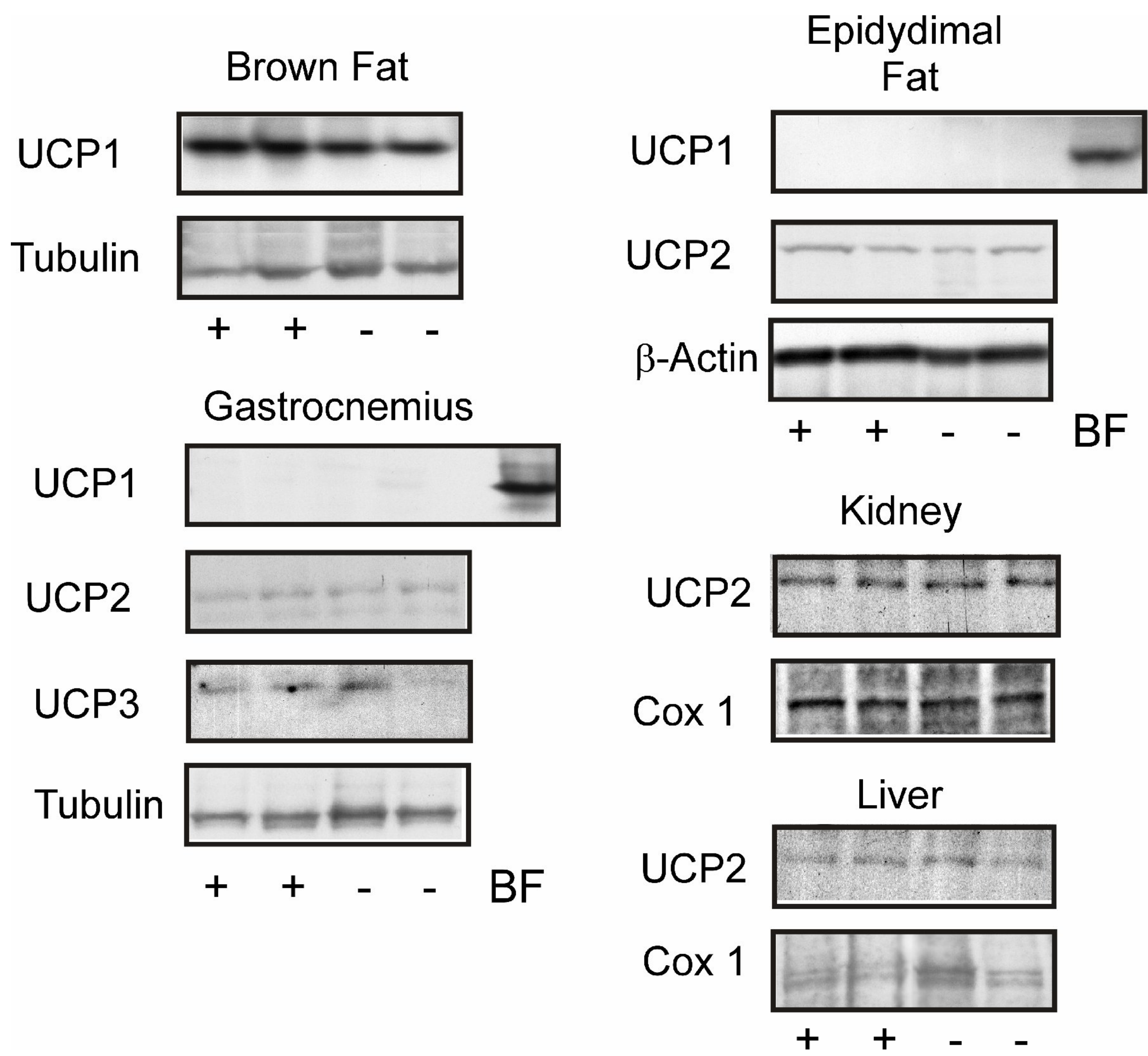


**Fig S3**